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PHYSIOLOGICAL CHANGES AND RESPONSES OF *PSEUDOMONAS AERUGINOSA* ATCC 9027 WHEN GROWN ON PETROLEUM COMPOUNDS.

by

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A thesis submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

Ph. D. Frank A. Pietrantonio Microbiology

PHYSIOLOGICAL CHANGES AND RESPONSES OF *PSEUDOMONAS AERUGINOSA* ATCC 9027 WHEN GROWN ON PETROLEUM COMPOUNDS.

Physiological and compositional changes in *Pseudomonas aeruginosa* (ATCC 9027) were monitored during growth on various petroleum compounds in a chemically-defined medium. Growth of *P. aeruginosa* was observed when furnace oil, kerosene, aviation fuel, light crude oil and hexadecane were used as carbon and energy sources. A variable and extended lag period before active growth was achieved was characteristic of petroleum-grown cells as compared to glucose-grown cells. Growth on the petroleum hydrocarbons, compared with that on glucose, resulted in changes in cell lipid composition, outer membrane proteins, cell-surface hydrophobicity, surface-tension, and pH changes in the growth medium during transition from early to late-log phase. Cell composition and physiology of cells grown in the petroleum mixtures varied due to differences in the chemical composition of the material. Production of an exopolymer (characterized as a peptidoglycolipid) was associated with petroleum-grown cells but not with glucose-grown cells. The above differences illustrate some of the dynamic and physiological and biochemical changes the microorganism undergoes to access its hydrophobic carbon and energy source.

RÉSUMÉ

Ph. D Frank A. Pietrantonio Microbiology

CHANGEMENTS ET RÉPONSES PHYSIOLOGIQUES DE *PSEUDOMONAS AERUGINOSA* ATCC 9027 EN CROISSANCE SUR DES COMPOSÉS PÉTROLIFÈRES.

Les changements de compositions et physiologiques de *Pseudomonas aeruginosa* (ATCC 9027) lorsque mis en croissance sur divers composés pétrolifères (dans un milieu chimiquement défini) ont été évalués. *P. aeruginosa* démontre une croissance sur les millieux de croissance suivants: huile de fournaise, kérosène, carburant d' avion, huile légère non raffiné, et sur hexadecane en tant que source d'energie et de carbones Des retards variés et prolongés ont été obtenu avant la croissance active, ceci reflétant les caracteristiques de la croissance des cellules en millieu pétrolifères comparé aux cellules en milieu à base de glucose. La croissance des cellules en millieu à base d'hydrocarbone de pétrole et de milieu à base de glucose révèlent des changements au niveau de la composition lipidique de la cellule, des protéines de la membrane externe, de l'hydrophobicité de la surface de la cellule, et de la tension de surface et des changements de pH du milieu de croissance du début à la fin de la consignation des données. La production d'un exopolymère est associée avec la croissance des cellules en milieu pétrolifères mais non en milieu à base de glucose. L'hétérogénéité des composantes à l'intérieur des melanges à base de pétrole résulte en différences visible dans la composition des cellules et de la physiologie entre les cellules en

cultures sur les différents milieux à base de pétrole. Les cellules en culture dans le glucose démontrent des différences uniques par rapport aux cellules en millieu pétrolifères. Ces différences illustrent certaines des changements moléculaires dynamiques et non statiques des cellules pour accéder à leurs sources d'énergie et de carbones.

DEDICATED

to the memory of

Dr. J. M. Ingram

who opened the doors to "Camp *Pseudomonas*" and by doing so,

opened my eyes to the world of science.

Thank you, SIR !!!!

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LIST OF CONTRIBUTIONS TO KNOWLEDGE

1. Cell growth was observed on different petroleum products (furnace oil, kerosene, aviation fuel) and light crude oil; this is the first reported instance of growth on all these petroleum substrates by *P. aeruginosa* and specifically this strain (ATCC 9027). Growth on small *n*-alkanes (hexane, heptane, and octane) grew marginally. Growth patterns differed amongst the different petroleum-grown cells. In all instances where petroleum hydrocarbons were the sole carbon and energy source, a pronounced lag period in the initial stages of growth and a requirement of 8-9 days to reach stationary phase was noted.

2. Growth on hexadecane showed different patterns in the outer membrane profile from the early to late stages of growth, while cells grown on glucose showed no change. Changes in the protein profile occurred in the 29-40-kDa range.

3. Distinct differences were noticed in the outer membrane protein profiles among the cells grown on the various petroleum hydrocarbons. Aviation fuel-grown cells revealed pronounced 45- and 30-kDa proteins. Overall, samples grown on hexadecane, furnace oil and kerosene showed similar protein profiles.

4. Growth on different petroleum compounds results in changes in cell-surface hydrophobicity and lowering in the surface-tension of the growth medium.

Differences in cell-surface hydrophobicity and surface-tension were observed amongst the different petroleum-grown cells. Furnace oil- and hexadecane-grown cells showed the highest increase in cell-surface hydrophobicity and largest drop in surface-tension.

5. Analysis of extractable-lipids of *P. aeruginosa grown* on different hydrocarbons showed the presence of four phospholipids (phosphatidylethanolamine, phosphatidyl -choline, *lyso*-phosphatidylethanolamine, and cardiolipin), neutral lipids, and two unknown lipids (Unk#1 and Unk#2). The amount of each lipid varied among the different petroleum-grown cells.

6. Different patterns in the lipid species as well as the fatty acid composition was observed from early- to late-log phase. As cells entered late-log an increase in the cyclopropane, *trans*-, and saturated fatty acid species was noted.

7. Cyclopropane fatty acids accounted for approximately 30-40% of the total fatty acid composition in each lipid of the petroleum-grown cells, whereas, in glucose-grown cells it accounted for 1%.

8. Release of a polymeric compound (exopolymer) into the liquid growth medium and on solid culture media was associated solely with petroleum-grown cells and not glucose-grown cells. Preliminary analysis of the exopolymer showed that the

compound consisted of protein, carbohydrate and lipid. The exopolymer was observed to be tightly bound to cells and the petroleum hydrocarbons substrate.

1. 0. INTRODUCTION

Pseudomonas aeruginosa ATCC 9027, has previously been shown to oxidize and utilize hexadecane as a sole carbon and energy source (Miguez *et al.*, 1986), however, there is no literature describing the growth of *Pseudomonas aeruginosa* ATCC 9027 on refined petroleum products (furnace oil, kerosene, and aviation fuel) and light crude oil. Gram-negative bacteria, such as *P. aeruginosa* are characterized by having two cell envelope membranes separated by a layer of peptidoglycan. The inner cytoplasmic membrane contains all the necessary oxidative enzymes associated with petroleum degradation, as well as many of the cell envelope enzymes and all the known transport systems. The outer membrane is distinguished by a unique component, lipopolysaccharide (LPS), and a distinct set of proteins and phospholipids (Sikkema *et al.*, 1995). The presence of the LPS causes the outer membrane to have a very low permeability towards hydrophobic compounds.

The ability of microorganisms to utilize water-insoluble substrates, such as petroleum hydrocarbons, has been attributed to their remarkable ability to adapt to the presence of such substrates, both at a physiological and molecular level (Hanson *et al.*, 1995). Microorganisms growing on hydrocarbons have been reported to undergo ultrastructural changes as well as to produce surface-active compounds (Hanson *et al.*, 1995). Questions arise as to how the hydrocarbon-utilizing microorganism deals with the hydrophobicity or lipophilicity of the substrate and consequently the transport of such substrates into the enzymatic and degradative centre of the cell (cytoplasmic membrane), and how the microorganism responds to some of the toxic components within the petroleum

mixture. Hydrocarbon uptake has been shown to occur via two processes: a direct interaction between the cell surface and the hydrocarbon (unmediated transport), or through the action of surface-active agents (mediated transport), by emulsifying the hydrocarbon (Hanson *et al.*, 1995).

The main goal of this research, was to provide a detailed documentation of changes in membrane composition associated with the growth of *Pseudomonas aeruginosa* ATCC 9027 on a number of different petroleum hydrocarbon substrates (furnace oil, kerosene, aviation fuel, and light crude oil) and to compare these changes to those observed during growth on a non-petroleum substrate, glucose. The results obtained here will be beneficial to the other researchers attempting to shed light on the mechanisms by which bacteria alter themselves to accommodate the utilization of hydrocarbon pollutants with limited water solubility.

2.0. LITERATURE REVIEW

2.1. PETROLEUM DEGRADATION

2.1.1. Petroleum in the environment

Petroleum enters the environment because of waste disposal practices, accidental spills, intentional application, atmospheric fallout, and natural seepage. The manufacture, distribution and transportation of petroleum have resulted in petroleum being a serious environmental pollutant in marine, freshwater, and soil ecosystems. Petroleum is used not only as a source of energy but also as a primary source for the production of lubricants, solvents, plastics, fibres, detergents, pharmaceuticals, cosmetics, and animal feed. The annual global release of petroleum by man into the environment, is estimated to be between 1.7 and 8.8 million metric tons per year (National Academy of Sciences, 1985).

The fate of hydrocarbons in the environment depends on both biotic and abiotic processes, which include chemical catalysis, adsorption, evaporation, solubilization, and biodegradation (Leahy and Colwell, 1990). Studies on the environmental fate of petroleum have shown a nearly ubiquitous distribution of hydrocarbon-metabolizing microorganisms (Atlas, 1988). Biodegradation of the hydrocarbons is determined by the structure of the components, the proportion of the degrader populations, the environmental factors that affect the activities of these microbial populations and the bioavailability of the hydrocarbon (Leahy and Colwell, 1990).

2.1.2. Predominant environmental factors affecting biodegradation of petroleum

Factors such as temperature, pH, inorganic nutrient concentrations, and oxygen availability are major physico-chemical influences on the biodegradation of hydrocarbons in the environment (Zhou and Crawford, 1995).

2.1.2.1. Temperature

Temperature affects the physical properties and the chemical composition of the oil, microbial metabolic rates, and the distribution and composition of the microbial community (Atlas, 1981). Temperature has a direct influence on the petroleum by affecting its viscosity, its water solubility, and the volatility of its toxic short-chain alkanes and small aromatic components (Atlas and Bartha, 1972a). Overall, a decrease in temperature results in decreased degradation due mainly to the reduced enzymatic activities of the microorganisms (Gibbs *et al.*, 1975). Nevertheless, biodegradation of petroleum does occur at low temperature (Whyte *et al.*, 1996; Colwell *et al.*, 1978; Huddleston and Creswall, 1976).

2.1.2.2. pH

Most heterotrophic bacteria and fungi favour a pH near neutrality, with the fungi being more tolerant of lower pH conditions (Atlas, 1988). Extremes in pH have a negative effect on the rate of biodegradation. Researchers have shown a near doubling in the rate of biodegradation, by adjusting the pH from 4.0 to 7.4 (Verstraete *et al.*, 1976). The soil pH will often determine the microbial population that can participate in hydrocarbon

biodegradation. The pH of the marine environment is somewhat uniform and consistent, and slightly alkaline (Bossert and Bartha, 1984).

2.1.2.3. Inorganic nutrients

The availability of nitrogen and/or phosphorous limits the microbial breakdown of hydrocarbons (Leahy and Colwell, 1990; Mills and Frankenberger, 1994). Studies have shown that low concentrations of available nitrogen and phosphorous influence greatly the extent of hydrocarbon degradation after most major oil spills (Swannell *et al.*,1976; Jobson *et al.*, 1974; Ward and Brock, 1976; Atlas and Bartha, 1972b). Rapid biodegradation of large quantities of oil requires high rates of inorganic nutrient replenishment, and these nutrients are generally lacking; therefore, nitrogen- and phosphorous-containing organic and/or inorganic fertilizers are often added to stimulate and enhance microbial hydrocarbon degradation (Swannell *et al.*,1996). Braggs *et al.* (1994) concluded that the application of inorganic nutrients accelerated the biodegradation of crude oil by a factor of three to five.

2.1.2.4. Oxygen availability

Oxygen plays an essential role in hydrocarbon degradation since the initial steps in the breakdown of aliphatic, cyclic and aromatic hydrocarbons by fungi and bacteria involve oxygenases and molecular oxygen (Singer and Finnerty, 1984; Perry, 1984; Cerniglia, 1984). Zobell estimated a theoretical oxygen demand value for the oxidation of oil, as one gram of oxygen for every 3.5 g of oil (Zobell, 1969). Zobell also calculated that the oxidation of one litre of oil required the dissolved oxygen in 3.2×10^5 l of sea water. Oxygen limitation

normally does not occur in the upper levels of the water column in marine and freshwater environments (Floodgate, 1984; Cooney *et al.*, 1985). Biodegradation may be severely hampered within anoxic basins, the hypolimnion of stratified lakes and benthic sediments (Atlas, 1981). The oxygen availability within soils relies on the rates of microbial oxygen consumption, the physical and chemical characteristics of the soil, and the presence of oxygen-depleting utilizable substrates that compete with the microbes (Song *et al.*, 1990; Song and Bartha, 1990; Bossert and Bartha, 1984).

Although rapid biodegradation of petroleum generally does not occur under anaerobic conditions (Atlas, 1981), the rate of biodegradation under anaerobic conditions can sometimes be significant (Grbic-Galic and Vogel, 1987; Milhekic and Luthy, 1988). Anaerobic degradation of petroleum hydrocarbons and aromatic compounds by bacteria and fungi has been documented (Villemur, 1995; Kameya *et al.*,1995; Berry *et al.*, 1987; Evans and Fuchs, 1988; Rueter *et al.*, 1994; Zeyer *et al.*, 1990, 1986; Grbic-Galic and Vogel, 1987; Atlas, 1981; Bertrand *et al.*, 1989; Ward and Brock, 1978). Some controversy as to the significance of the role of anaerobic degradation of alkanes exists. Schink (1989) remains skeptical as to the possibility of anaerobic alkane degradation, while Bertrand *et al.* (1989), state that this process may occur despite the fact that there is a lack of convincing proof that this is a real phenomenon. Further, as there is evidence for aromatic hydrocarbon degradation under anaerobic conditions, one should not discard the possibility of anaerobic degradation of other hydrocarbons involving the aliphatic hydrocarbons.

2.1.3. The effect of chemical composition of the petroleum on biodegradation

Petroleum is an extremely complex mixture of hydrocarbons (Atlas, 1981). Petroleum can be fractionated via silica gel chromatography into three fractions: a saturated or aliphatic fraction, an aromatic fraction, and an asphaltic or polar fraction (Brown *et al.*, 1969). Others (Colwell and Walker, 1977) have separated the petroleum mixture into four fractions: the saturates, the aromatics, the asphaltenes (phenols, carboxylic acids, ketones, esters and porphyrins), and the resins (pyridines, quinolones, carbazoles, sulfoxides and amides).

Fuels contain a major proportion of aliphatic hydrocarbons and in some cases aromatics (which may make up to 25 % of the fuel). Other components in the fuel may include oleofins, cyclic compounds, mercaptans, and additives such as antistatic, antioxidant, antiicing or corrosion inhibitors (Hill, 1984). Song *et al.* (1989) and Perry (1984) have quantitated the amount of aromatic, alicyclic, and paraffin in various fuel products which are presented in Table 1. The relative amount of components within the refined fuels is largely dependent upon the origin and the nature of the petroleum crude (Perry, 1984).

The petroleum hydrocarbons have been ranked in the following order of susceptibility to biodegradation: *n*-alkanes > branched alkanes > low molecular weight aromatics > alicyclics (Perry, 1984; Fussey and Oudot, 1984; Jobson *et al.*, 1972; Walker *et al.*, 1976a). Hydrocarbons susceptibility to biodegradation depends on the structure and size of the hydrocarbon molecule (Atlas, 1981). Short-chain alkanes (C< 10) tend to be toxic to microorganisms because of their water solubility and their interactions with lipid

Table 1: Composition of various refined fuel products

	% Composition		
Fuel Product	**Aromatics**	**Alicyclics**	**Paraffins**
Gasoline	10-20	50-60	15-20
Aviation Fuel	20	20-30	50-60
Furnace Oil	33	2	60-70
Kerosene	40	← 60-70 →	
Diesel	← 30-40→		60-70

membranes (Pfaender and Buckley, 1984; Klug and Markovetz, 1971; Teh and Lee, 1974), but tend to evaporate from slicks rapidly (Atlas, 1981). Although biodegradation of *n*-alkanes ranging in size up to 44 carbon units has been documented (Haines and Alexander, 1974), microorganisms exhibit a marked preference for *n*-alkanes of intermediate chain length (C_{10}-C_{24}). As the chain length of the alkane increases, there is a tendency for the alkane to be increasingly resistant to biodegradation. Branched alkanes are degraded slowly since the protruding carbon atoms may interfere or block the degradative mechanisms of the microorganisms (Watkinson and Morgan, 1990; Fall *et al.*, 1974; Pirnick, 1977). Schaeffer *et al.* (1979) found that the terminal branching of some alkanes inhibits biodegradation. Methyl branching at the beta position has also been shown to affect the biodegradation of an alkane by blocking β-oxidation (Singer and Finnerty, 1984; Schaeffer *et al.*, 1979).

Substituted alicyclic compounds (or cycloparaffins or cycloalkanes) appear to be degraded more readily than unsubstituted forms. Microbial attack on the substituted alicyclic compounds generally occurs on the aliphatic side-chain, especially if the carbon chain is long (Perry, 1977). Degradation of alicyclic compounds requires the cooperation of at least two microbial strains with complementary metabolic capabilities (Atlas, 1981). Complex alicyclic compounds are often the most resistant and persistent components of oil spills (Perry, 1984; Atlas *et al.*, 1981).

The primary enzymatic attack on the light aromatic hydrocarbons is at the level of the alkyl side group or directly on the ring (Gibson, 1971). Extensive methyl substitution of light aromatic hydrocarbons can affect the initial oxidation by the microorganisms (Atlas, 1981). Condensed ring aromatic hydrocarbons are very resistant to enzymatic attack (Smith,

1990). Investigators have observed biodegradation of structures containing more than four condensed rings; however, degradation involved cooperation between microbes (Smith, 1990; Atlas, 1981). Complete biodegradation of fluoranthene by pure cultures of *Pseudomonas paucimobilis* was observed by Mueller *et al.* (1990).

The metabolic pathways for the degradation of the asphaltenic fraction of petroleum mixtures are the least understood (Atlas, 1981; Pendrys, 1989), and no common degradation pathway has been found. Although researchers had, in the past, considered the asphaltenic and resinous fractions to be very resistant to microbial degradation (Walker *et al.*, 1975a), all fractions of crude oil including the asphaltenes and resins are degraded often involving co-oxidation (Rontani *et al.*, 1985, Bertrand *et al.*, 1983; Horowitz and Atlas, 1977). The co-oxidation biodegradation of asphaltenic compounds in a mixed bacterial culture was highly dependent upon the presence of *n*-alkanes (C_{12} to C_{18}), (Rontani *et al.*, 1985).

One would therefore expect that the rate and degree of biodegradability of petroleum mixtures will be influenced by their qualitative hydrocarbon composition. Walker *et al.*(1976b) compared the biodegradation of two fuel and two crude oils. Their biodegradation study revealed that the decrease in the various fractions (saturate, aromatic, resinous and asphaltenic hydrocarbons) was highly variable among the four oils. Jobson *et al.* (1974) compared the degradation of a "high quality" crude oil to one of lower quality and found a better biodegradation rate with the higher quality crude. They believed that the higher levels of sulfur, aromatics, asphaltenes and resins in the lower quality crude may have accounted for the decreased rate.

2.1.4. Microbial degradation of hydrocarbons in the environment

A large and diverse group of bacteria and fungi can degrade petroleum (Atlas and Cerniglia, 1995; Cerniglia *et al.*, 1992; Atlas, 1981). The distribution of these hydrocarbon-degrading microorganisms varies from milieu to milieu (marine, freshwater and soil) (Jones *et al.*, 1970; Pinholt *et al.*, 1979; Hollaway *et al.*, 1980), and their numbers within the microbial community are highly influenced by the level of hydrocarbon in the environment; less than 1% and up to 100 % of the microbial community in unpolluted and polluted ecosystems, respectively (Atlas, 1981).

2.1.5. Microbial contamination of refined petroleum products

The microbial spoilage of refined petroleum products has economic implications (Hill, 1984). Three types of petroleum product spoilage have been documented (Hill, 1984); i.e., biodegradation of fuel oils, breakdown of lubricating oils, hydraulic oils and straight cutting oils, and spoilage of oil-in-water emulsions used in cooling and lubricating during metal working processes.

The problems associated with microorganisms contaminating petroleum products include: blockage of screens, filters, etc. caused by the physical presence of the proliferating microorganisms; interference by the electrical properties of the microorganisms with the capacitance fuel probes; production of surfactants preventing fuel and water separation; formation of corrosive metabolic by-products (organic acids and sulfides); microbial uptake of oxygen resulting in a corrosive electrochemical oxygen gradient cell; corrosion due to microbial depolarization; degradation of protective coatings in tanks (Hill, 1984).

2.1.5.1. Contamination of fuel, hydraulic, and lubricating oils

Microbial contamination problems have been described in gasoline, kerosene, aviation fuels, diesel and gas oil for road vehicles, rail locomotive fuels, ships' light fuel oils, and heating oils (Hill, 1984; Walker *et al.*, 1976b; Hill and Thomas, 1975; Jamison *et al.*, 1975; Patel *et al.*, 1985). Contamination of fuel oils in road and rail vehicles, power and heat-generating equipment and ships is widespread and results in fewer drastic consequences than in airplanes. The symptoms of microbial contamination are a lowering of interfacial tension, filter plugging and injector fouling (Hill, 1984). In airplanes, filter plugging and corrosion of the aluminum alloys are the major problems associated with microbial contamination of the aviation fuel (Hill and Thomas, 1975). Gasoline spoilage is not an issue as the hydrocarbon range (C_5 to C_9) and the additives within the gasoline are less likely to support microbial growth due to their toxicity (Hill, 1984).

Microbial growth on hydraulic and lubricating oils differs from that of fuel oils. Pressure additives, antifoaming agents, and corrosion and oxidation inhibitors within the fuel oils may act as reservoirs of nitrogen, phosphorous and sulphur (Hill, 1984). Microbial growth on the oils (also referred to as "mineral oils") results in changes in lubrication capacity, loss of effectiveness of the additives and an increased rate of corrosion (Hill, 1984). Microbial growth on hydraulic oils results in the plugging and malfunctioning of valves, filters and orifices of the hydraulic system and a decrease in the viscosity of the mineral oil (Hill and Al-Haidary, 1976). The overall result of microbial contamination of the hydraulic oil is inadequate hydraulic pressure. Microbial growth on lubricating oils may be due, in a large part, to a change in the composition of the components within the lubricant.

The addition of nutrient-rich additives, changes in the base oils (less naphthenic in nature) and generally less maintenance of the hydraulic system are contributing factors leading to spoilage. The effects of spoilage of the lubricating oils result in decreased load bearing properties, additive depletion, filter plugging, and engine seizures due to pitting and rusting of journals, cams and bearings (Hill, 1978b). *Pseudomonas aeruginosa* and *Pseudomonas* spp. were found to initiate the breakdown (attack) of the oils (Naylor *et al.*, 1982). Some fungi as well as sulphate-reducing bacteria have also been implicated in the breakdown of the hydraulic and lubricating oils (Hill, 1978b; King and McKenzie, 1977; Hill and Al-Haidary, 1976).

2.2. HYDROCARBON UPTAKE IN MICROORGANISMS

The majority of hydrocarbons are essentially water-insoluble. Compounds with a high molecular weight and/or a high degree of saturation have a very low solubility in water (Sikkema *et al.*, 1995). Aromatic hydrocarbons are more soluble in water than the long-chain alkanes (Klevens, 1950). The hydrocarbon-utilizing microorganisms must in some way achieve an initial contact with the water-insoluble hydrocarbon in order to transport the carbon and energy source into the cell. There have been several proposed mechanisms of hydrocarbon transport into microbial cells (Singer and Finnerty, 1984): (1) interaction of the microorganism with the hydrocarbon substrate dissolved in the aqueous phase; (2) direct contact of the microbial cells with large hydrocarbon droplets (larger than one μm in diameter); and (3) interaction of the microbial cells with "solubilized" or "accommodated" hydrocarbon droplets smaller than the cell (smaller than one μm in diameter). For the first

mechanism, a general consensus exists among investigators that the rate of dissolution of long-chain alkanes and higher molecular weight compounds in the aqueous medium by physical processes of solubilization is relatively low so as not to support an observable growth rate of microorganisms. There have been, however, reports supporting this mechanism for more soluble alkanes, aromatics and gaseous hydrocarbons (Wodzinski and Bertolini, 1972; Wodzinski and Coyle, 1974; Goma *et al.*, 1973; Yoshida *et al.*, 1971). In the second proposed mechanism, microbial cells adhere to the surface of the hydrocarbon droplets (which are larger than the cell), and the hydrocarbon substrate is thought to enter the cell through diffusion or active transport at the point of contact (Goswani and Singh, 1991; Miura *et al.*, 1977; Rosenberg and Rosenberg, 1981; Velankar *et al.*, 1975). Increased substrate surface area for cell attachment raises substrate uptake (Witholt *et al.*, 1990; Sikkema *et al.*, 1995). The production of surface-active compounds by hydrocarbon-utilizing microorganisms results in the emulsification of the hydrocarbons, which in turn results in better dispersion of the hydrocarbon substrate in the aqueous medium and a concomitant increase in surface area (Kennedy *et al.*, 1975; Miura *et al.*, 1977; Nakahara *et al.*, 1977), but it cannot fully account for hydrocarbon uptake and microbial growth (Goswami and Singh, 1991). In the third mechanism, microbial cells interact with small-diameter particles of "solubilized" or "microemulsified" hydrocarbon. These particles attach to the surface of the microbial cells instead of the cell attaching to the surface of the hydrocarbon droplet. An increase in hydrocarbon uptake is expected with smaller particle size because of the greater surface area between the hydrocarbon substrate and the water. Nakahara *et al.* (1977) and Gutierrez and Erikson (1977) have shown that when *Candida lipolytica* grows on *n*-

hexadecane, both the second and third proposed mechanisms play an equally vital role in hydrocarbon uptake. Goswami and Singh (1991) reported two species of *Pseudomonas* (N1 and M1) which differed in the hydrocarbon uptake mechanism. *Pseudomonas* N1 was observed to utilize the third mechanism while *Pseudomonas* M1 used the second mechanism when grown on n-hexadecane. *Pseudomonas* N1 not only grew faster than M1, but also produced an extracellular biosurfactant-bioemulsifier, and hexadecane solubilizing factor.

2.2.1. Biosurfactants

Surface-active compounds are generally produced by microorganisms when they are grown on insoluble or immiscible compounds (carbon and energy sources, Desai and Banat. 1997; Banerjee *et al.*, 1983). The surface-active compounds are termed "biosurfactants" or "bioemulsifiers". The surfactant molecule is amphipathic in nature, i.e. possesses both a hydrophobic region and a hydrophilic region. This characteristic allows these molecules to lower the surface tension of water which then allows the insoluble compound to remain in a single phase with the medium (Desai and Banat. 1997; Cooper, 1986). Biosurfactants can be grouped into several component chemical categories such as carbohydrate- or amino acid-containing, or as a phospholipid-, fatty acid-, or neutral lipid-containing (Desai and Banat. 1997; Cooper and Zajic, 1980).

P. aeruginosa produces several types of glycolipid (rhamnolipid) biosurfactants when grown on hydrocarbons or on glucose (Robert *et al.*, 1989 ; Hisatsuka *et al.*, 1971; Guerra-Santos *et al.*, 1986: Itoh and Suzuki, 1972) these include: monorhamnolipid acid, monorhamnolipid methyl ester, dirhamnolipid acid, dirhamnolipid methyl ester and

rhamnolipids with alternative fatty acids (Ochsner *et al.*, 1995). The group of Ochsner *et al.* (1994) have studied the genetics of rhamnolipid synthesis in *P. aeruginosa*. The *rhlABR* gene cluster was found to be responsible for the synthesis of RhlR regulatory protein and a rhamnosyltransferase, both necessary for rhamnolipid synthesis. Zhang and Miller (1992) demonstrated that the biodegradation of a hydrocarbon (octadecane) was enhanced by the addition of rhamnolipid. Similar glycolipids are seen with *Pseudomonas* sp. DSM 2874 (Syldatk *et al.*, 1985), *Aerobacter aerogenes*, *P. fluorescens* and *E. coli* (Shaw, 1970). Other carbohydrate-containing surfactants described in the literature are trehalose lipids (Li *et al*, 1984), sophorose lipids (Hommel *et al.*, 1994), diglycosyldiglycerides (Shaw, 1970, 1974), and polysaccharide-lipid complexes (Kapplei and Fiechter, 1976, 1977).

Lipopeptides, amino-containing biosurfactants, have been isolated from many bacteria and yeasts. Subtilysin (or surfactin), a well-known cyclic lipopeptide and a very effective biosurfactant, is produced by *Bacillus subtilis* ATCC 21332 (Arima *et al.*, 1968). *Serratia marcescens* NS 38 (Mutsuyama *et al.*, 1985), *Corynebacterium lepus* (Cooper *et al.*, 1978) as well as *Streptomyces canus* (Heineman *et al*, 1953) are among many microorganisms known to produce lipopeptides. Wilkinson (1972) isolated a a single amino acid (ornithine)-containing lipid with emulsifying capabilities from *Pseudomonas rubescens*.

Fatty acids, neutral lipids, and phospholipids excreted into the medium have been observed to lower the surface tension (Desai and Banat, 1997; Zajic and Seffens, 1984). Release of phospholipid by some *Aspergillus* spp. (Kappeli and Finnerty, 1979), *P. aeruginosa* 44TI (Robert *et al.*, 1989), *Thiobacillus thiooxidans* (Schaeffer and Umbreit, 1963) and *Rhodococcus erythropolis* (Kretschener *et al.*, 1982) into culture media during

growth on alkanes have been observed to lower the surface tension of the culture broth.

2.2.2. Cellular barriers of hydrocarbon-degrading microorganisms

Both eukaryotes and prokaryotes degrade petroleum. The physical barrier of prokaryotes (cell envelope) to petroleum will be discussed.

As the majority of hydrocarbon-metabolizing enzymes are found within the cell, the petroleum hydrocarbon must enter the cell to be metabolized. The cell wall and/or the hydrophilic components of the outer membrane (Nikaido and Vaara, 1985), however, impede direct contact between the hydrocarbon and the hydrophobic part of the cell membranes (Fig. 1). The envelope components varies not only with the organism but also through physiological adaptations to environmental conditions under which the organisms are grown (Rose, 1989; Russel, 1989).

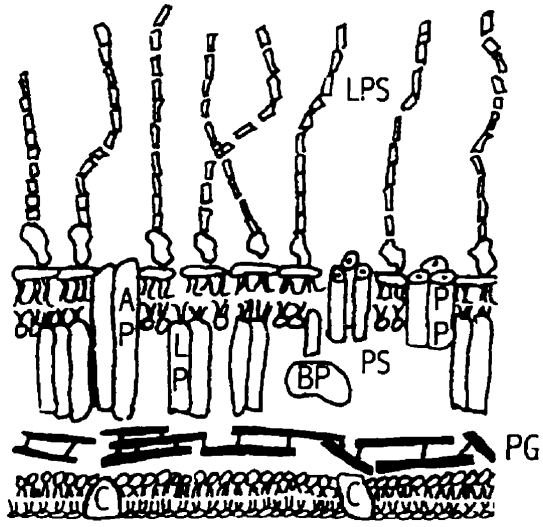
2.2.2.1. Outer membrane

The outer membrane of Gram-negative bacteria functions as a molecular filter, excluding molecules with a molecular mass of greater than 600 to 1000 Da (Decad and Nikaido, 1976; Nakae and Nikaido, 1975). Intrinsic selective properties of the outer membrane are due to the action of porin proteins (Nakae, 1976). The outer membrane of *P. aeruginosa* has very special sieving properties which make this microorganism in comparison to other microorganisms very resistant to most antibiotics (Yoshimura and Nikaido, 1982; Angus *et al.*, 1982). The outer monolayer of the outer membrane contains lipopolysaccharide (LPS) as its major lipid while the inner leaflet contains phospholipids

Figure 1. Schematic presentation of the cell envelope of gram-negative bacteria. PP, porin; C, cytoplamic membrane-embedded protein; BP, binding protein; PS, periplasmic space; A, outer membrane protein; LP, lipoprotein; PG, peptidoglycan (Adapted from Sikkema et al., 1995).

Outer Membrane

Cytoplasmic Membrane



(mostly phosphatidylethanolamine and small amounts of phosphatidylglycerol and cardiolipin) (Lugtenberg and van Alphen, 1983; Nikaido and Nakae, 1979). The outer membrane also contains, excluding the porins, a small fraction of proteins given the designation "major" proteins (Benz and Bauer, 1988). These "major" proteins are present in high copy numbers in the cell and have a role in the structure and stability of the outer membrane (Lugtenberg and van Alphen, 1983; Nikaido and Nakae, 1979). The outer membrane proteins are situated in both surfaces of the membrane with some of them spanning the entire width of the membrane (Nikaido and Nakae, 1979).

Despite the presence of porins which have a low affinity for hydrophobic compounds, studies done by researchers have shown some permeability of these compounds into the cell (Nikaido *et al.*, 1983; Nikaido and Vaara, 1985). This low level of permeability has been linked to changes in the lipopolysaccharide (LPS) in the outer membrane. Plésiat and Nikaido (1992) have also reported that some steroid probes could pass through the outer membrane with ease in *P. testosteroni*, *P. acidovorans* and *Acinetobacter calcoaceticus*. The outer membrane of some *Brucella* strains has been reported to be an ineffective barrier against hydrophobic compounds (Martinez de Tejada and Moriyón, 1993).

2.2.2.1.1. Lipopolysaccharide (LPS)

The lipopolysaccharide (LPS) is a unique constituent of the outer membrane. The molecule of LPS is amphipathic. The lipid A, also known as endotoxin, comprises the biologically active hydrophobic region of LPS. The O- specific chain, which comprises part of the hydrophilic region in the LPS, consists of repeating units of oligosaccharides which

exhibit a strain-specific structural diversity (Mayer *et al.*, 1985). This is the outer-most region of the LPS that is exposed to the environment and may play a significant role in the entry of components into the cell.

2.2.2.1.2. Outer membrane proteins

A large proportion of the membrane proteins are thought to be solely located in the outer membrane; however, some proteins are found in both the outer and cytoplasmic membranes (Nikaido and Vaara, 1985). Osborn *et al.*(1972) reported that approximately half the mass of the outer membrane in a *Salmonella* species consisted of protein. Extensive studies on the membrane proteins have been performed by investigators on *Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas* spp. Studies done by researchers on the outer membrane proteins (Opr) of *P. aeruginosa* have identified several proteins, these include: Opr I , Opr L, Opr H, Opr B, Opr P, Opr D, Opr G, Opr C, Opr F, and some minor proteins such as iron-repressible outer membrane proteins (IROMP), esterase and pilin (Siehnel *et al.*, 1990; Hancock *et al.*, 1990; Yoshira and Nakae, 1989; Chamberland *et al.*,1989; Kroprinski *et al.*, 1987; Woodruff *et al.*, 1986; Nikaido and Hancock, 1986; Angus and Hancock, 1983; Watts *et al.*, 1983; Angus *et al.*, 1982; Hancock *et al.*, 1981; Mizuno and Kageyama, 1979a; Mizuno and Kageyama, 1979b; Hancock and Carey, 1979; Inouye *et al.*, 1972). Some of these proteins may play a role in the selective entry of hydrophobic components into the cell.

2.2.3. Cytoplasmic (inner) membrane

Cytoplasmic membranes consist of a phospholipid bilayer in which enzymes and transport proteins are embedded. The cytoplasmic (plasma) membrane (CM) is a multifunctional structure in that it has a participatory role in energy generation, biosynthesis, transport and secretion as well as acting as a relatively impermeable barrier (Sikkema *et al.*, 1995). The fluidity of the cytoplasmic membrane is monitored and adjusted to any external stimuli by altering the fatty acid species present (Sikkema *et al.*, 1995; Sinewsky, 1974). Cytoplasmic membranes are relatively impermeable to polar and charged molecules. Non-polar compounds such as hydrocarbons can easily enter the lipid bilayer, most likely by passive diffusion. Bateman *et al.* (1986) demonstrated that the uptake of naphthalene by a *Pseudomonas* species did not require an energized membrane nor ATP. Lieb and Stein (1986) showed that the size and the hydrophobicity of the solute determined its permeability into the cytoplasmic membrane. The CM is the final destination for the hydrocarbons. Catabolic processes within the CM will then act upon the hydrocarbons, and convert these substrates into energy and cellular components.

2.2.3.1. Microbial petroleum degradative mechanisms

Since there are so many different compounds, from the simple to the more complex, to discuss all the probable degradative mechanisms would be overwhelming. Therefore this section will discuss the mechanisms of only some of the less complex hydrocarbons. To date, there are still many unidentified compounds within petroleum. In gaining a better understanding of the nature of the degradation of the less complex alkane, aliphatic, and

aromatic hydrocarbons, the knowledge obtained can be used to explain the probable biodegradative pathways of the more complex hydrocarbons.

A large number of studies have indicated that the catabolic genes involved in alkane and aromatic oxidation are plasmid encoded (Takizawa *et al.*, 1994; Tam and Fong, 1993; Irie *et al.*, 1984; Winstanley *et al.*, 1987; Tsuda and Iino, 1987; You et al., 1988; Chakrabarty *et al.*, 1973), however research into the genetics of the alicyclic oxidation system is limited (Perry, 1984). Not much is known about whether the alicyclic oxidation enzymes are encoded on a plasmid or a chromosome.

2.2.3.1.1. Microbial alkane, alicyclic and aromatic oxidation

The *n*-alkanes are generally the components within a petroleum mixture that are the most readily degraded (Oudot *et al.*,1989; Kennicutt, 1988; Perry, 1984). Biodegradation of both short- and long-chain alkanes proceeds by a monoterminal attack by which the alkanes get oxidized to the corresponding alcohol, aldehyde and monocarboxylic acid (fatty acid). The initial oxidation of *n*-alkanes to *n*-alkanols is carried out by the alkane hydroxylase system, a three-protein component system consisting of ω-hydroxylase, rubredoxin and rubredoxin reductase. Alkane hydroxylase, or ω-hydroxylase, is an integral cytoplasmic membrane monooxygenase (van Beilen *et al.*, 1994). Rubredoxin and rubredoxin reductase are localized in the cytoplasm (Benson *et al.*, 1977). The role of rubredoxin reductase during alkane oxidation is to deliver reducing equivalents supplied by NADH to the membrane hydroxylase via the electron carrier rubredoxin. The monocarboxylic acid derived from the successive oxidation steps, is then primed to undergo further oxidations via β-oxidation

resulting in the formation of acetate (even-numbered alkanes) (Herringa *et al.*, 1961) or propionate (odd-numbered alkanes). The acetate (acetyl-CoA) then enters the Kreb's cycle, generating four carbon units and energy (via the glyoxylate bypass). The glyoxylate bypass is induced in alkane-growing cells (Singer and Finnerty, 1984). The preferred route of attack on the alkane is at the terminal methyl group; however, in some cases both terminal methyl groups are oxidized (Atlas, 1981), and this process is referred to as diterminal oxidation or ω-oxidation (Blasig *et al.*,1988, 1989; Jurtshuk and Cardini, 1971). Some microorganisms attack alkanes subterminally, with the formation of a secondary alcohol and with further consecutive oxidations forming a ketone, an ester and, finally, a primary alcohol and a fatty acid (Rehm and Reiff, 1981; Markovetz, 1971). The oxygen is inserted within the alkane chain rather than at the termini.

Alicyclic hydrocarbons are usually more resistant to microbial degradation than alkanes of equivalent molecular weight. Due to their higher boiling point, alicyclic hydrocarbons tend to be resistant to volatilization (Perry, 1984). Unsubstituted alicyclic hydrocarbons are biodegraded by a mechanism similar to subterminal oxidation in alkanes. The cycloalkane (alicyclic hydrocarbon) is hydroxylated by a broad-acting monooxygenase to an alicyclic alcohol. Further oxidations of the hydroxyl groups result in the formation of a dicarboxylic acid that is metabolized via β-oxidation. Alkyl-substituted alicyclics can be oxidatively attacked at either of two positions, on the side-chain or on the ring. The initial site of attack on the substituted alicyclic is highly dependent on the characteristics of the compound and the type of microorganism (Feinberg *et al.*, 1980). Degradation of substituted alicyclics appears to occur more readily than the degradation of unsubstituted alicyclics

(provided that the *n*-alkane side group is of the appropriate length) (Perry, 1984).

Crude oil and the majority of refined products contain numerous aromatic hydrocarbons and heterocyclic compounds that range from monocyclic to large fused ring structures. The larger polycyclic aromatic hydrocarbons (PAH) with more than five rings are recalcitrant to biodegradation and tend to remain in the environment (Foght and Westlake, 1988). Persistence of the PAHs within the environment is mainly due to their low water solubility (Cerniglia, 1993). The recalcitrant aromatic hydrocarbons can cause potential toxic effects to higher life forms since most are chemical carcinogens (Gundlach *et al.*, 1983; Vandermeulen, 1981). Microorganisms such as bacteria, fungi, yeasts and algae are enzymatically able to metabolize aromatic hydrocarbons ranging in size from benzene to benzo(a)pyrene (Cerniglia, 1981; Gibson and Subramanian, 1984). Condensed ring structures are thought to undergo similar microbial degradation as do the simpler monoaromatic hydrocarbons (Dean-Raymond and Bartha, 1975; Gibson, 1975). Three *Pseudomonas* strains were observed to degrade PAHs (phenanthrene and anthracene) other than naphthalene (Sanseverino *et al.*, 1993). The ability of microorganisms to metabolize small aromatics (benzene, toluene, and naphthalene) is well known (Gibson and Subramanian, 1984). In bacteria initial oxidation of aromatic hydrocarbons is via an aromatic dioxygenase, which incorporates two atoms of molecular oxygen and two hydrogen atoms into the aromatic ring producing a *cis*-dihydrodiol (Gibson and Subramanian, 1984; Gibson, 1977). The multi-component dioxygenase consists of three components: a flavoprotein (ferredoxin reductase), an iron-sulphur protein, and a ferredoxin (Crutcher and Geary, 1979; Yeh *et al.*, 1977). The *cis*-dihydrodiol is further metabolized (in bacteria)

through dehydrogenation reactions to produce a catechol. The dihydroxylated aromatic rings are then cleaved by means of aromatic ring-cleavage dioxygenases (Atlas and Cerniglia, 1995; Dagley, 1971). Catechols can be oxidized through two different pathways. The "*ortho*" pathway results in cleavage at the bond between the two hydroxyl groups, yielding *cis*, *cis*-muconic acid. The *cis*, *cis*-muconic acid is then metabolized to β-ketoadipic acid which is subsequently oxidatively cleaved to form the TCA intermediates, succinic acid and acetyl-CoA. The alternate pathway, "*meta*" pathway, involves cleavage of the ring adjacent to rather than between the hydroxyl groups, yielding 2-hydroxy-*cis*, *cis*-muconic semi-aldehyde. This is further oxidized to formic acid, pyruvic acid and acetylaldehyde. Non-substituted aromatic hydrocarbons such as benzene are principally oxidized via the "*ortho*" pathway. Methyl- substituted catechols are usually oxidized via the "*meta*" pathway (Nozaki, 1970).

2.2.3.2. Membrane lipid changes associated with growth on organic solvents

Many biotransformation and bioremediation processes are impeded by the toxic effects of organic solvents on whole cells (Heipieper *et al.*, 1994). Many organic solvents partition into the cell membrane and affect the integrity and permeability of the membrane (Sikkema *et al.*, 1995). Several reports have demonstrated the resistance or tolerance of bacteria to toxic solvents (Tsubata *et al.*, 1997; Heipieper and deBont, 1994; Weber *et al.*, 1994; Keweloh *et al.*, 1991). The major adaptive responses of the bacterial cells are alterations in the composition of the membrane, specifically changes in, the fatty acid composition, phospholipid head groups, and in the protein content (Heipieper *et al.*, 1994;

Pinkart *et al.*, 1996; Chen *et al.*, 1995). Alteration in the fatty acid composition of the membrane lipids is a prerequisite for bacteria to protect them from membrane-active substances (Heipieper and de Bont, 1994). Membrane-active substances that partition in the cell membrane can cause expansion of the membrane and impairment of membrane function (Sikkema *et al.*, 1994). Alterations in *Pseudomonas putida* have been observed to directly influence the fluidity of the membrane and cell membrane hydrophobicity (Tsubata *et al.*, 1997; Weber *et al.*, 1994). Mechanisms such as increasing acyl chain length of the fatty acids, increasing synthesis of saturated fatty acids, and converting *cis* unsaturated fatty acids to *trans* isomers decrease the fluidity and increase the transition temperature of the membrane (Weber *et al.*, 1994). Chen *et al.* (1995) have shown that membrane lipids containing *trans*-unsaturated fatty acids have a higher lipid transition temperature (or lower membrane fluidity) than membrane lipids containing the *cis* isomers. Resistance of several *Pseudomonas* strains to toxic organic solvents such as, phenolic compounds (Heipieper *et al.*, 1992), toluene, styrene (Heipieper *et al.*, 1994) and small *n*-alkanes (van Beilen *et al.*, 1996) have been shown to be associated with the conversion of the *cis*-unsaturated fatty acids to the *trans* isomers. Formation of the *trans* species appears to play an important role in the growth of *Pseudomonas* strains on organic solvents (van Beilen *et al.*, 1996). Diefenbach and Keweloh (1994) have demonstrated that in a phenol-degrading *P. putida*, conversion of the *cis* species to the *trans* species was due to direct isomerization of the double bond of the lipids and not by de novo synthesis of fatty acids. Isomerization of the double bond, from the *cis* to *trans* configuration, decreases the membrane fluidity by removing the bent steric structure and allowing the fatty acids to align themselves. The *trans*

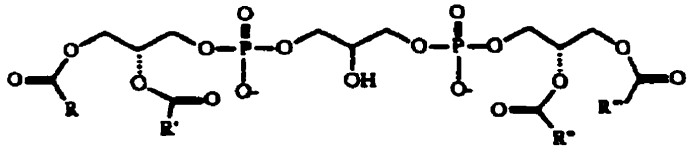
isomer has a steric configuration which is similar to that of saturated fatty acids Growth on toxic organic solvents such as xylene, toluene, and phenol results in an apparent increase in the degree of saturation of the lipids (Heipieper and de Bont, 1994; Pinkart *et al.*, 1996; Heipieper *et al.*, 1992). Saturated fatty acids are known to increase the transition temperature of the membrane (decrease fluidity) by an increase in lipid ordering and packing in the membrane (Weber *et al.*, 1994). Increased lipid ordering opposes the partitioning of lipophilic compounds into the membrane of the cell (Atunes-Madiera and Madiera, 1989). Growth on octane by *P. oleovorans* was demonstrated by Chen *et al.* (1995) to alter the membrane phospholipid fatty acid composition and this was due to the induction of *alkB* (on the OCT plasmid) and synthesis of octanol. Plasmid-cured variants of *P. oleovorans* did not undergo any fatty acid changes during growth in the presence and absence of octane, implying involvement of the OCT plasmid-encoded functions. When the *alk* system of *P. oleovorans* is expressed in *E. coli* W3110, significant changes in phospholipid metabolism of the host is seen (Nieboer *et al.*, 1996). Expression of AlkB was shown to enhance net phospholipid synthesis and change the composition of the phospholipid head groups in the membrane and the fatty acid composition of the membrane (Nieboer *et al.*, 1996; Nieboer *et al.*, 1993). Growth on alkanes and the subsequent induction and expression of the genes in the *alk* system (oxidative enzymes) results in changes in the lipids of the membrane; modification of the membrane composition is thought to facilitate the insertion and function of membrane-bound Alk proteins (Chen *et al.*, 1995).

2.2.3.2.1. Phospholipids

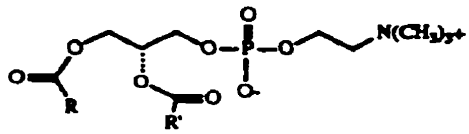
The total phospholipids in Gram-negative bacteria are distributed within the cell envelope. The phospholipids can be classified into two groups based upon the characteristics of the side groups attached to phosphoric acid: acidic nitrogen-free phospholipids and alkaline nitrogen-containing phospholipids (Váczi, 1973). The most common acidic nitrogen-free phospholipids in bacteria consist of phosphatidic acid (PA), phosphatidylglycerol (PG), cardiolipin (diphospatidylglycerol, DPG), and phosphatidylinositol (PI). Phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) (lecithin) are the most common alkaline nitrogen-containing phospholipids (Fig. 2). In most bacteria, phospholipids may represent 70 to 90 % of the total lipid content of the cell (Váczi, 1973; Hancock and Meadows, 1969). Qualitative and quantitative differences exist between the phospholipids of various bacterial species as well as among different strains. Ames (1968) reported that the phospholipid content in *Salmonella typhimurium* was higher than the 5% content present in *Escherichia coli*. Approximately 60 % of the total phospholipid in *E. coli* is present within the outer membrane, and the remaining 40 % is found in the cytoplasmic (inner) membrane (Finnerty and Makula, 1975; Silbert, 1975). In a *P. aeruginosa* strain, the phospholipid content differed from *E. coli* in that the phospholipid was equally distributed between the inner and outer membranes (Sinha and Gaby, 1964). Sinha and Gaby (1964) reported that the phospholipid content in a *P. aeruginosa* strain was 90 to 95 % PE, 3 % PC and trace amounts of PS. In *E. coli* several studies have shown that the phospholipid composition is: 70 to 80 % PE, 5 to 15 % PG, 5 to 15 % C, and approximately 1 % PA (Ames, 1968;

Figure 2. Different classes of phospholipids. Fatty acyl chains are represented by R and R' (adapted from Matreya Inc., 1995).

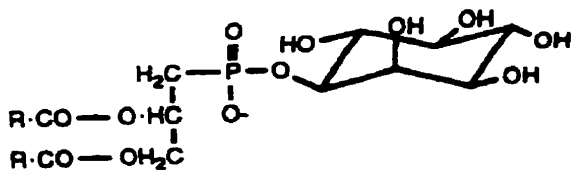
Cardiolipin (Diphosphatidyl Glycerol) **(C)**



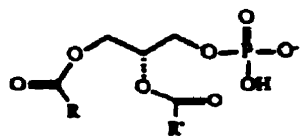
Phosphatidyl Choline (PC)



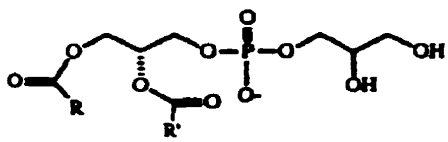
Phosphatidyl Inositol (PI)



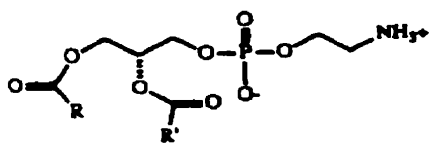
Phosphatidic Acid (PA)



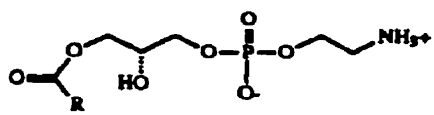
Phosphatidyl Glycerol (PG)



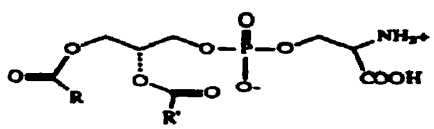
Phosphatidyl Ethanolamine (PE)



Lyso-Phosphatidyl Ethanolamine (*l*-PE)



Phosphatidyl Serine (PS)



DeSiervo, 1969). Differences in the phospholipid species have also been observed between the membranes of the cell envelope (Osborn *et al.*, 1972; Lugtenberg and Peters, 1976; Rottem *et al.*, 1975; Diedrich and Cota-Robles, 1974). Phospholipid content and fatty acid composition have been documented to vary depending on growth conditions (DeSiervo, 1969; Sinensky, 1972). Changes in temperature, age of culture and the composition of the growth medium are known factors (Lugtenberg and Peters, 1969).

The synthesis of phospholipds in bacteria starts with a two-step acylation of glycerol-3-phosphate to give rise to PA and mono-acylglycerol-3-phosphate (Zajic and Mahomedy, 1984; Gottshalk, 1979). Phosphatidic acid is esterfied with an alcohol and CTP to produce CDP-diacyglyceride. Displacement of CMP by alcohols, like serine, inositol, and glycerol, through the action of specific enzymes on CDP-diacylglyceride, produce PE, PI, and PG (Gottshalk, 1979). Decarboxylation reactions on PS yield PE which can then be further methylated to produce PC. Neutral lipids can be formed through the hydrolytic removal of phosphate from PA and consequent reaction with a third acyl carrier protein (ACP) (Gottshalk, 1979).

2.2.3.2.2. Fatty acids

As previously mentioned, environmental and physiological factors (eg. temperature, pH, etc.) as well as the genetic makeup of the cell, determine the type and characteristics of the lipid by influencing the metabolism and synthesis of fatty acids, and hence the biological properties of the cell. Classical experimental research on fatty acid synthesis was done on *E. coli* and yeast in the 1950's and 1960's (Zajic and Mahomedy, 1984). This research clearly

defined the separate steps by which two-carbon fragments are added from malonyl-CoA to a primer molecule until a fatty acid of the required length was achieved. Fatty acid synthesis consists of successive cycles of condensation of activated acceptor (ACP) with malonyl-CoA, reduction, dehydration, and an additional reduction until the required chain length is required. The fatty acids in most bacteria can be divided into five groups: saturated fatty acids with straight carbon chains; fatty acids with unsaturated carbon chains; fatty acids with branched carbon chains; fatty acids with cyclopropane rings; and hydroxy fatty acids (Váczi, 1973).

Saturated fatty acids in bacteria generally range between ten and twenty carbons, hexadecanoic acid ($C_{16:0}$) being the most prevalent fatty acid species (Shaw, 1974). The function of the saturated fatty acids is mostly structural (Váczi, 1973). Branched fatty acids are isolated from Gram-positive bacteria, though they have been found in Gram-negative bacteria as well (Kaneda, 1977). Branched fatty acids generally play a structural role and are important in the maintenance of the membrane fluidity, since they render the membrane more flexible (Váczi, 1973). The unsaturated fatty acids play a significant role in membrane fluidity. The majority of unsaturated fatty acids are in the *cis* configuration. Biosynthesis of mono-unsaturated fatty acids can occur via two pathways, one aerobic and the other anaerobic (Zajic and Mahomedy, 1984). The aerobic synthesis of mono-unsaturated fatty acids, which is associated with some members of the bacterial orders *Pseudomonodales* and *Eubacteriales*, occurs through removal of water from a preformed acyl chain by a special dehydratase (Zajic and Mahomedy, 1984; Gottshalk, 1979). The anaerobic pathway produces mono-unsaturated fatty acids through dehydration and double bond isomerization, however,

this does not occur until the ten carbon level is reached (Gottshalk, 1979). Biosynthesis of *trans*-unsaturated fatty acid is through the action of a membrane-bound isomerase on a *cis*-unsaturated fatty acid (Heipieper et al., 1992). The biological importance of the hydroxy fatty acids is the least known. They may serve as energy supplies; however, they have a structural role in being components of glycolipids and lipopolysaccharides. Cyclopropane fatty acids (CFA) are found in the phospholipids of many bacteria (Golfine, 1972; Law, 1971). Despite the widespread occurrence of CFAs, it still remains a mystery as to what their physiological role is in bacteria. One proposed role for cyclopropanes is to prevent the peroxidation of unsaturated fatty acids (Jungkind and Wood, 1974). Oxygen limitation has also been observed to promote CFA accumulation in *E. coli* and *P. fluorescens* (Cullen *et al.*, 1971). In *P. aeruginosa* the reverse occurs: low oxygen reduces cyclopropane fatty acid accumulation (Hancock and Meadow, 1969). Another possible function proposed for CFA may be to stabilize the phospholipid molecule by preventing degradation of the fatty acyl chain at a time when resynthesis would be difficult due to a sluggish metabolism (stationary-phase growth) (Cronan, 1968). The major CFAs in bacteria are *cis*-9-10-methylene hexadecanoic acid, cis-9-10-methylene octadecanoic acid (sterulic acid) and cis-11-12-methylene octadecanoic acid (lactobacilic acid). The CFAs are formed by the transfer of a methylene group from *S*-adenosyl-*L*-methionine to the *cis* double bonds of the unsaturated fatty acid chains of membrane phospholipids forming a *cis* cyclopropane ring (Law, 1971). The enzyme CFA synthetase was responsible for catalysing the membrane modification. This soluble enzyme was found in the cytoplasm loosely associated with the cytoplasmic membrane in *E. coli* (Wang *et al.*, 1992; Taylor and Cronan, 1979). Phospholipids are

necessary for activity, suggesting that the CFA synthetase must have an active site within the hydrophobic core of the membrane. The level of the enzyme varies little from log phase to stationary phase, that is, synthesis of the enzyme occurs throughout the growth cycle (Law, 1971). Activity of the enzyme, however, increases from log to stationary phase (Wang and Cronan, 1994). The gene encoding CFA synthetase (*cfa*) was sequenced and cloned (Grogan and Cronan, 1984; Wang *et al.*, 1992). Wang and Cronan (1994) reported that the gene encoding CFA synthetase (*cfa*) was transcribed from two promoters. One promoter was found to be active throughout the growth cycle while the second only became active during late-log to stationary phase. The activation of the second promoter increases the level of CFA synthetase, augmenting CFA production.

3. 0. MATERIALS AND METHODS

3. 1. ORGANISM AND GROWTH CONDITIONS

Pseudomonas aeruginosa ATCC 9027 was grown on a chemically defined medium (CDM) (Miguez *et al.*, 1986). The CDM contained 2.3 mM K_2HPO_4, 20 mM $MgSO_4$, 30 mM KCl, 50 mM Tris-HCl, 25 mM NH_4NO_3, 0.02 mM $Fe(NH_4)_2(SO_4)\cdot H_2O$, and 10 mM $CaCl_2\cdot 2H_2O$. The pH of the medium was adjusted to 7.5 with 6N KOH. Stock cultures of *P. aeruginosa* ATCC 9027 were maintained at 30° C on slants containing the CDM, 2% (wt/vol) agar (Difco Laboratories, Detroit, Mich.) and a carbon energy source (either hexadecane, kerosene, furnace oil, aviation fuel, light crude oil or glucose).

Starter cultures were grown in 100 ml of CDM containing 1% (wt/vol) glucose or 1% (vol/vol) petroleum hydrocarbon (furnace oil, hexadecane, aviation fuel and kerosene) in 500-ml flasks with shaking (250 rev·min^{-1}) at 30° C. In all experiments the ratio of the medium:volume of the Erlenmyer flask was 1:5. Large batch cultures were grown in 10 l. fermentation vessels with a magnetic stir bar, and constant filtered air supply passed through plastic tubing of .3175 cm I.D. (83 kPa) at 30° C. Growth was measured as an increase in optical density at 660 nm with a Beckman DU-70 spectrophotometer (path length (l) = 1 cm). To minimize possible interference by micro-emulsions, culture samples were first kept at rest (1 min) to allow any emulsion to rise to the top and then OD readings were taken below the oil-water interface. Glass cuvettes were pre-soaked in chromic acid for approximately 4 h (in order to remove interfering contaminants) and then rinsed several times. In all experiments, early-log petroleum-grown cells were harvested at day 4, and late-

log cells at day 7 of the growth cycle. Early- and late-log glucose-grown cells were harvested after 6 h and 16 h respectively.

3. 2. CHEMICALS

Hydrocarbons consisted of *n*- hexadecane (Sigma Chemical Co., St. Louis, MO), furnace oil (Petro Canada Ltd., Montreal, QC) kerosene, Canadian light crude, Iranian light crude, and aviation fuel (Shell Canada, Montreal, QC), hexane, heptane and octane (Fisher Scientific Co., Pittsburgh, PA). All chemicals used were of reagent-grade quality or better.

Glycine, TEMED (N,N,N,N'-methylethylenediamine), acrylamide, low molecular weight protein standards, bis (N,N'-methylenebisacrylamide), bromophenol blue, Coomassie Blue and β-mercaptoethanol were obtained from Bio-Rad Laboratories, Richmond, CA. BSA (bovine serum albumin), DNase, RNase and lysozyme were obtained from Sigma Chemical Co., St. Louis, MO. Acetic acid, methanol, Hepes, Tris-HCl ([hydroxymethyl] aminomethane-hydrochloride) and ultra pure sucrose were from Fisher Scientific Co., Pittsburgh, PA. Solvents for thin layer chromatography, including: glass-distilled chloroform, methanol, *n*-hexane and acetone were purchased from Caledon Labs Ltd., Georgetown, Ont.

The bacterial fatty acid methyl esters mix (10 $mg \cdot ml^{-1}$) was obtained from Matreya, Inc., Pleasant Gap, PA. The fatty acid and phospholipid standards were obtained from Sigma Chemical Co., St. Louis, MO.

Thin layer chromatography plates used were plastic Polygram SIL G /UV, with 0.25 mm silica gel layer (Macherey-Nagel, Straße, Germany).

3.3. ANALYTICAL METHODS

3.3.1. Extraction of readily-extractable lipids from whole cells

Three 100-ml cultures of cells grown on glucose or petroleum hydrocarbons were centrifuged (12 000 x g for 20 min) and washed three times with 0.30 mM Tris-HCl buffer (pH 8.0).

The free and loosely bound lipids of pelleted cells were extracted by a modification of the technique of Folch *et al.* (1953). Three ml of chloroform/methanol/acetic acid (50:100:5, vol/vol) were added to the pellet. After vortexing, the suspension was sonicated for 10 min (Branson 1200 Sonicator, Branson Co., Shelton, CT). The cell suspension was then centrifuged for 3 min at high speed in a clinical centrifuge and the supernatant collected. Residual lipids in the pellet were extracted as above with 1 ml of solvent. The extracts were then combined, and 1 ml of 1 M KCl and 1 ml of chloroform added. The mixture was vortexed and centrifuged for 2 min at high speed in a clinical centrifuge to separate phases. The upper aqueous phase was discarded. Chloroform/methanol/water (1.5 ml, 3:48:47, vol/vol) was added to the remaining chloroform-lipid mixture. This mixture was vortexed and centrifuged and the upper phase removed as before. The final chloroform-lipid phase within the tubes was then dried under a constant stream of nitrogen with mild heating (approximately 35° C). The lipid residue was redissolved in 1 ml of chloroform, sealed under N_2 in Teflon-lined screw-capped test tubes. Triplicate samples were collected and placed in a freezer (-22° C) for storage.

3.3.2. Lipid separation and fatty acid analysis

3.3.2.1. Thin-layer chromatography

The components of lipid extracts were separated by thin-layer chromatography (TLC) using a double solvent system according to the method of Sparace and Mudd (1982), except that the second solvent system consisted of chloroform/methanol/water (65:25:4, vol/vol). The first solvent mixture, acetone/acetic acid/water (100:2:1, vol/vol), was used to resolve the more neutral lipids towards the top of the TLC plates. The more polar lipids remaining at the origin were resolved on the lower two-thirds of the plate by the second solvent system.

The lipid samples were applied to dried TLC plates (previously run with the first solvent system) as 1.5-cm bands 1 cm apart. Approximately 30 μg of lipid standards (section 3.3.2.3) were applied to separate lanes for identification purposes. The TLC plates were then allowed to dry for 20-30 min. Dried TLC plates with standards and lipid samples were developed in glass tanks containing the first solvent mixture (acetone/acetic acid/water). The solvent was allowed to run to the top of the TLC plate (approximately 1 h). The plate was then removed and allowed to dry in a separate tank for about 2 h under flowing N_2. The dried TLC plate was then placed in a tank containing the second solvent mixture (chloroform /methanol/water). The solvent mixture was allowed to run two-thirds of the way from the bottom (approximately 45 min). The TLC plates were removed and dried for 2 h under flowing N_2. The dried plates were cut in order to separate lanes containing standards

from those containing lipid samples. Lipid standards were visualized with iodine vapour. After visualization, standard lanes were re-aligned with sample lanes, and zones corresponding to each standard were marked in pencil. The sample zones on the silica plates were scraped off with a razor blade and placed in Teflon-lined screw-capped test tubes containing 1 ml of chloroform. Fatty acids of individual lipids were trans-esterified (section 3.3.2.2), identified and quantified by gas-liquid chromatography (section 3.3.2.4).

3.3.2.2. Preparation of fatty acid methyl esters

Methyl esters of fatty acids were prepared according to the method of Morrison and Smith (1964). Fifty μg of methylpentadecanoate (C15:0) an internal fatty acid standard, were added to the stored extracted lipid samples (with 1 ml of chloroform), and mixed. The mixture (lipid and standard) was placed under flowing N_2 to remove solvent. Two ml of commercially prepared 14% borontrifluoride (BF_3) in methanol were added to the dried lipid sample. The test tubes were firmly capped and the sample was thoroughly mixed to dissolve the lipids. Samples were then heated in a water bath (65° C) for 30 min. The test tubes were cooled on ice for approximately 5 min. To the cooled samples, 3 ml of distilled water were added. After mixing, 3.5 ml of *n*-hexane were added and the mixture vortexed to obtain an emulsion. The sample was centrifuged for 3 min at high speed in a clinical centrifuge as before, to separate the phases. The upper layer (hexane containing fatty acid methyl esters) was removed with a Pasteur pipette and transferred to a new Teflon-lined screw-capped test tube. The addition and removal of hexane was repeated twice, each time combining the second and third hexane layers to the first hexane extract. The pooled hexane extract was

dried under flowing N_2, redissolved in 1 ml of hexane, sealed under flowing N_2 and stored in the freezer (-22° C) until analysis by gas-liquid chromatography.

3.3.2.3. Lipid standards for thin-layer chromatography

Lipid standards used were phosphatidylethanolamine (PE), phosphatidylcholine (PC), *lyso*-phosphatidylethanolamine (LPE), cardiolipin [diphosphatidylglycerol] (C), phosphatidylglycerol (PG), phosphatidylserine (PS), triacylglycerol and oleic acids as neutral lipids (NL).

3.3.2.4. Gas chromatography analysis

Fatty acid methyl esters were analyzed with a Varian Model 3400 gas chromatograph equipped with a split injector, a flame ionization detector and a fused silica capillary column (Chromatographic Specialties, Inc., Brockville, Ont.) (30 m x 0.25 mm internal diameter) coated with 0.25 μ Durabond 225 as the stationary phase. Helium served as the carrier gas with a flow rate of approximately 2 ml • min^{-1}. Injector and detector temperatures were held constant at 275 and 300° C, respectively. Column temperature programming consisted of an initial temperature of 180° C (without holding) that was increased at the rate of 2° C • min^{-1} to 200° C, and finally held at that temperature for 10 min. Analysis of the petroleum hydrocarbon substrates was performed on the same column however initial temperature of the column was set to 140 ° C.

3.3.2.5. Calculation of fatty acids

The fatty acid profiles reflect the fatty acid composition. The fatty acid composition was calculated as a weight % based on the mass of individual fatty acids recovered in each lipid. Lipid composition represents mass % based upon the total weight of fatty acids recovered in each individual lipid, using C15:0 as an internal standard.

3.3.3. Protein estimation

Proteins concentration was determined using the Peterson method (1977) with BSA as the standard.

3.3.4. Separation of outer membrane proteins

Membrane proteins were extracted from early or late log phase cells as described by Hancock and Nikaido (1978). Cells (3 L) were harvested by centrifugation at 12 000 x g for 10 min at 4° C. The pellet was washed twice in one-tenth the original volume with 30 mM Tris-HCl (Tris buffer, pH 8.0). The washed pellet was resuspended in 15 ml of 20% (wt/vol) ultra-pure sucrose in Tris buffer (pH 8.0), to which 1 µg of DNase and 1 µg of RNase were added. Cells were passed twice through an ice-cold French pressure cell at 1 x 10^5 kPa (15 000 psi). Fifty µl of lysozyme were added (10 mg·ml^{-1}) to the French pressed material, and the mixture was left to incubate for 10 min at room temperature. The incubated mixture was centrifuged (3 000 x g for 10 min at 4° C) to remove whole cells and debris. The supernatant was retained, and Tris buffer (pH 8.0) was added to a final volume of 42 ml. Six ml were layered onto a sucrose (ultra-pure) density gradient containing a bottom layer of 1 ml 70%

(wt/vol) and a 5-ml top layer of 15% (wt/vol) sucrose in Tris buffer. The sample-containing gradients were then centrifuged at 100 000 x g for 60 min at 4° C in a Beckman SW 41 rotor. The bottom 2 ml of each gradient, containing membrane proteins, were removed and homogenized with a glass homogenizer and Teflon pestle (at 4° C). The homogenate was layered onto a second sucrose density gradient (containing 1 ml of 70% (wt/vol), 3 ml of 64% (wt/vol), 3 ml of 58% (wt/vol) and 3 ml of 52% (wt/vol) sucrose). The gradient was centrifuged at 100 000 x g for 16 h at 4° C in a SW 41 rotor. The individual bands at each interface were removed with a Pasteur pipette. The bottom band (containing the outer membrane) was collected, homogenized, and stored at 4° C. The homogenate was diluted with double glass distilled water and centrifuged twice (120 000 x g) for 30 min at 4° C using a Ti 55.2 rotor. The final pellet was resuspended in 2 ml of double glass distilled water and frozen at -22° C.

3.3.5. SDS-PAGE

SDS-polyacrylamide gel electrophoresis procedures were similar to those described by Laemmli (1970). The sample buffer (SDS reducing buffer) consisted of 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% (vol/vol) glycerol, 0.001% (wt/vol) bromophenol blue, 5% (vol/vol) β-mercaptoethanol and 0.1 M $MgCl_2$. The electrode buffer (running buffer) consisted of 0.025 M Tris-base buffer, 0.19 M glycine and 0.1% (wt/vol) SDS at an adjusted pH of 8.3. A two-tiered gel system was used for the electrophoresis of the early and late log-phase outer membrane proteins, a 4% (wt/vol) polyacrylamide stacking gel and a 12% (wt/vol) polyacrylamide separating gel. The gel thickness was 1.5 mm.

Bio-Rad (Bio-Rad Laboratories, Richmond, CA) low molecular weight protein standards were used for direct comparison to identify and estimate the molecular weight of the outer membrane polypeptides. Proteins present in the standard mix ranged from 94 000-14 300 Da.

Outer membrane protein samples were placed in a digestion buffer within Eppendorf tubes and heated at 100° C for 5 min in a glycerol Temp-block module heater (CANLAB). The sample was then allowed to cool to room temperature. The digested protein samples (ranging from 40-60 µg of protein) and low molecular weight standards were loaded into the electrode buffer-filled wells in the stacking gel with a 50 µl Hamilton syringe.

The samples were electrophoresed for 12-15 h at a constant 15 mA. The gel was run until the tracking dye exited the bottom of the separating gel. The circulated water within the radiator core was maintained at a temperature between 10-15° C for ideal electrophoresis. At the end of the electrophoresis run, the gels were carefully removed and fixed overnight in an acetic acid/methanol (10:30, vol/vol) solution. The following day, the fixed gel was stained with acetic acid/methanol/Coomassie blue (10:30:0.1, vol/vol) at 65° C for 1 h and de-stained in 10% acetic acid (vol/vol) overnight

3.3.6. Hydrophobicity (adherence) test

The hydrophobicity test used was a modified version of that of Rosenberg *et al.* (1980). Tests were performed at various times in the growth cycle. To rule out ionic interferences, three different wash/resuspension solutions were used, these included: a phosphate-saline buffer, pH 7.4; a 0.05 M Hepes, pH 7.4; and a minimal salts medium

(CMSM) according to Miguez *et al.* (1986).

Optical density readings at 660 nm were taken before removing cells from the growth culture. One to 200 ml of culture from a 10 L fermenter were centrifuged (13 000 x g, 4° C) and pelleted cells washed twice with one of the three wash/resuspension solutions. Washed cells were resuspended in the same buffer, optical densities recorded, and 1.2 ml added to each of the 5 replicate test tubes (10 mm I.D.). To this, 2 ml of hexadecane were added and the mixture kept at 30° C for 15 min. The tubes were stoppered, placed on a slant rack, and agitated on a reciprocal shaker (slow speed) for 2-3 min. After shaking, the tubes were held upright for 15 min for phase separation. The oil layer was discarded and the optical density (660 nm) of the aqueous layer measured.

Hydrophobicity was measured as a "decrease in the % absorbance" and calculated as follows (Rosenberg *et al.*, 1980):

$$\frac{(\text{Resuspended Cells OD} - \text{Aqueous Phase OD}) \times 100}{\text{Resuspended Cells OD}}$$

3.3.7. Surface tension analysis

Surface tension of quadruplicate samples of growth medium taken at different phases in the growth cycle were measured using the du Noüy method (1919) with a Fisher Surface Tensiometer Model 20 (Fisher Scientific Co., Pittsburgh, PA). Liquid cultures were centrifuged (12 000 x g, 15 min, 4° C) and 10 ml of the supernatants removed to separate test tubes (16 x 76 mm, Quik Seal centrifuge tubes, Beckman Instruments Inc., Palo Alto,

CA). After 10 min standing to allow any residual petroleum hydrocarbon to rise to the top 8 ml were removed for measurement. The bottom aqueous phases were carefully removed and deposited in plastic Petri plates (35 x 10 mm, Becton Dickinson and Co., Cockeysville, MD), and surface tension measured. Residual hydrocarbon was removed from the platinum-iridium ring after every trial by first immersing the ring in benzene, then immersing it in acetone and, finally, flaming the ring.

3.3.8. Exopolymer extraction

The exopolymer aggregate (oil-exopolymer-cells) on the surface of the growth medium (present at approximately day 4-5) was collected by suction. The white-beige compound was allowed to stand overnight at 4°C in a beaker. The white-beige compound was then removed on the following day and centrifuged several times using a Ti 55.2 rotor (100 000 x g , 1 h, 4° C) and a SS34 rotor (3 000 x g, 1 h, 4° C). After each spin, the cell pellet and the oil layer were removed. This procedure was repeated (approximately 10 times) until only a white layer remained. Protein, carbohydrate and lipid analyses were then performed on the exopolymer.

3.3.9. pH Measurement

The pH of the growth medium was monitored throughout the growth cycle. Following optical density readings (660 nm) of the culture medium, cells were removed by centrifugation, and the supernatant was collected. The pH of the supernatant was then measured (at room temperature) on a Corning [Model 200] pH meter

4.0. RESULTS

4.1. Growth as related to a change in energy sources

4.1.1. Growth of *P. aeruginosa* on various petroleum products and glucose

Hexadecane is found in many petroleum mixtures. Growth of *P. aeruginosa* on 1% (vol / vol) hexadecane, furnace oil, kerosene, aviation fuel, light crude oil, and small n-alkanes was monitored using both absorbance and viable plate count measurements.

In all instances where petroleum hydrocarbons were the sole carbon source, a pronounced lag period (2-3 d) in growth was observed (Figs. 3A-3C). Growth of *P. aeruginosa* on petroleum hydrocarbons required 7-8 d incubation for stationary phase to be reached. With glucose as a substrate, the stationary phase of growth was reached within one day. Maximum viable cell counts were observed when cells were grown on hexadecane (1.5×10^{11} CFU·ml^{-1}) followed by that of furnace oil (9.1×10^{10} CFU·ml^{-1}); kerosene (6.9×10^{10} CFU·ml^{-1}); aviation fuel (6.0×10^{10} CFU·ml^{-1}, Table 2). Stationary growth of glucose-grown cells yielded 1.1×10^{11} CFU·ml^{-1} (Table 2). Growth on hexadecane and glucose were similar to those observed by Miguez *et al.* (1986). *P. aeruginosa* did not grow well on *n*-alkanes (Fig. 3B). A longer lag period, slower "growth rate" and a longer time to reach stationary phase were observed with growth on the crude fractions (Fig. 3C).

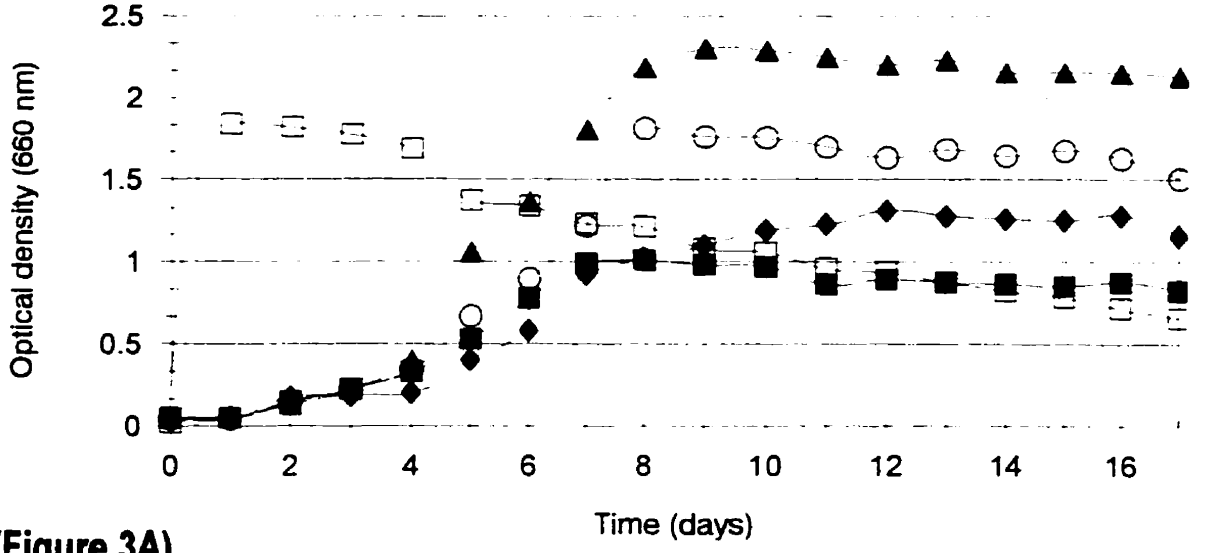
4.1.2. Hydrophobicity of cells grown on glucose and petroleum

The hydrophobicity of cells grown on petroleum changed with time (Fig. 4); that of

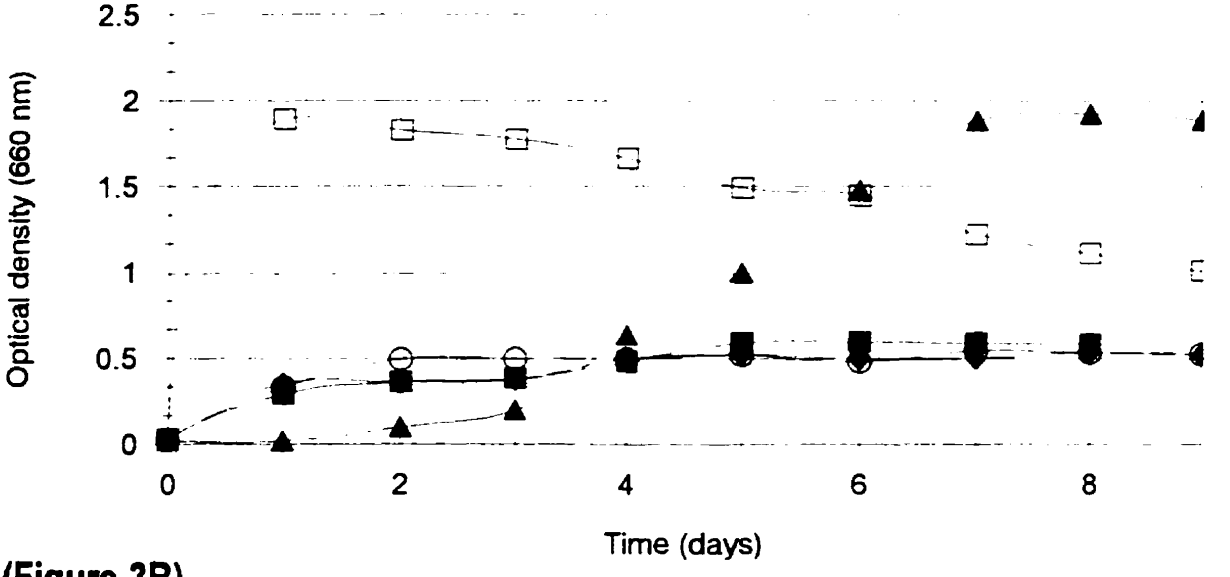
Figure 3A. Growth of *Pseudomonas aeruginosa* on various carbon sources in a minimal-salts medium (30° C, 250 rev·min^{-1}). glucose (□), hexadecane (▲), furnace oil (○), aviation fuel (♦), and kerosene (■).

Figure 3B. Growth of *Pseudomonas aeruginosa* on various carbon sources in a minimal-salts medium (30° C, 250 rev·min^{-1}). *n*-alkanes [octane (●), heptane (◆), hexane (⊖)], glucose (□) and hexadecane (▲).

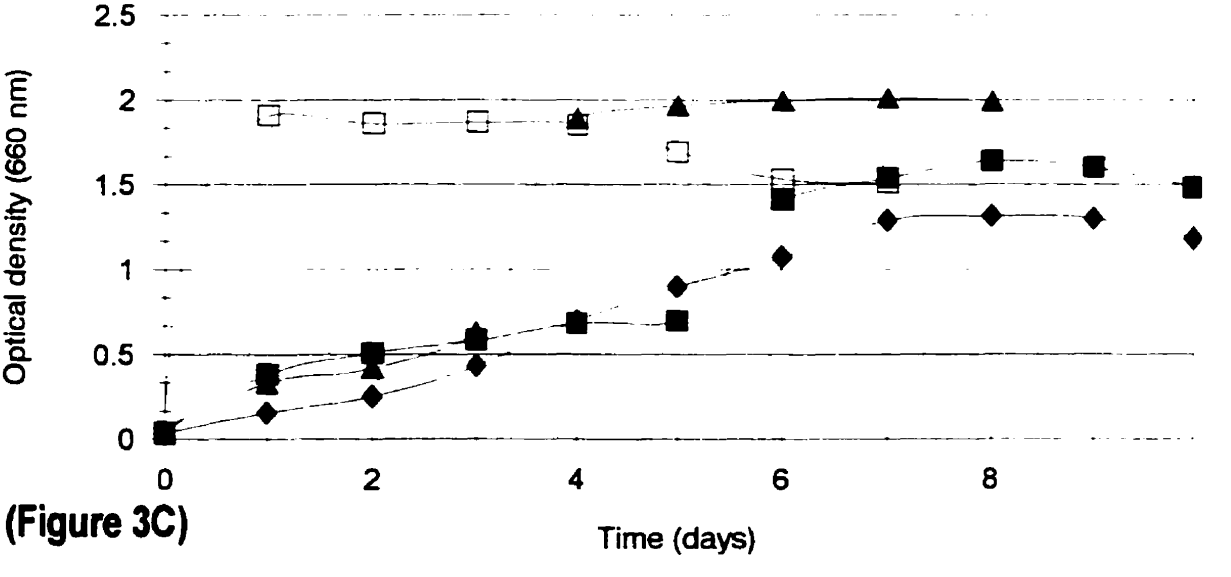
Figure 3C. Growth of *Pseudomonas aeruginosa* on various carbon sources in a minimal-salts medium (30° C, 250 rev·min^{-1}).Canadian light crude oil (■), Iranian light crude (◆), glucose (□), and hexadecane (▲).



(Figure 3A)



(Figure 3B)



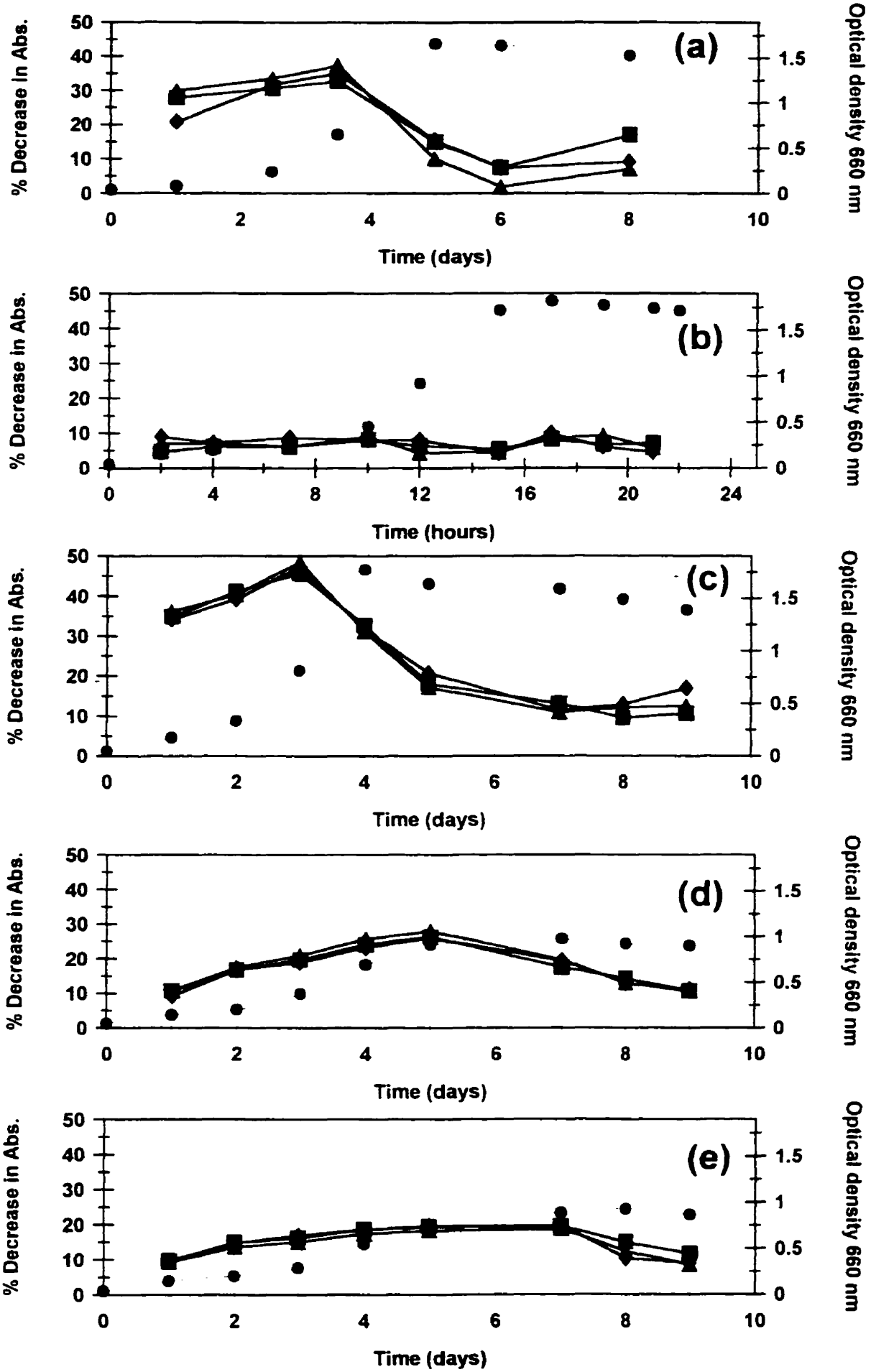
(Figure 3C)

Table 2: Viable cell count measurements of early log and late log growth of *P. aeruginosa* on various carbon sources

	Early-log		Late-log	
Carbon Source	Incubation Time	[1]CFU·ml^{-1}	Incubation Time	CFU·ml^{-1}
Hexadecane	4 days	2.0×10^{7}	7 days	1.5×10^{11}
Kerosene	4 days	1.2×10^{7}	7 days	6.9×10^{10}
Furnace Oil	4 days	1.7×10^{7}	7 days	9.1×10^{10}
Aviation Fuel	4 days	1.0×10^{7}	7 days	6.0×10^{10}
Glucose	6 hours	1.9×10^{7}	16 hours	1.1×10^{11}

[1] CFU·ml^{-1} = colony forming units·ml^{-1}

Figure 4. Measurement of hydrophobicity with time (measured as a % decrease in absorbance) of *Pseudomonas aeruginosa*: (a) hexadecane-grown cells; (b) glucose-grown cells; (c) furnace oil-grown cells; (d) kerosene-grown cells; (e) aviation fuel-grown cells. Re-suspension media consist of PBS (◆), HEPES (■) and CMSM (▲). Growth (OD_{660}) represented by (●). Plotted values are the means of 5 separate replicates.


(a)
% Decrease in Abs.
Optical density 660 nm
Time (days)
(b)
% Decrease in Abs.
Optical density 660 nm
Time (hours)
(c)
% Decrease in Abs.
Optical density 660 nm
Time (days)
(d)
% Decrease in Abs.
Optical density 660 nm
Time (days)
(e)
% Decrease in Abs.
Optical density 660 nm
Time (days)

cells grown on glucose remained constant at a low level. With hexadecane and furnace oil, cell hydrophobicity increased sharply initially and then decreased when the cells reached their mid-log phase of growth. Changing the medium in which hydrophobicity of the cells was measured (phosphate-saline buffer, 0.05 M Hepes, or minimal salts medium) had no influence.

4.1.3. Surface tension analysis

Cells grown on furnace oil (Fig. 5c) reduced the surface tension from of the minimal-salts medium (MSM) by approximately 30 dynes·cm^{-2}. Reduction in the surface tension of the MSM was also observed when cells were grown on hexadecane (23 dynes·cm^{-2}), kerosene (18 dynes·cm^{-2}), and aviation fuel (6 dynes·cm^{-2}). Cells grown on glucose (Fig. 5b) produced little or no drop in the initial surface tension of the growth medium (78 dynes·cm^{-2}). Once again, as with cell hydrophobicity changes, decreases in the medium surface tension occurred during the mid-log phase of growth.

4.1.4. Outer membrane protein changes of cells grown on petroleum and glucose

As cells grew on hexadecane and proceeded from early log to late log phase of growth, changes in outer membrane proteins (OMP) were observed (Fig. 6). The changes occurred primarily in proteins in the 29-40 kDa range. In the early log phase, OMP included the 33 and 30 kDa varieties. As the cell progresses to the late log phase of growth, the 33 kDa protein concentrations decreased, the 30 kDa protein disappeared, and new proteins appeared, viz. 34- and 29 kDa and several proteins ranging from 34-39 kDa. The OMP

Figure 5. Surface tension of media during growth of *Pseudomonas aeruginosa*: grown on (a) hexadecane (b) glucose (c) furnace oil (d) kerosene (e) aviation fuel cells. (●) Growth (OD_{660}); (◆) surface tension (dynes·cm^2)

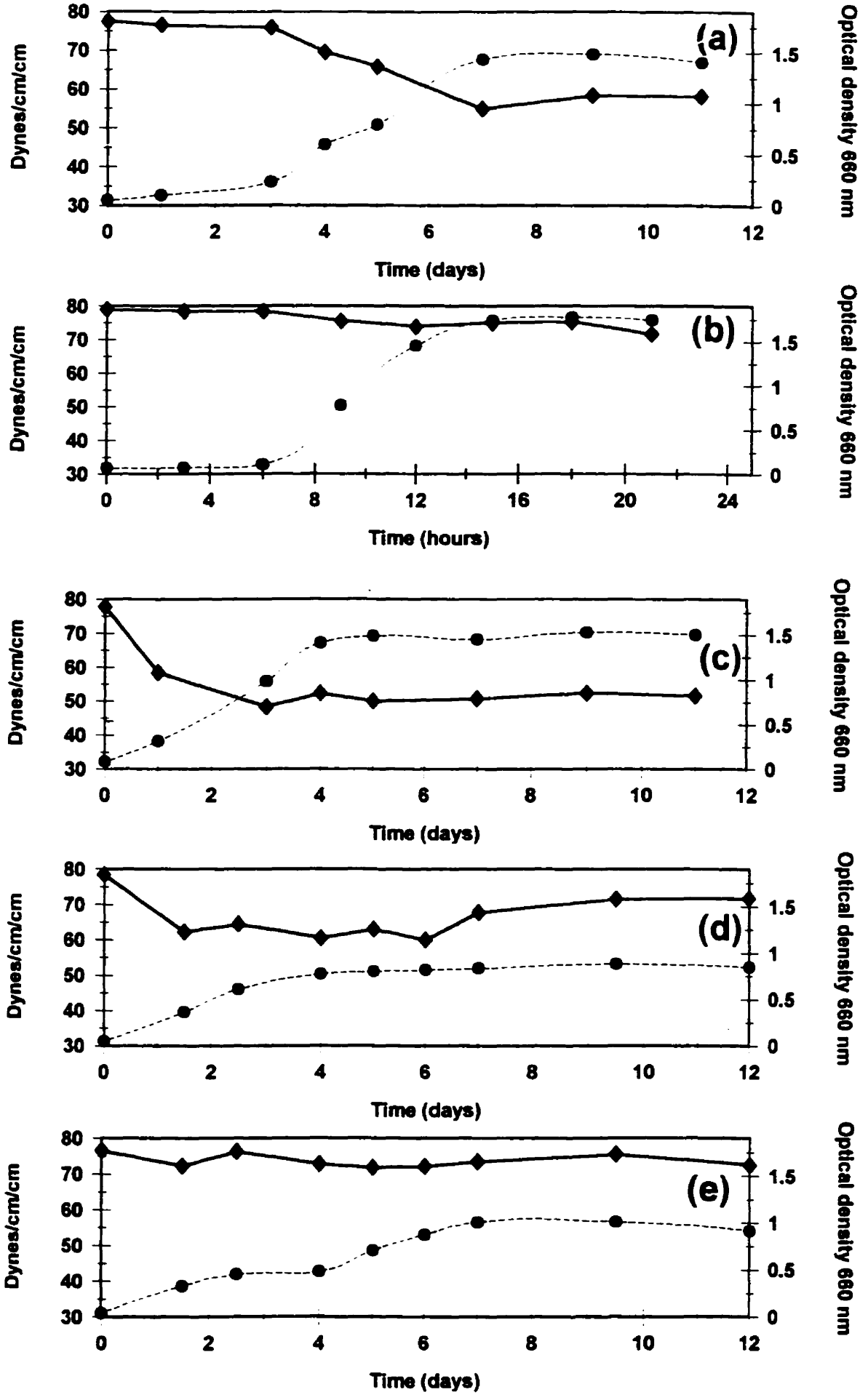
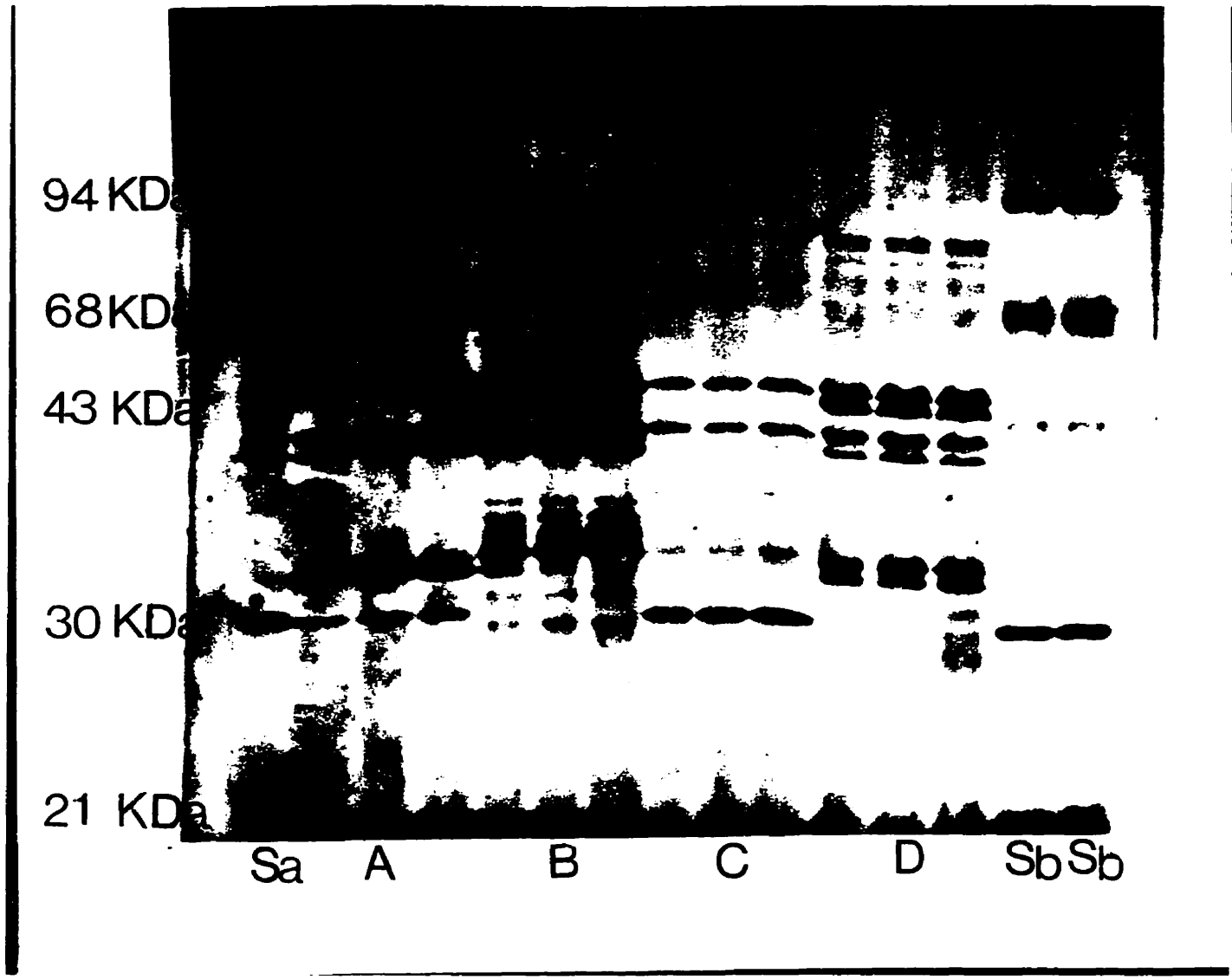

(a)
Dynes/cm/cm
Optical density 660 nm
Time (days)
(b)
Time (hours)
(c)
(d)
(e)

Figure 6. SDS-PAGE of outer membrane proteins from cells of *P. aeruginosa* grown on hexadecane: (A) early-growth phase cells; (B) late-growth phase cells; (C) trapped cells; and (D) on glucose, late-growth phase. Protein concentration in wells: 50, 60, and 70 µg. Protein standards (Sa= 10 µg, Sb= 5 µg); 94-21 kDa.


94 KDa
68 KDa
43 KDa
30 KDa
21 KDa
Sa
A
B
C
D
Sb
Sb

diversity and concentrations, with the exception of the 30 kDa protein, was lower in cells that were trapped in the exopolymer-hexadecane matrix found at the surface of the growth medium (Fig. 6, lane C). Glucose-grown cells elicited unique 44- and 32 kDa OMP (Fig. 7). Although changes were observed as cells grew older (early to late log), these changes were not as marked as those observed with hexadecane-grown cells. The increase in 37 and 34 kDa OMP as cells grew older was accompanied with a decrease in the 22 kDa OMP.

Differences were noticed in the outer membrane protein profiles among the cells grown on various petroleum hydrocarbons (Fig. 8). The OMP of glucose-grown cells showed a pronounced band at approximately 44 kDa and an absence of a 70-kDa doublet band in comparison to the OMP of cells grown on petroleum hydrocarbons. The aviation fuel outer membrane protein profile revealed pronounced 45- and 30-kDa bands which were not present in the other samples. The furnace oil and kerosene shared similar characteristics in that there was an almost complete absence of proteins in the 32-35 kDa range and in that they revealed a strong presence of 3 bands (22, 23 and 25 kDa) which were not observed in the other cell samples. The higher molecular weight proteins (> 48 kDa) varied amongst all samples. Overall, the OMP of cells grown on hexadecane, furnace oil and kerosene were quite similar, and the OMP of cells grown on either glucose or aviation fuel contained additional proteins.

Figure 7. SDS-PAGE of outer membrane proteins from cells of *P. aeruginosa* grown on glucose. E- early log phase cells; L- late log phase cells. Protein concentration in wells was 50 µg. Standard proteins are represented in lane (S).

Note: Due to age of standard some markers have degraded.

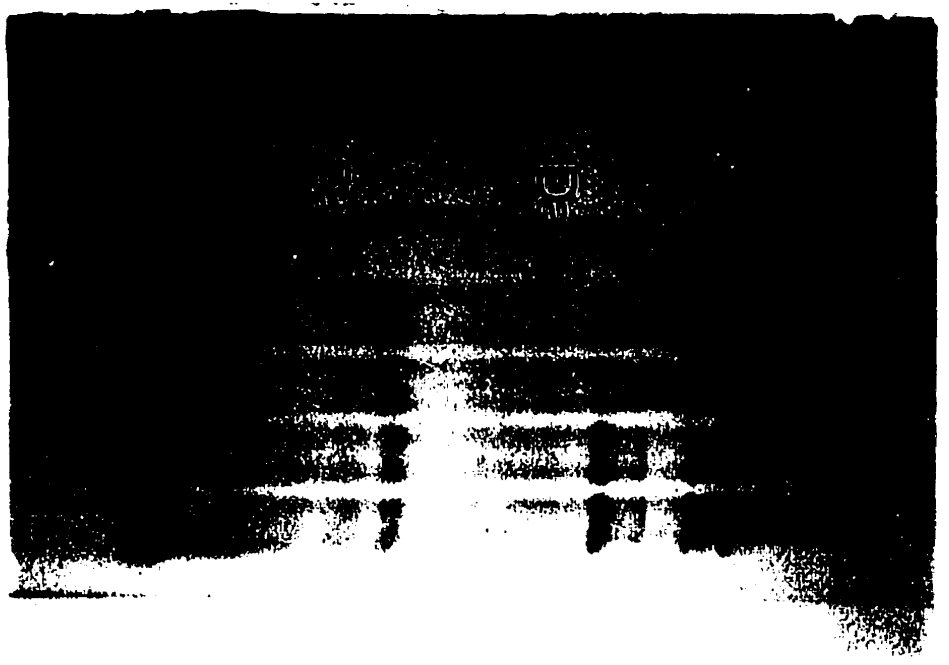
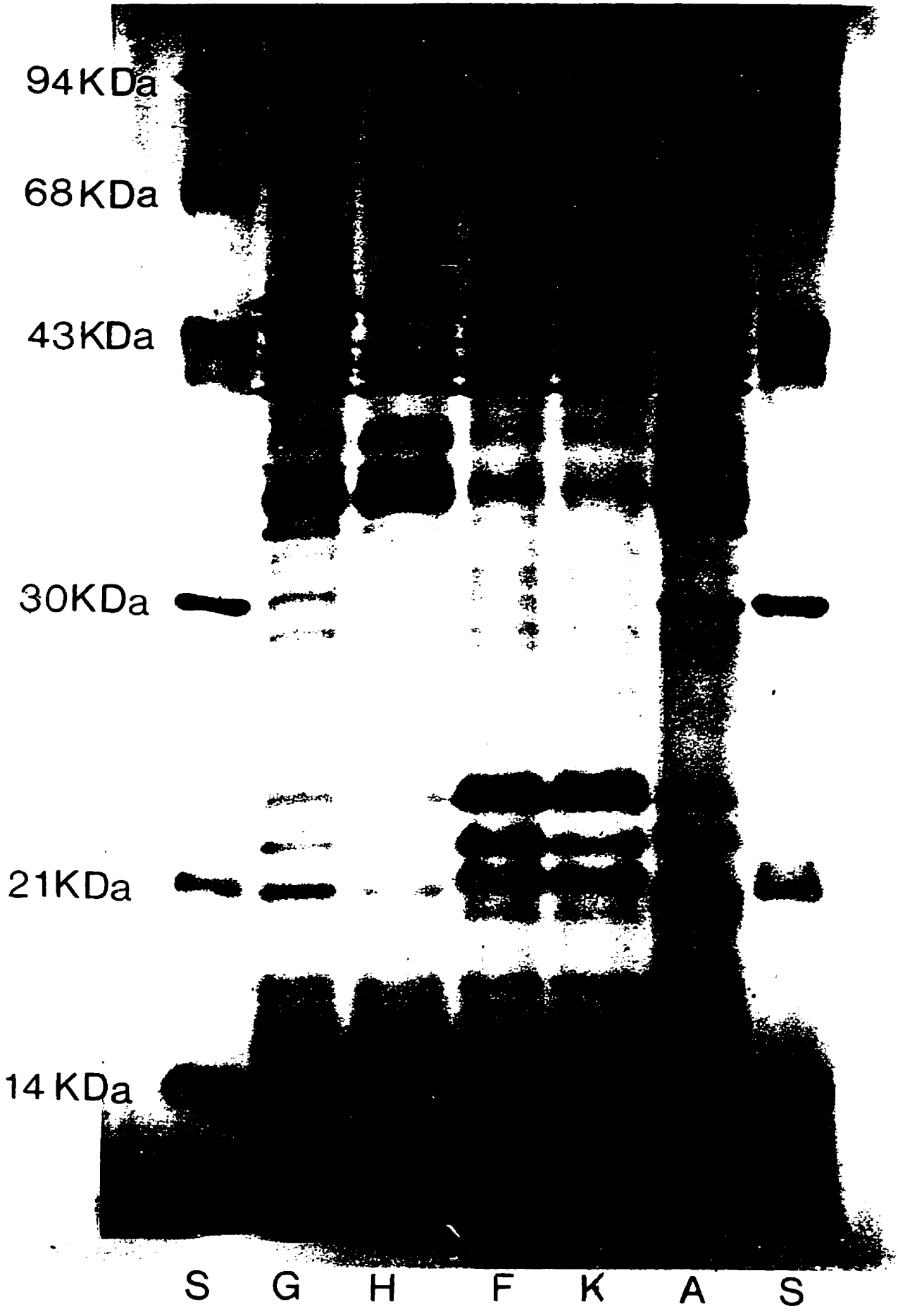


Figure 8. SDS-PAGE of outer membrane proteins from late-log cells of *P.aeruginosa* grown on five different carbon sources: (G) glucose; (H) hexadecane; (F) furnace oil; (K) kerosene; and (A) aviation fuel. Protein concentration in wells was 50 µg. Protein standards (S):94-14 kDa.


94KDa
68KDa
43KDa
30KDa
21KDa
14KDa
S
G
H
F
K
A
S

4.1.5. Cell membrane lipid changes

4.1.5.1. Identification of readily-extractable lipids by thin-layer chromatography

Separation of the readily-extractable lipids (loosely bound lipids) by thin-layer chromatography (TLC) revealed seven distinct components. Five of these components were tentatively identified (by comparison with known standards) as phosphatidylethanolamine (PE), phosphatidylcholine (PC), neutral lipids (NL), lyso-phosphatidylethanolamine (LPE) and cardiolipin (C). Two of the components were not identified and were referred to as unknown #1 (Unk #1) and unknown #2 (Unk #2). The order of separation of the individual lipids (low R_f to increasing R_f) was the following: (Unk #1), (PC), (LPE), (PE), (Unk #2), (C) and (NL).

4.1.5.1.2. Early-log phase total lipids

The cells grown on hexadecane and kerosene contained (PE) as the major lipid fraction (*ca.* 70 %); whereas, cells grown on furnace oil had PE and LPE as their major lipids (Fig. 9). Lipid composition in aviation fuel- and glucose-grown cells was similar and Unk #1 lipid was the major component.

4.1.5.1.3. Fatty acid analysis of early-log phase lipids

The total lipid fatty acid profiles (mass %) are similar among the cells grown on petroleum hydrocarbons and different from that of cells grown on glucose (Fig. 10). The degree of saturation of the lipids in cells grown on petroleum products is 4-6 times that of

Figure 9. Total lipid (% composition) of early-exponential phase cells grown on various hydrocarbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene. Total lipids consisted of seven fractions: (PE) phosphatidylethanolamine; (LPE) *lyso*-phosphatidyl ethanolamine; (PC) phosphatidylcholine; (Unk #1) unknown #1; (C) cardiolipin; (NL) neutral lipids; and (Unk #2) unknown #2.

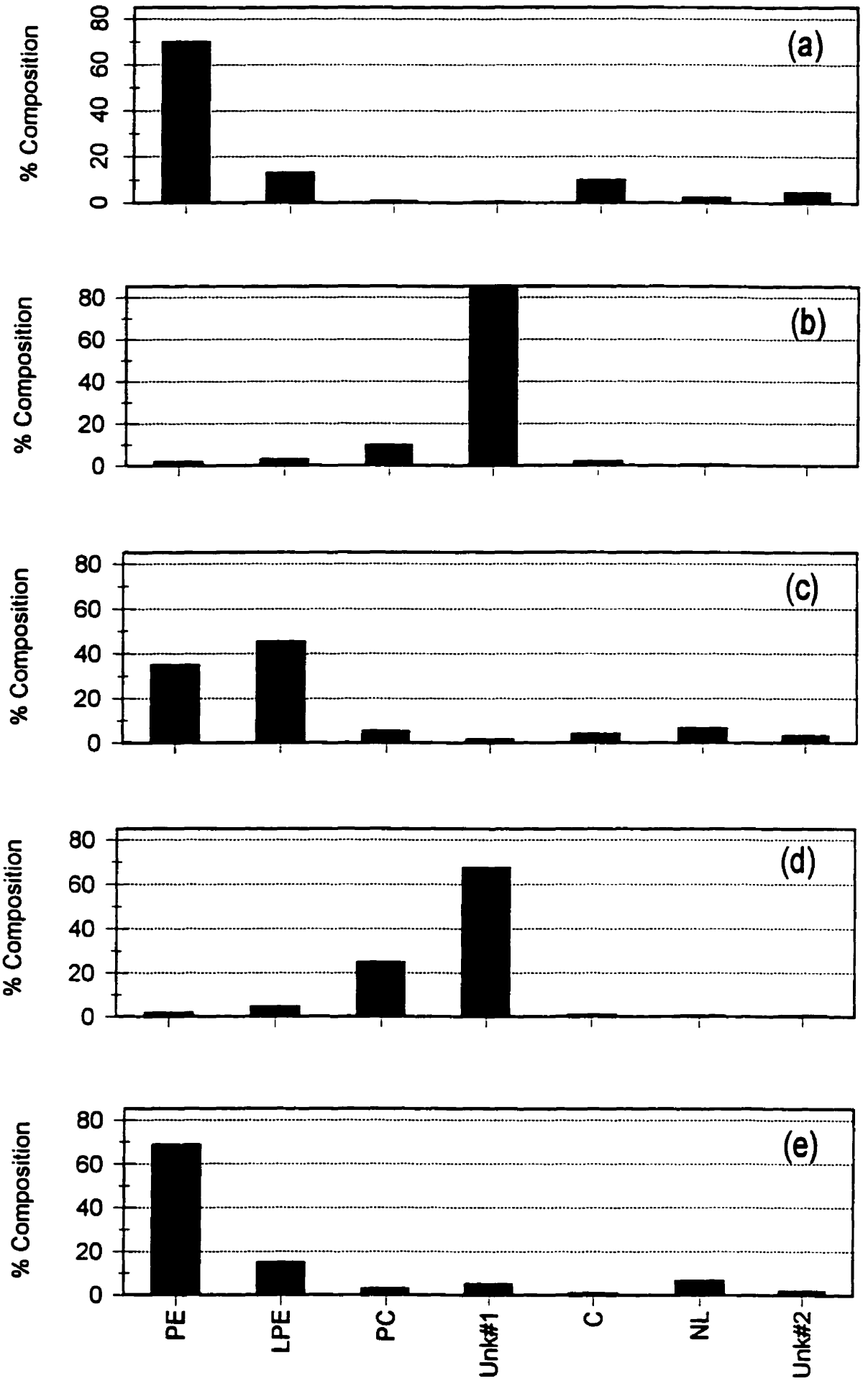

% Composition
0
20
40
60
80
(a)
(b)
(c)
(d)
(e)
PE
LPE
PC
Unk#1
C
NL
Unk#2

Figure 10. Total lipid fatty acid profiles of early-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.

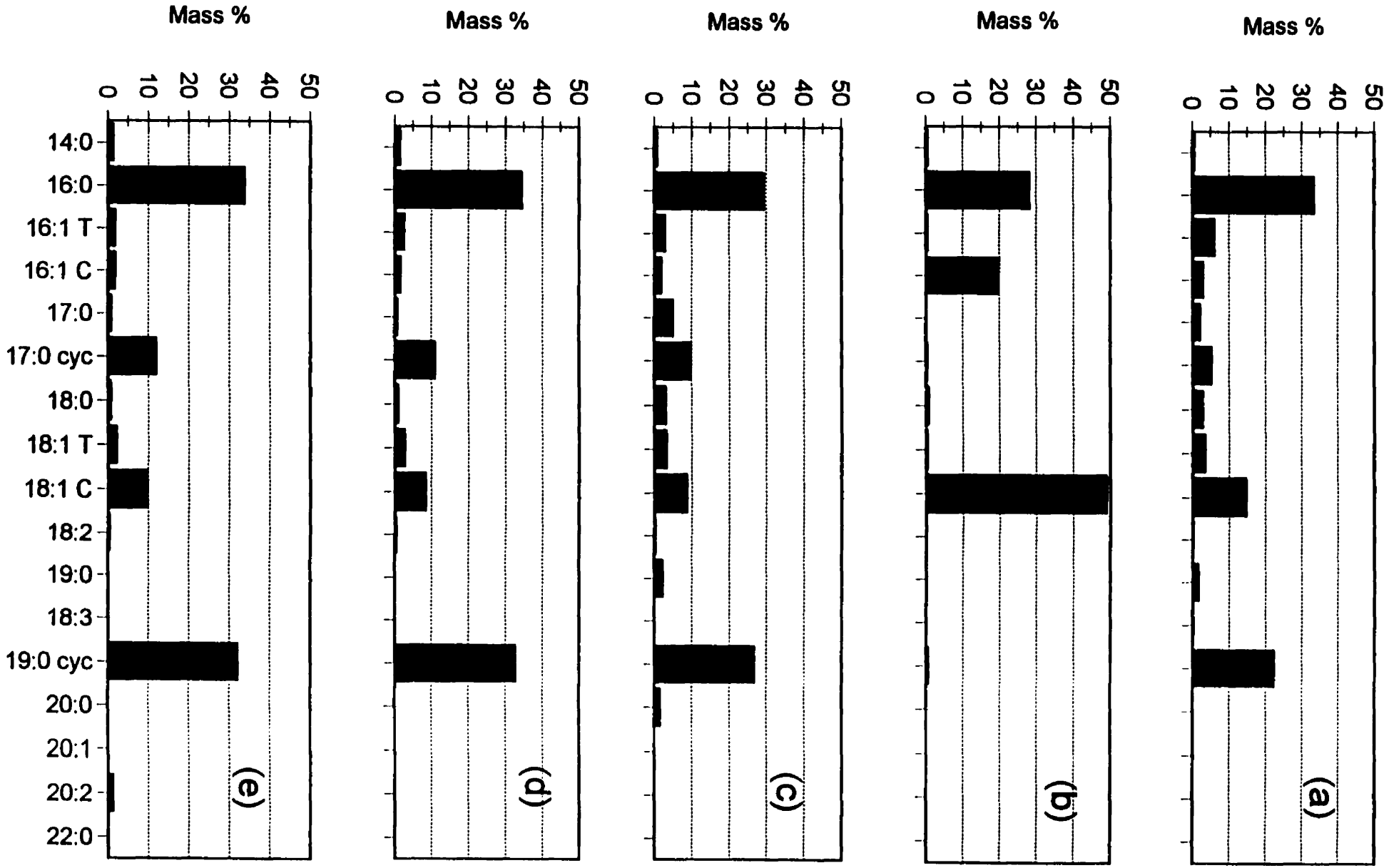

Mass %
0
10
20
30
40
50
14:0
16:0
16:1 T
16:1 C
17:0
17:0 cyc
18:0
18:1 T
18:1 C
18:2
19:0
18:3
19:0 cyc
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

TABLE 3: Differences in the fatty acid profile of *P. aeruginosa* grown on various carbon sources (early-log).

CARBON SOURCE	TOTAL LIPIDS	PE [1]	LPE	PC	UNK #1	UNK #2	C	NL
				Trans/cis ratio				
Hexadecane	0.535	0.497	0.458	1.79	0.506	0.241	0.738	0.617
Glucose	0.00989	0.0177	0.0120	0.00954	0.00868	0.130	0.0237	0.0621
Furnace Oil	0.580	0.556	0.483	0.411	0.511	0..403	0.330	0.715
Aviation fuel	0.517	0.541	0.941	1.20	1.17	0.544	0.601	0.140
Kerosene	0.341	0.494	0.327	0.0433	0.0327	0.262	0.364	0.487
				Degree of Saturation (Sat./ Unsat. Fatty acids)				
Hexadecane	1.47	1.06	1.36	2.07	0.799	0.682	1.41	1.26
Glucose	0.422	0.334	0.396	0.409	0.416	0.297	0.420	0.594
Furnace Oil	2.79	1.43	1.02	1.47	1.50	1.18	1.31	1.77
Aviation fuel	2.24	1.52	0.959	2.29	2.36	1.40	1.04	0.573
Kerosene	2.19	2.18	2.81	0.610	0.446	1.01	0.484	0.710
				Total % [3] Cyclopropane Fatty Acids (C17:0 + C19:0))				
Hexadecane	27.3	27.3	35.0	36.0	27.7	29.9	36.0	42.9
Glucose	0.864	0.792	0.763	0.803	0.818	N.A [2].	1.09	1.27
Furnace Oil	36.1	37.5	37.8	43.4	31.1	42.1	46.1	48.4
Aviation fuel	43.5	34.5	34.2	43.6	43.9	14.3	7.28	11.2
Kerosene	43.8	45.0	39.8	13.4	4.06	17.4	4.38	7.60

1) PE = phosphatidyl ethanolamine; C = cardiolpin; LPE = lyso-phosphatidyl ethanolamine; NL = neutral lipids; PC = phosphatidyl choline; Unk #2= unknown #2 lipid; Unk #1 = unknown #1 lipid

2) N.A.= None available

3) %= Per Cent

cells grown on glucose (Table 3). The highest degree of saturation was recorded for lipids from cells grown on furnace oil (Table 3). The cyclopropane fatty acids C17:0 *cyc* (*cis*-9,10-methylene-hexadecanoic acid) and C19:0 *cyc* (*cis*-9,10-methylene-octadecanoic acid) were present in significant quantities in all lipids from cells grown on petroleum hydrocarbons (*ca.* 36%) but in negligible amounts in glucose-grown cells (0.09 %) The *trans cis* ratio of the fatty acids in the lipids isolated from cells were below one. The petroleum-grown cells had *trans cis* ratios of *ca.* 0.5; whereas, the ratio of glucose-grown cells was 0.010 (Table 3). The lower degree of saturation for glucose was the result of a higher content of C18:1 and C16:1 fatty acids (Fig. 10). The cells grown on glucose had C18:1 *cis* (*cis*-9-octadecenoic acid), C16:0 and C16:1 *cis* (*cis*-9-hexadecenoic acid) as the major fatty acids. ; whereas, in petroleum-grown cells C16:0 and C19: cyc were the dominant fatty acid species present (Fig. 10). Analyses on the distribution and characteristics of the fatty acid components of the individual lipids reveal large differences (Table 3, Appendix A, and Appendix C).

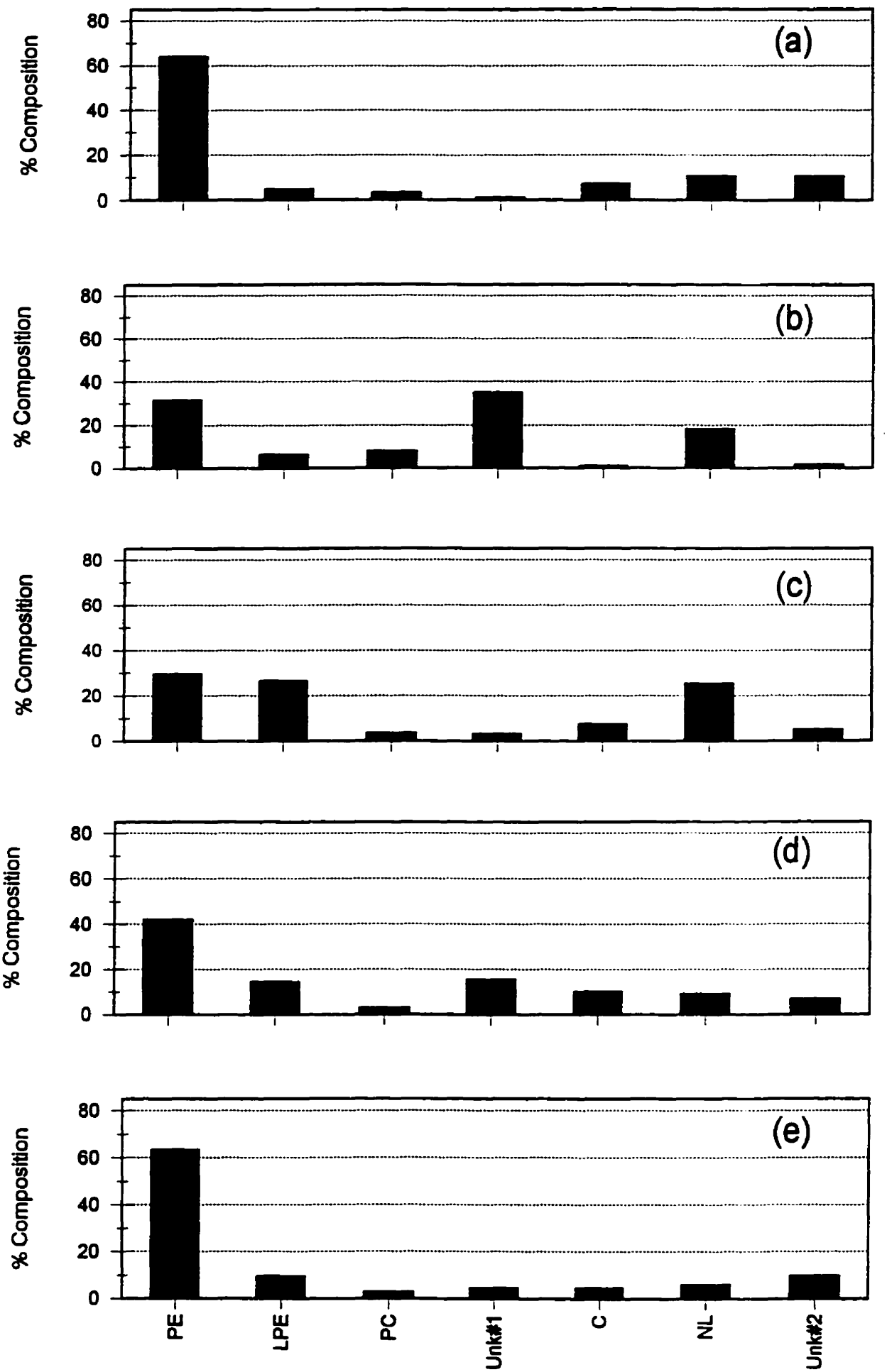
4.1.5.1.4. Late-log phase total lipids

PE appears as one of the major lipids in all the late-log petroleum- and glucose-grown cells. In addition, the glucose-grown cells have large amounts of Unk #1, and the furnace oil-grown cells have large amounts of the LPE and NL (Fig. 11).

4.1.5.1.5. Fatty acid analysis of late-log phase lipids

Petroleum-grown cells contain large amounts of C16:0, C19:0 *cyc* and C17:0 *cyc*

Figure 11. Total lipid (% composition) of late-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene. Total lipids consisted of seven fractions: (PE) phosphatidyl ethanolamine; (LPE) *lyso*-phosphatidyl ethanolamine; (PC) phosphatidyl choline; (Unk #1) unknown #1; (C) cardiolipin; (NL) neutral lipids; and (Unk #2) unknown #2.


% Composition
80
60
40
20
0
(a)
(b)
(c)
(d)
(e)
PE
LPE
PC
Unk#1
C
NL
Unk#2

fatty acids: whereas, glucose-grown cells contain C18:1 *cis* and C16:0 as the major fatty acid fractions (Fig 12). Cyclopropane fatty acids were present in small amounts in the glucose-grown cells, *ca.* 8%, however, in cell grown on petroleum the cyclopropane fatty acids accounted for *ca.* 42% (Table 4). The degree of saturation of the lipids in cells grown on the petroleum products was 3 times that of cells grown on glucose (Table 4). Analyses on the distribution and characteristics of the fatty acid components of the late-log individual lipids reveal large differences (Table 3, Appendix B, and Appendix C).

4.1.5.1.6. Differences between early- and late-log phase total lipids

Significant changes occurred in the glucose and aviation fuel-grown cells in the transition from early to late log phase of growth (Fig. 9, 11). In the glucose and aviation fuel-grown cells approximately two-thirds of the Unk #1 lipids disappeared from early log and was replaced in the late-log phase of growth by other lipid species, PE being one of the significant lipids. The cells grown on the other hydrocarbons did not vary substantially in terms of quantity (Fig. 9, 11). There also appeared to be an increase in the proportion of neutral lipids and the Unk #2 lipid in all cultures except the kerosene-grown cells, from early to late exponential phase.

4.1.5.1.7. Differences between the fatty acids of early- and late-log lipids

The most significant differences observed between the total lipids of the early- and late-exponential phase cells were a decrease in the C18:1 *cis* and C16:1 *cis* fatty acids and an increase in C19:0 *cyc* fatty acids (Table 3, 4). The total *trans/cis* ratio shows an increase

Figure 12. Total lipid fatty acid profiles of late-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.

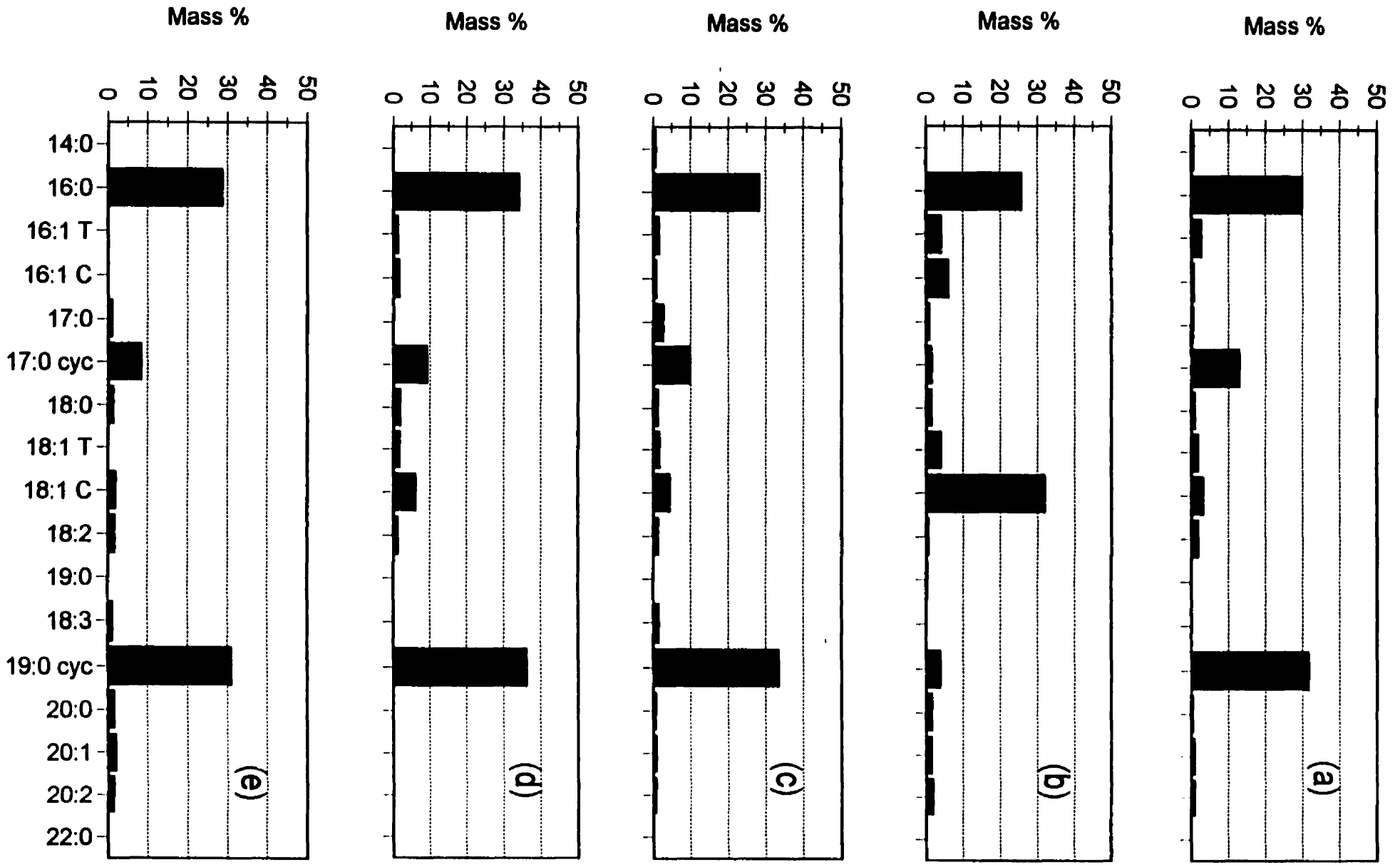

Mass %
0
10
20
30
40
50
14:0
16:0
16:1 T
16:1 C
17:0
17:0 cyc
18:0
18:1 T
18:1 C
18:2
19:0
18:3
19:0 cyc
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

TABLE 4: Differences in the fatty acid profile of *P. aeruginosa* grown on various carbon sources (late-log).

CARBON SOURCE	TOTAL LIPIDS	PE [1]	LPE	PC	UNK #1	UNK #2	C	NL
				Trans/cis ratio				
Hexadecane	1.19	1.15	1.03	N.A[2].	N.A[2].	0.521	0.679	0.500
Glucose	0.207	0.0914	0.122	0.118	0.415	0.252	N.A[2].	0.137
Furnace Oil	0.622	1.38	1.10	0.729	8.64	2.56	1.66	0.137
Aviation fuel	0.408	0.275	12.7	3.37	27.9	11.6	8.04	2.01
Kerosene	N.A [2].	0.334	0.194	0.109	0.285	0.278	0.225	0.208
				Degree of Saturation (Sat./ Unsat. Fatty acids)				
Hexadecane	2.73	2.12	4.00	3.03	1.55	1.99	1.78	1.86
Glucose	0.603	0.570	1.82	0.495	2.08	1.13	0.581	0.476
Furnace Oil	3.39	2.83	3.22	2.25	0.441	0.451	1.24	0.944
Aviation fuel	3.08	1.82	0.241	0.373	0.497	0.208	0.101	0.845
Kerosene	3.96	1.89	1.46	1.02	5.37	2.49	2.01	5.02
				Total % [3] Cyclopropane Fatty Acids (C17:0 + C19:0))				
Hexadecane	44.2	50.8	59.5	15.7	16.8	44.5	54.7	50.2
Glucose	5.08	4.79	5.29	7.31	2.13	11.7	10.6	5.00
Furnace Oil	43.0	48.4	47.0	41.8	14.1	27.0	46.1	11.8
Aviation fuel	45.2	45.5	23.5	7.70	20.8	17.7	20.5	24.8
Kerosene	39.1	43.0	39.1	31.0	10.6	24.9	39.5	41.9

1) PE = phosphatidyl ethanolamine; C = cardiolpin; LPE = lyso-phosphatidyl ethanolamine; NL = neutral lipids; PC = phosphatidyl choline; Unk #2= unknown #2 lipid; Unk #1 = unknown #1 lipid
2) N.A.= None available
3) %= Per Cent

from early to late exponential phase which is indicative of an overall decrease in the amount of C18:1 *cis* and C16:1 *cis* fatty acids (Table 3,4, Figs.10, 12). All cells grown on the hydrocarbons excluding kerosene displayed an overall increase in the total amount of cyclopropanes (Table 3, 4). A transitional change in the characteristics of the outer cell wall membranes from being slightly saturated to being increasingly more saturated in the final stages of growth was observed. An increase in the amount of saturated fatty acid species from early to late log (almost two-fold) was exhibited by growth on all the hydrocarbon sources (Figs. 10, 12). The saturation index (degree of saturation) of the lipids from cells grown on petroleum hydrocarbons was 5-6 times that of glucose-grown cells; this is due to glucose-growing cells containing a higher proportion of unsaturated fatty acids (Table 3, 4).

4.1.6. Exopolymer production

The appearance of a creamy-white compound was observed at approximately mid-way in the growth cycle of *P. aeruginosa* ATCC 9027 when grown on petroleum hydrocarbons. This compound, however, was not produced when the organism was grown on glucose. The compound aggregated at the surface of the growth medium and was easily removed by suction for analysis. Aggressive centrifugation resulted in three separate layers: (1) top layer (clear, consisting solely of a petroleum hydrocarbon); (2) middle layer (white exopolymer); and (3) bottom layer (beige, consisting of cells). The compound initially removed at the surface was a mix of oil, cells and exopolymer. The white middle layer (exopolymer) was tightly bound to the oil and cell layers and therefore may be amphipathic in nature. The white exopolymer was also observed in solid media cultures (agar slants, Fig.

Figure 13. Starter culture slants with various carbon sources: (A) aviation fuel; (G) glucose; (H) hexadecane; (F) furnace oil; and (K) kerosene. Exopolymer are white areas on slant (arrows).

13). It was embedded in and around the agar slants at the periphery of growth. This polymeric substance was only seen with slants that contained a petroleum hydrocarbon as a carbon and energy source. Preliminary analysis of the composition of the exopolymer revealed that it was not a rhamnolipid, however, one µl of exoploymer consisted of 0.5120 µg of lipid, 3.119 µg of protein and 1.210 µg of carbohydrate.

4.1.7. pH differences between glucose and petroleum-grown cells

In Figure 14, the cells grown on glucose showed the greatest decrease in pH when compared to the petroleum-grown cells (pH 3.9). The lowest pH obtained by the petroleum-grown cells was 5.0. The greatest drop in pH with respect to time was seen in the kerosene- and aviation fuel-grown cells. The cells grown on hexadecane took the longest (seven days) to reach a minimum pH. The other petroleum-grown cells required two to three days to achieve the minimum value.

Figure 14. Measurement of pH with time of *P. aeruginosa* grown on: (a) hexadecane; (b) glucose; (c) furnace oil; (d) kerosene; (e) aviation fuel.Growth (OD_{600}) on respective substrates is represented by (●) and pH is represented by (◆).

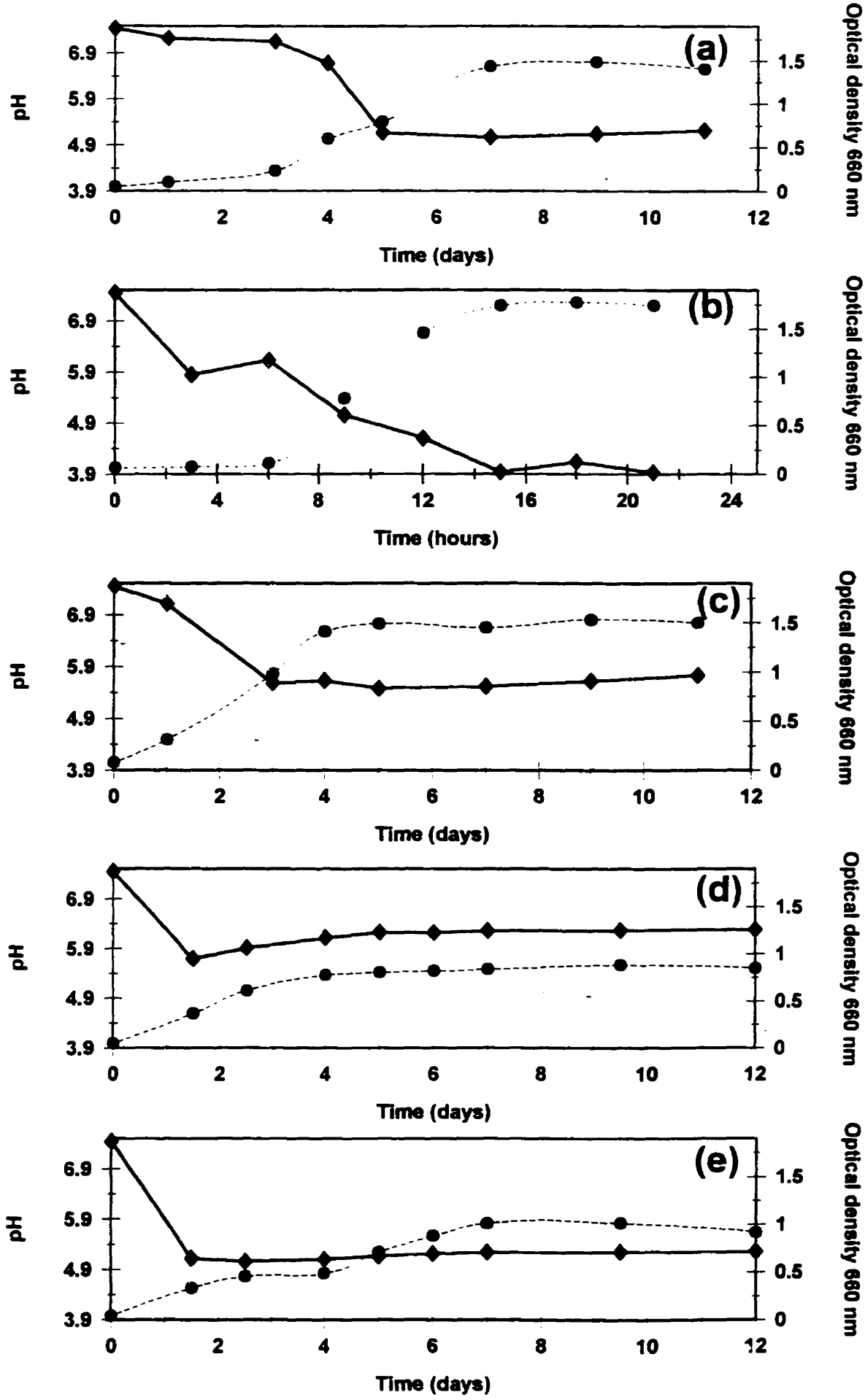
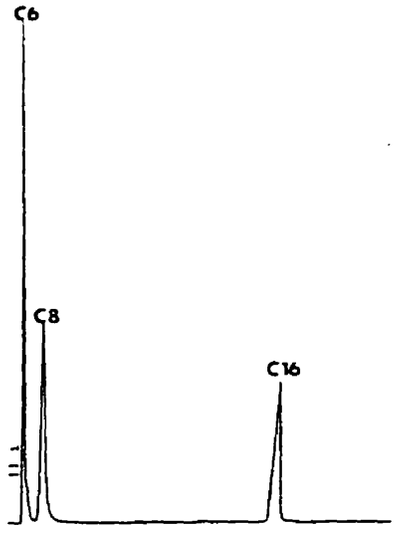
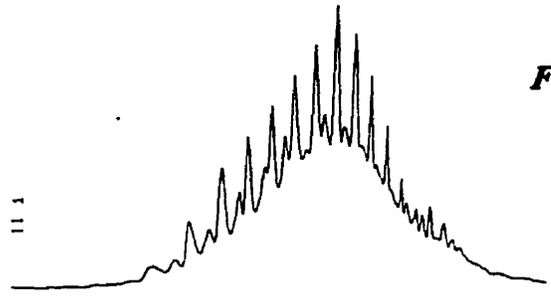

(a)
pH
Optical density 660 nm
6.9
5.9
4.9
3.9
1.5
1
0.5
0
0
2
4
6
8
10
12
Time (days)
(b)
pH
Optical density 660 nm
6.9
5.9
4.9
3.9
1.5
1
0.5
0
0
4
8
12
16
20
24
Time (hours)
(c)
pH
Optical density 660 nm
6.9
5.9
4.9
3.9
1.5
1
0.5
0
0
2
4
6
8
10
12
Time (days)
(d)
pH
Optical density 660 nm
6.9
5.9
4.9
3.9
1.5
1
0.5
0
0
2
4
6
8
10
12
Time (days)
(e)
pH
Optical density 660 nm
6.9
5.9
4.9
3.9
1.5
1
0.5
0
0
2
4
6
8
10
12
Time (days)

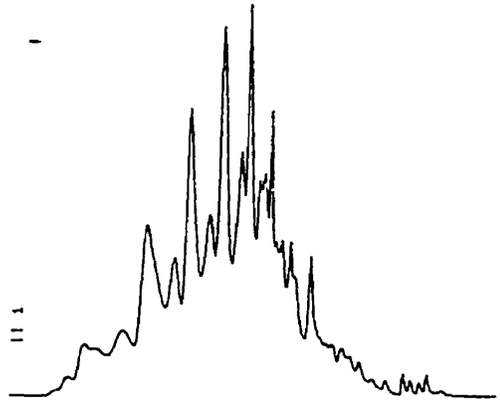
Figure 15. Gas chromatograms of various refined petroleum products (furnace oil, kerosene, and aviation fuel) and *n*-alkanes (hexane, octane, and hexadecane)..



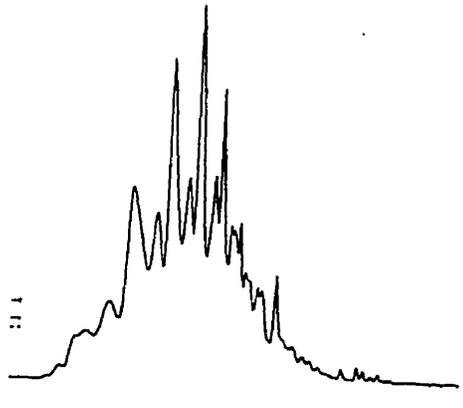
ALKANE MIXTURE



FURNACE OIL



AVIATION FUEL



KEROSENE

5. 0. DISCUSSION

Pseudomonas aeruginosa (ATCC 9027) grows on hexadecane as a carbon and energy source (Miguez *et al.*, 1986). However, growth of this strain on other petroleum hydrocarbons (furnace oil, aviation fuel, kerosene, and light crude oil) has not been fully documented until this study. Collectively, the data obtained after examining the growth characteristics (Figs. 3A-3C) show that *P. aeruginosa* can use and metabolize a variety of petroleum hydrocarbons as its sole source of carbon and energy. The microorganism, , has difficulty, however utilizing small chain alkanes (C6-C8) (Fig. 3B) and thus growing. This is due to the deleterious solvent effects on its outer cell wall (Pfaender and Backley, 1984; Atlas and Cerniglia, 1995). In addition, it has been noted that toxicity of hydrocarbons increases as the viscosity of the hydrocarbons decreases (Sikkema *et al.*, 1995).

Variability and extended lag periods characteristically describe growth on the petroleum hydrocarbons. The initial lag period is somewhat representative of hydrocarbon-degrading microorganisms (Feinberg *et al.*, 1980; Lindley and Heydeman, 1985). This characteristic lag period is due to physiological responses to the hydrocarbon substrate involving compositional changes or production of the necessary enzymes for hydrocarbon utilization. Thus during this period energy is diverted to these processes rather than toward growth. Key enzymes during the initial stages of growth are those that facilitate hexadecane or other petroleum sub-components' accessibility to degradative enzymes within the cell and those which are degradative enzymes themselves (Breuil and Kushner, 1980).

The different petroleum mixtures vary in the complexity and diversity of their

components (Fig. 15 and Perry 1984). One or more of these components may affect the microbial cell by causing changes in the membrane lipids and fatty acids, outer membrane proteins, cell surface hydrophobicity, and growth medium surface tension and pH. Depending on the actions of the various components, some may support growth; others, hinder growth and this could account for the variability in microbial growth on the different petroleum mixtures.

P. aeruginosa growth on the petroleum mixtures decreased in the following order: hexadecane > furnace oil > kerosene > aviation fuel (Figs. 3A-3C). The lower growth on kerosene and aviation fuel is attributed to their higher concentrations of aromatic and alicyclic compounds. *P. aeruginosa* was also able to grow on two different light crude oils, though growth was not as pronounced as growth on hexadecane or furnace oil (Fig. 3C). This observation was of interest since crude oils tend to contain components within the hydrocarbon mixture that are sub-optimal for growth (since many sub-components are not easily degraded) (Walker *et al.*, 1969). Differences in growth on the two light crude oils are a result of the different sub-components within the two light crudes. Leahy and Colwell, (1990) stated that no two crude oils are necessarily similar.

SDS-PAGE of the outer membrane proteins reflected the changes that are occurring at the outer cell wall due to growth on the different petroleum mixtures (Fig. 8). Several new proteins were synthesized as the cell proceeded from the early log phase of growth to the late log (Fig. 6). Overall, growth on hexadecane, kerosene, and furnace oil resulted in similar protein profile changes. With aviation fuel there were two distinct proteins (45 and 30 kDa) found in the cell's membrane. These may have been produced in response to the higher

concentrations of aromatics and alicyclics found in the fuel. These changes in the membrane protein profile during growth on petroleum mixtures indicate ultrastructural changes in the outer membrane which could allow the organism to better control and facilitate entry of the different sub-components in the petroleum mixtures into the cell (Hanson *et al.*, 1994). In contrast, cells grown on a glucose-carbon source did not reveal any new proteins from early to late log (Fig. 7), however, significant differences among the protein profiles of cells grown on glucose and petroleum hydrocarbons were noted (Fig. 8). The differences in the protein profiles of the glucose-grown cells to that of the petroleum-grown cells are due to the involvement of different proteins in transport and catabolism.

The ability of *P. aeruginosa* to physiologically respond to different refined petroleum products does not rest solely on the microorganism's ability to change the proteins in the cell envelope; it can also be attributed to the modification of the membrane lipids. The observed modifications during growth on the various petroleum hydrocarbons and glucose included changes in the type of lipids and the composition and distribution of the fatty acids within the lipids in the cell. The cell lipid and fatty acid profiles were distinct for glucose and each individual petroleum mixture. The extractable lipids examined did not include the lipids of the lipopolysaccharide (LPS) layer, since these are firmly bound and only extractable after saponification (Hancock and Meadows, 1969). Large amounts of unknown lipids were observed in aviation fuel- and glucose-grown cells (Fig. 9). The unknown (Unk #1) lipid accounted for approximately 60 to 80 % of the total weight of the lipids, in the early-log cells of the aviation fuel- and glucose-grown cells. This large amount of Unk#1 lipid is due to the hydrophilic nature of glucose and maybe some of the lesser hydrophobic sub-

components within aviation fuel. Phosphatidylethanolamine (PE) was present in significant amounts in the hexadecane-, furnace oil- and kerosene-grown cells (Fig. 9) and appears to be essential in the earlier stage of growth. Phosphatidylethanolamine is thought to have a role in minimizing packing defects around membrane proteins, in activating membrane-bound enzymes, and in optimizing membrane functions (Chen *et al.*, 1995). In addition to PE and Unk #1, the extractable lipids consisted of several other phospholipids (including phosphatidylcholine, cardiolipin, and *lyso*-phosphatidylethanolamine), neutral lipids and a second unknown lipid (Unk#2). Changes in the lipids and fatty acid profiles were also detected from early to late growth. It was interesting to note that as the glucose- and aviation fuel-grown cells entered late-log (Fig. 11), there was a disappearance of approximately 40 to 50 % of the Unk #1 lipid. The disappearance of the unknown lipid can be attributed to the microbial cell having catabolized this lipid or converted it to another lipid species in response to changes in the culture medium and in the case of the aviation fuel-grown to changes in the carbon source (sub-components). With this apparent decrease in the Unk#1 lipid, there was an increase in the phospholipid, PE. This phospholipid appears to play a vital role in the later stages of growth (late log to stationary phase) in order to protect the cell from some of the remaining toxic sub-components (within the hydrocarbon mixtures) and metabolic waste products present in the growth medium. The substitution or the production of PE in the later stage of growth may be related to the activity of the alkaline nitrogen-containing polar head group. The polar head group would play a role in buffering the cell from the low pH of the culture medium. Replacement of the Unk#1 lipid with PE also directly affects the fluidity of the lipid membrane and thus would control entry of

compounds into the cytoplasmic membrane. Researchers have shown that replacement of membrane phospholipids with PE results in a membrane that is more gel-like than liquid as a consequence of the higher ordering and lateral compression of the head groups (Perly *et al.*, 1985).

Significant changes occurred in the composition of the fatty acids within the different classes of lipids as the microbial cells aged and when they grew on different carbon sources. Growth of the cells on the petroleum mixtures as compared to glucose-grown cells, results in lipids whose fatty acid profiles contained a larger proportion of saturated fatty acids, cyclopropane fatty acids (CFAs), and *trans* species of unsaturated fatty acids. Chen *et al.*(1995) have demonstrated that induction of the *alk* system (on the OCT plasmid) by an alkane- degrading *Pseudomonas* results in changes in the membrane phospholipid fatty acid composition, presumably due to the activities of enzymes involved in fatty acid and phospholipid syntheses. The changes in the fatty acid profiles are indicative of the changes in the cell membrane fluidity (Sikkema *et al.*, 1995). The *cis* to *trans* conversion and the change to saturated fatty acids result in a membrane that is more viscous (gel-like) (Weber *et al.*, 1994; Heipieper *et al.*, 1992). This homeoviscous modification mechanism allows vital membrane processes to continue unabated and to offset any physical or chemical changes imposed by the cell environment (Diefenbach *et al.*, 1992, Sinensky, 1974). The observed change in the fatty acids from *cis* to *trans* and from unsaturated to saturated allows the cells to oppose the partitioning of lipophilic solutes into the lipid bilayers of the cell as a result of the tighter packing of the fatty acids (Sikkema *et al.*, 1995; Weber *et al.*, 1994). Resistance to several toxic organic solvents by several *Pseudomonas* strains was associated

with the conversion from the *cis* to the *trans* unsaturated fatty acid isomer (Heipieper *et al.*,1994; van Beilen *et al.*, 1996). These physiological changes in the fatty acids of the cell lipids allow the cell to control the accumulation of lipophilic (or hydrophobic) compounds within the cytoplasmic membrane. The large accumulation of lipophilic molecules can result in a loss of membrane integrity (due to swelling of the lipid bilayers), a decrease in membrane-embedded enzyme activity (due to interference with protein-protein and protein-lipid interactions), and an increase in the permeability to protons and other ions (Sikkema *et al.*, 1995).

In all the early-log cells grown on petroleum hydrocarbons, CFAs initially accounted for 30 to 40 % of the total fatty acids in the total extracted lipids (Tables 4-8). In contrast, in the early-log glucose-grown cells, CFAs accounted for only 1 % of the total fatty acids. The large amount of CFAs present initially within the petroleum-grown cells suggests that the CFAs are an integral cellular component. Having a substantial amount of CFAs is necessary in order for the microorganism to grow on the petroleum hydrocarbons. All phospholipids and lipids extracted contained CFAs. Depending upon the petroleum mixture, the distribution of CFAs was not the same in all the individual lipids extracted. Phospholipid bound unsaturated fatty acyl groups are substrates for cyclopropane fatty acid synthetase (CFA synthetase) (Wang and Cronan, 1994; Taylor and Cronan, 1979). The presence of CFAs in the neutral lipids and the unknown lipid from the extractable cellular lipids in our samples suggests that there may be more than one mechanism for CFA production and that the phospholipid moiety is not a prerequisite. The increase in CFAs in the later stage of growth by petroleum- and glucose-grown cells is in agreement with other researchers that

have stated that the production of CFAs occurs from late-log to stationary phase (Wang and Cronan, 1994; Taylor and Cronan, 1976; and Cronan, 1968). The CFAs may have several functional roles in the cell. The initial CFAs can play a role in controlling the uptake of the petroleum hydrocarbon into the cell by virtue of affecting membrane fluid dynamics (McGarrity and Armstrong, 1981; George et al., 1995). The observation by Jungkind and Wood (1974) that oxidation of CFAs did not advance past the cyclopropane ring, leads one to assume that CFAs can additionally serve to protect the fatty acid acyl chains in the lipids of the cytoplasmic membrane from degradation by the oxidative enzymes involved in petroleum hydrocarbon oxidation. The concomitant increase of the CFAs in the final stages of growth also serves as a protective mechanism against inhibitory metabolic end-products and substrates (Cronan, 1968). To date the exact function of CFA has not been determined, even though there are many hypotheses for its role (Wang and Cronan, 1994). As mentioned earlier, the CFAs are not equally distributed in all the different lipids. This suggests that the role of CFA's are expressed more in certain lipids than others. What is also evident is that the hydrocarbon makeup within the petroleum mixtures may in some way directly influence this disproportionate CFA distribution.

Compositional changes in the outer cell wall can result in a cell with a more hydrophobic or lipophilic characteristic. This is substantiated by the results of the hydrophobic test performed (Fig. 4), where the petroleum-grown cells maintain a more hydrophobic nature in the initial stages of growth (in some cases 2 to 3 times higher than glucose-grown cells) which ultimately decreased in the final stages of growth. Qualitatively, the pattern illustrated in Fig. 4, describes the petroleum-grown cells as dynamic cells

(undergoing constant change) while the glucose-grown cells are relatively passive throughout the growth cycle in terms of hydrophobicity. Since the cell wall (outer membrane) is the region of the cell that makes initial contact with the petroleum hydrocarbon, it is important for the cell surface to be hydrophobic. Though examination of the LPS in petroleum-grown cells was not part of this study, LPS plays an important role in cell-surface hydrophobicity. The change in the hydrophobic nature of the outer cell wall has previously been documented and was shown to be in part due to changes in the lipopolysaccharide (LPS) layer of the cell wall (Sikkema *et al.*, 1995; Miguez *et al.*, 1986). Miguez *et al.* (1986) noted a 30 to 40 % decrease in the carbohydrate content of the LPS in *P. aeruginosa* when grown on hexadecane as compared to growth on glucose. Decreasing the amount of carbohydrate in the LPS in the outer membrane and modifying the outer membrane proteins (more hydrophobic), would increase the affinity of *P. aeruginosa* toward petroleum hydrocarbons. Some sub-components within the petroleum mixtures enter the cell easily, therefore requiring a lesser amount of change in the outer cell wall, while other sub-components, due to their lower permeability, require the hydrophilic outer cell wall to be modified. The higher hydrophobicity of hexadecane- and furnace oil-grown cells (Fig. 4) suggests a higher loss of hydrophilic components. In the case of the cells grown on hexadecane and furnace oil, modifying its outer cell wall in order to obtain its carbon and energy source seems essential for the cell, whereas the cells grown on kerosene and aviation fuel require a lesser degree of modification. The decrease in hydrophobicity as cells enter mid-log phase of growth suggests that the cells may have the full complement of hydrophilic outer cell wall components, and that maintaining a hydrophobic cell surface is not necessary.

This non-hydrophobic requirement can be attributed to daughter cells that have already incorporated petroleum hydrocarbon donated from the mother cells; thus, it is not a prerequisite for the cells to change the surface cell properties since the petroleum hydrocarbons are already within the cells.

Surface tension studies revealed that the largest drop in surface tension of the growth medium of *P. aeruginosa* ATCC 9027 was observed when the microorganism was grown on furnace oil and hexadecane (Fig. 5). The increase in cell-surface hydrophobicity and surface tension lowering of the growth medium by *P. aeruginosa* may have a direct relationship with each other. Cells grown on furnace oil and hexadecane had the highest cell-surface hydrophobicity and were also associated with the greatest drop in surface tension. The drop in surface tension may be due to the release of biosurfactants or bioemulsifiers by the microorganism into the growth medium. Biosurfactants and bioemulsifiers enhance the aqueous dispersion of organic compounds having limited water solubility. *P. aeruginosa* ATCC 9027 and other strains have been known to produce rhamnolipids that lower surface tension (Zhang and Miller, 1992; Mulligan *et al.*, 1988; Zajic and Seffens, 1984; Itoh *et al.*, 1971). Other cellular components such as lipopolysacharides, lipopetides, phospholipids, fatty acids, lipids, glycolipids, and proteins, have also been shown to act as bioemulsifiers or biosurfactants (Desai and Banat, 1997; Zajic and Seffens, 1984; Cooper, 1986). *P. aeruginosa* ATCC 9027 released a white compound into the growth medium only when grown on petroleum mixtures and not on glucose. The white compound (exopolymer) was also observed in solid media cultures (agar slants) (Fig. 13). Analysis of the composition of the exopolymer revealed that it consisted

of protein, carbohydrate, and lipid. The exopolymer was tightly associated with the microbial cell and the petroleum substrate and required aggressive centrifugation to separate the two. The literature describes the involvement of extracellular factors such as LPS and a protein-like activator protein (PA) that facilitates *n*-hexadecane assimilation (Hardegger *et al.*, 1994; Oschner *et al.*, 1994). Other studies, describe the role of a number of polymeric biosurfactants from microbial cells consisting of protein and carbohydrate and/or lipid (Desai and Desai, 1993). Koronelli *et al.* (1983b) have described a peptidoglycolipid compound similar to the exopolymer described above for *P. aeruginosa* ATCC 9027. The peptidoglycolipid produced by *P. aeruginosa* P-20, was shown to have bioemulsifier activity and was also comprised of 52 amino acids, fatty acids, and rhamnolipid (Koronelli *et al.*, 1983a). Burd and Ward (1996) have also observed in a *Pseudomonas* sp. (*Pseudomonas marginalis* PD-14B) production of an extracellular surface-active agent (PM-factor) which contained cell wall components (LPS).These results illustrate that the lowering in the surface tension of the growth medium may not be solely associated with rhamnolipid production, but due also to sloughed-off cell wall components. An increase in cell-surface hydrophobicity therefore, reflect the release of outer cell wall components into the medium, and these components in turn, lead to a decrease in the surface tension of the growth medium by acting as biosurfactants or bioemulsifiers. The exact chemical structure and surface tension studies of the exopolymer still needs to be determined.

Growth on the petroleum mixtures and glucose were both accompanied by a lowering in the pH of the growth medium. The lowest pH obtained by the petroleum-grown cells was 5.0, whereas, the pH of the medium with cells grown on a glucose carbon source

was 3.9. The pH values reflect the differences in the energy coupling mechanisms associated with the uptake of the carbon and energy source. *P. aeruginosa* employs an active transport system for the uptake of glucose rather than a phosphotransferase system which is associated with glucose transport in *E. coli* (Gottshalk, 1979). The low pH of the growth medium represents the increase in the H^+ concentration associated with proton-motive transport. The higher pH values of the growth medium by the petroleum-grown cells show that a different mechanism is involved in transport of the petroleum substrate. Transport of the substrate into the petroleum-grown cells is via passive diffusion (Bateman *et al.*, 1986). Bateman *et al.* (1986) using metabolic inhibitors, ionophores and conditions of low ATP, showed that it was unlikely that an electrochemical gradient, an ATP-driven transport process, or a protein carrier were required for movement of naphthalene into the cell of a *P. putida* sp. These researchers also concluded that transport of the petroleum hydrocarbon was via simple diffusion and that no specific association with the naphthalene was required. The large amount of LPS molecules and proteins reduce the hydrophobicity of the outer surface of the membrane. A decrease in the amount of LPS and the hydrophilic nature of the proteins can increase simple diffusion of the petroleum sub-components. Glucose-grown cells do not require extensive changes in the periphery of the cell, due to the water-soluble nature of glucose and the different kinetics involved in glucose transport (Eagon and Phibbs, 1971). Additionally, the lower pH associated with the glucose-grown cells may also be attributed to the higher production and release of organic acids into the medium (via catabolism of glucose through the Entner-Doudoroff pathway).

The overall result of all the experiments revealed the inherent ability of *P.*

aeruginosa (ATCC 9027) to physiologically respond to changes in the carbon and energy source. *P. aeruginosa* (ATCC 9027) possesses an innate ability to alter the structural cellular components and enzymatic machinery to adjust to the available carbon and energy source. Growth on the various petroleum compounds revealed that the nature of the sub-components within the petroleum mixtures highly influenced the adaptation dynamics at all levels of the microbial cell. The physiological processes included modifications in: the lipid composition of the membranes, the outer cell wall components, the activities of the cellular enzymes, the membrane fluidity, and the cell-surface hydrophobicity. These changes in turn, protected the cell and facilitated entry of the substrate. To date, the exact physiological trigger or switch which induces these changes to occur in the cell are still unknown.

Many questions still remain unanswered. In order to obtain a better understanding of this microorganism, future research should examine the following: the chemical signal that induces compositional changes in the cell; the detection of the genes encoding catabolism of the sub-components within the petroleum mixture; the molecular processes involved in hydrocarbon transport into the cell; the enhancement of the microorganism in order to improve their capacity to degrade petroleum pollutants; the biodegradation of petroleum hydrocarbons in the environment (field studies); and determination of the exact chemical composition of the exopolymer.

In summary, the work presented in this thesis on the physiological changes by *Pseudomonas aeruginosa* ATCC 9027 when grown on various petroleum hydrocarbons illustrates the remarkable responsiveness of this microorganism to grow on a variety of petroleum products. This thesis provides important information on some of the physiological

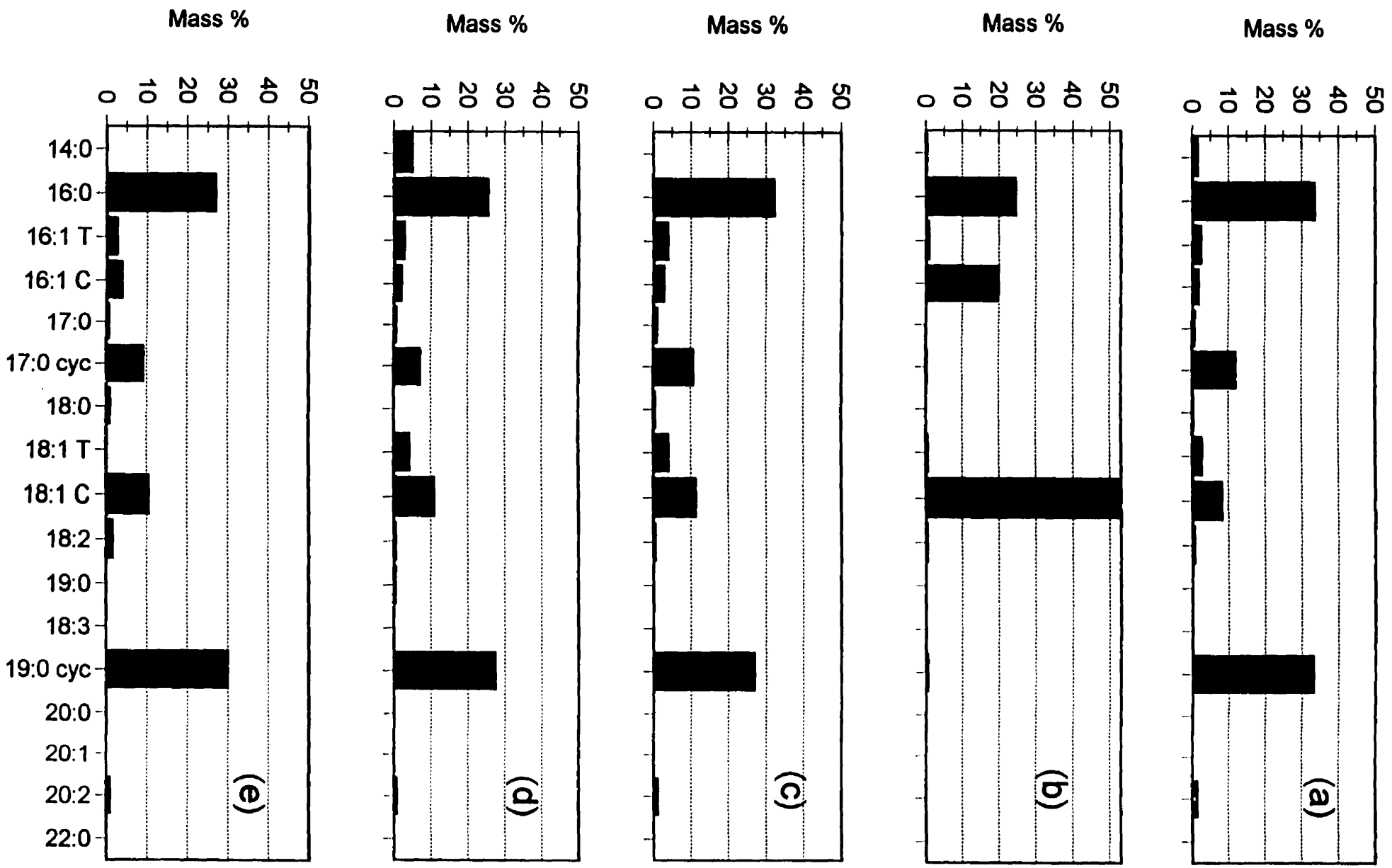
intricacies associated with growth on petroleum, including aspects of membrane dynamics and the interactions of the microbial cell surface with hydrophobic or lipophilic substances outside the cell. Thus, a better understanding of the cell dynamics related to growth on petroleum will improve the existing knowledge about the biodegradation of petroleum pollutants in the environment.

Appendix A: Early-log phase lipids of *P. aeruginosa* grown on various hydrocarbons.

Figure A-1. Phosphatidylethanolamine fatty acid profiles of early-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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18:0
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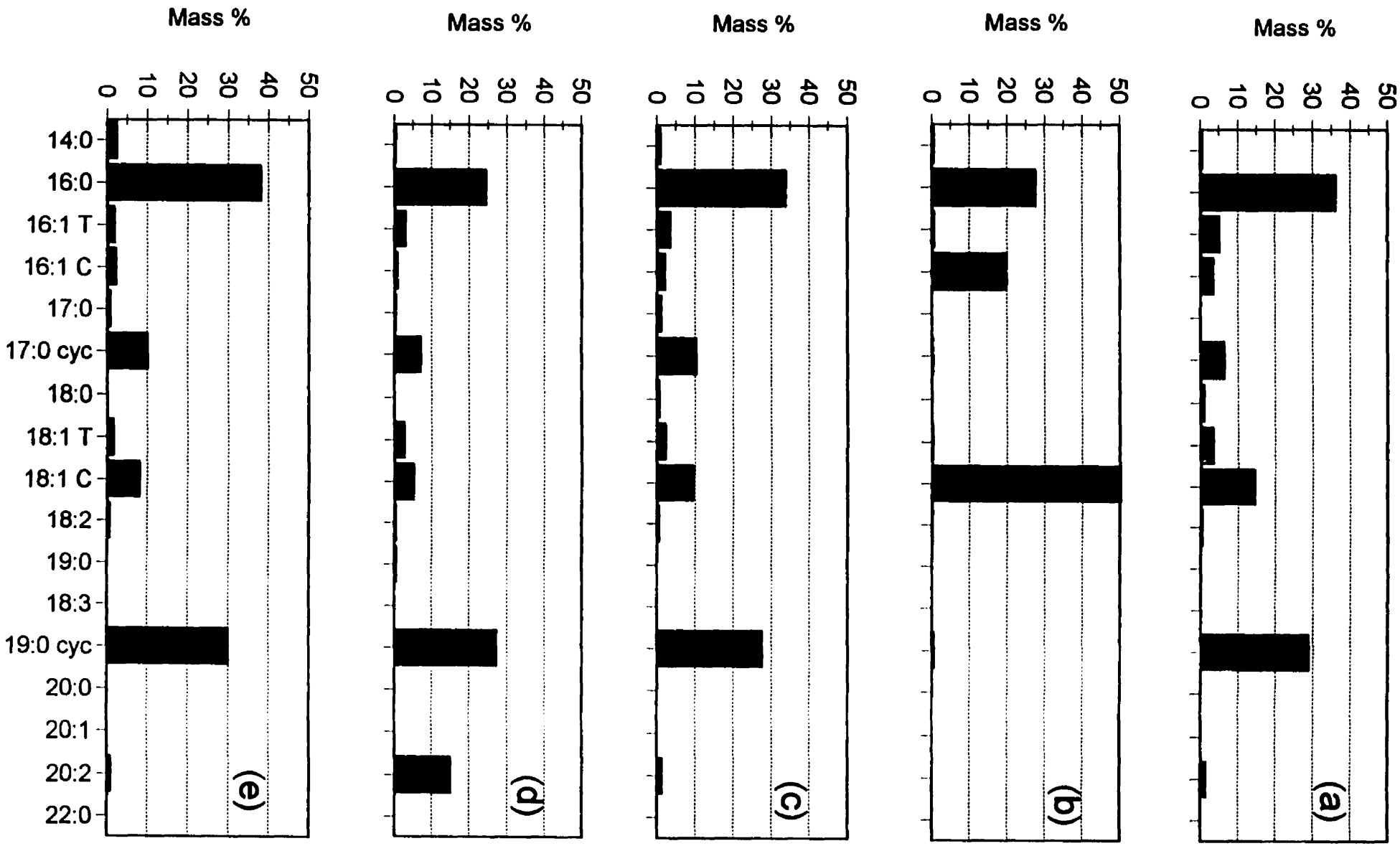
18:1 C
18:2
19:0
18:3
19:0 cyc
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

Figure A-2 *Lyso*-phosphatidylethanolamine fatty acid profiles of early-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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17:0
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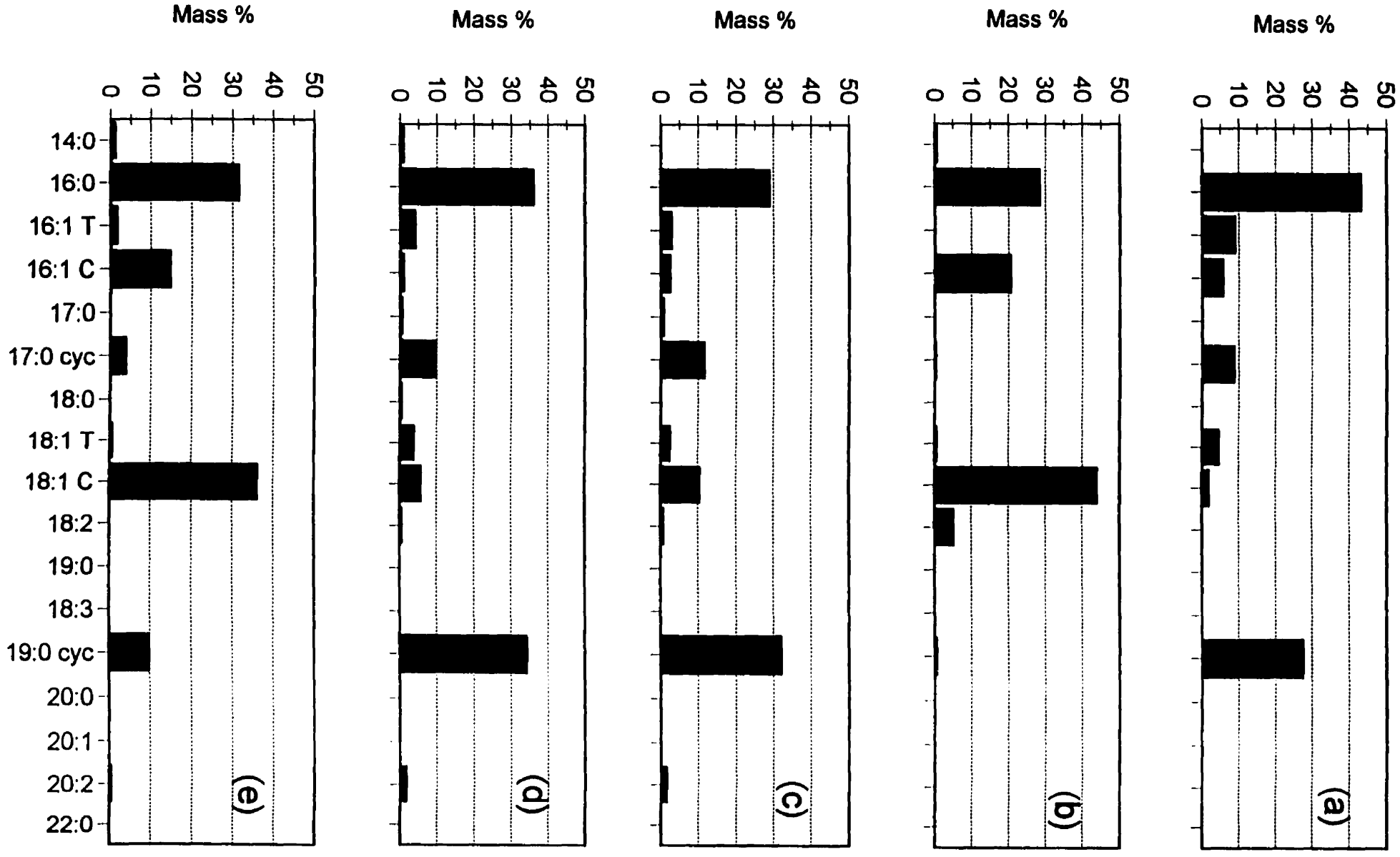
18:0
18:1 T
18:1 C
18:2
19:0
18:3
19:0 cyc
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

Figure A-3. Phosphatidylcholine fatty acid profiles of early-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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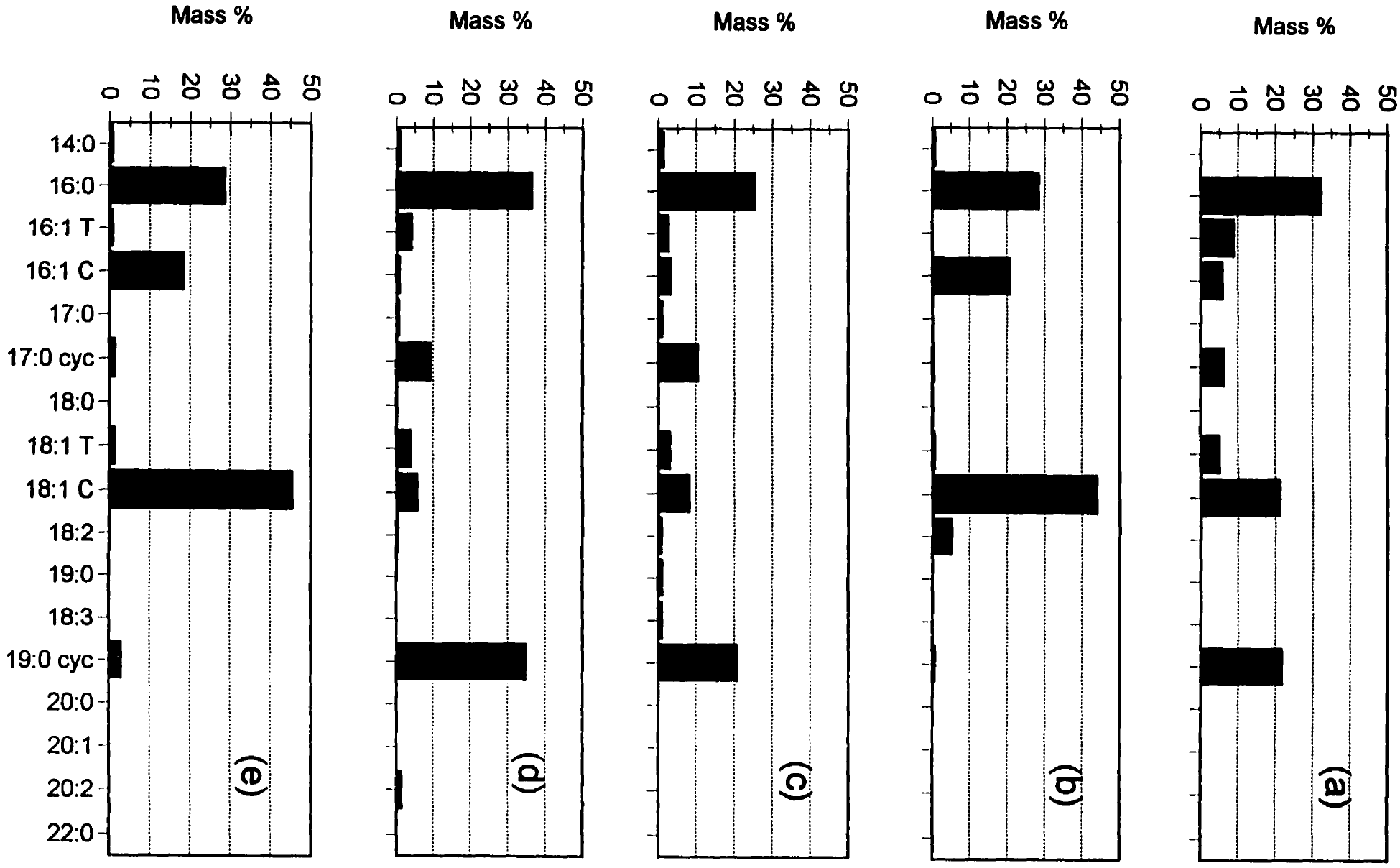
18:1 T
18:1 C
18:2
19:0
18:3
19:0 cyc
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

Figure A-4. Unknown #1 lipid fatty acid profiles of early-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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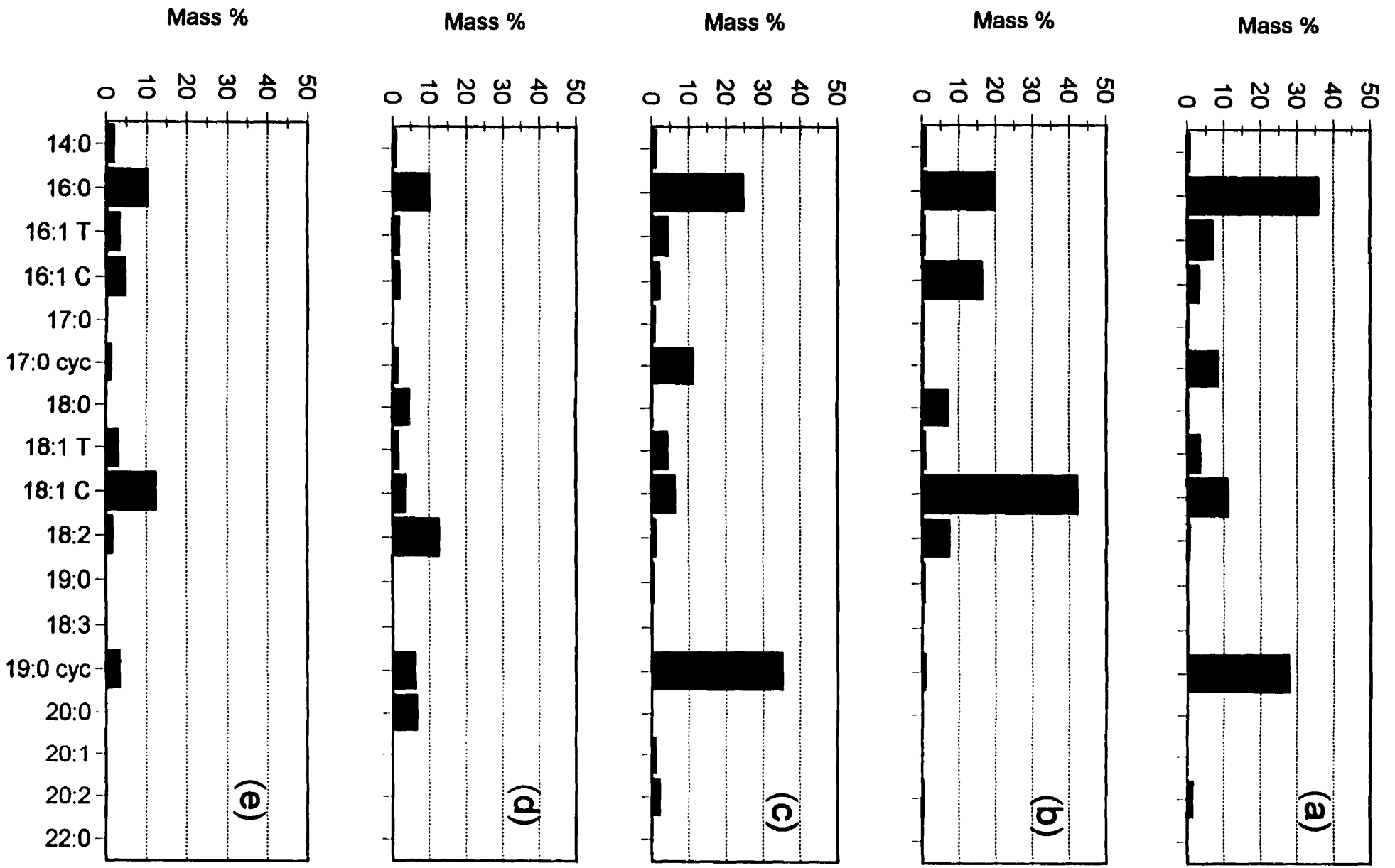
18:2
19:0
18:3
19:0 cyc
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

Figure A-5. Cardiolipin fatty acid profiles of early-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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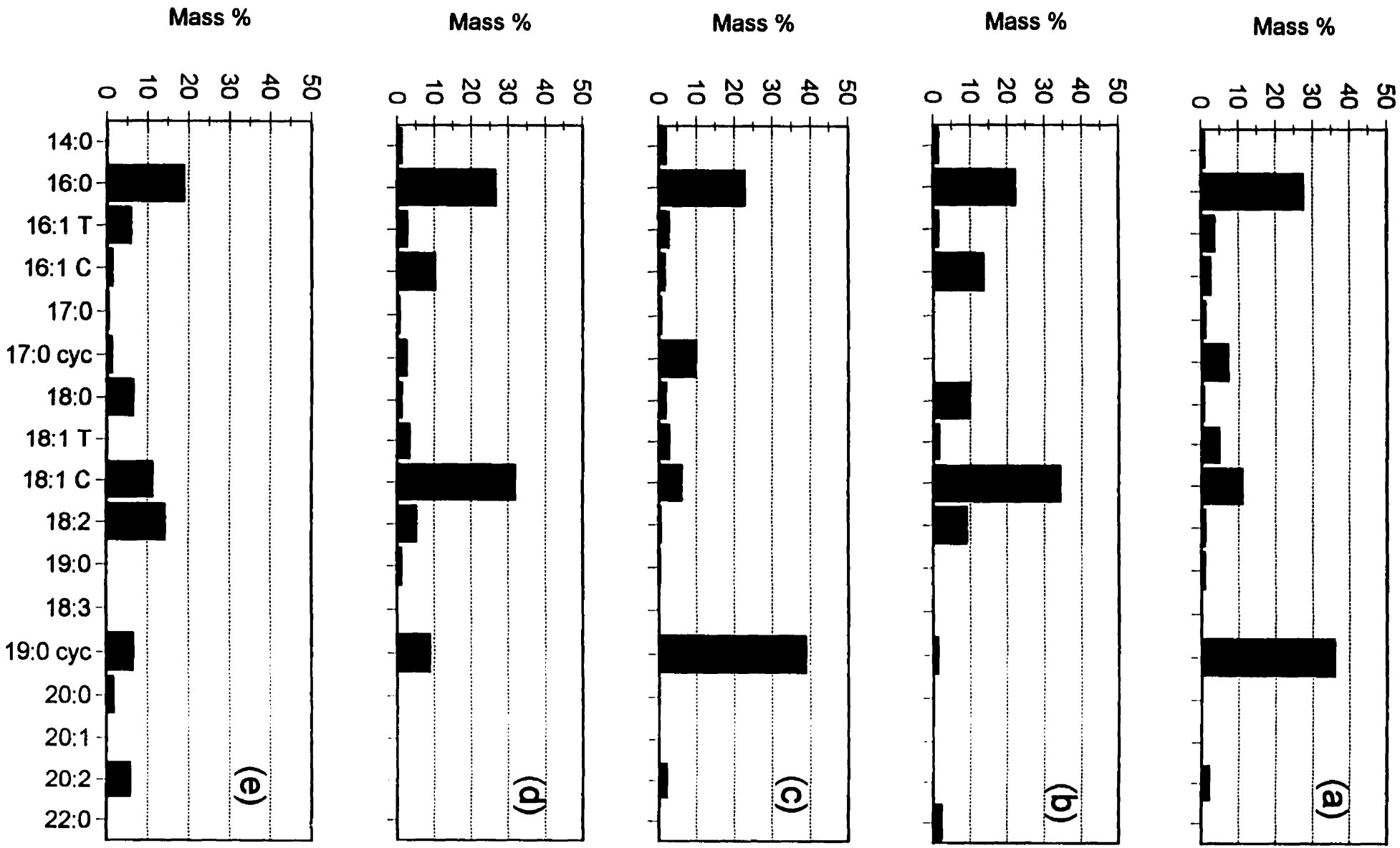
18:1 C
18:2
19:0
18:3
19:0 cyc
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

Figure A-6. Neutral lipids fatty acid profiles of early-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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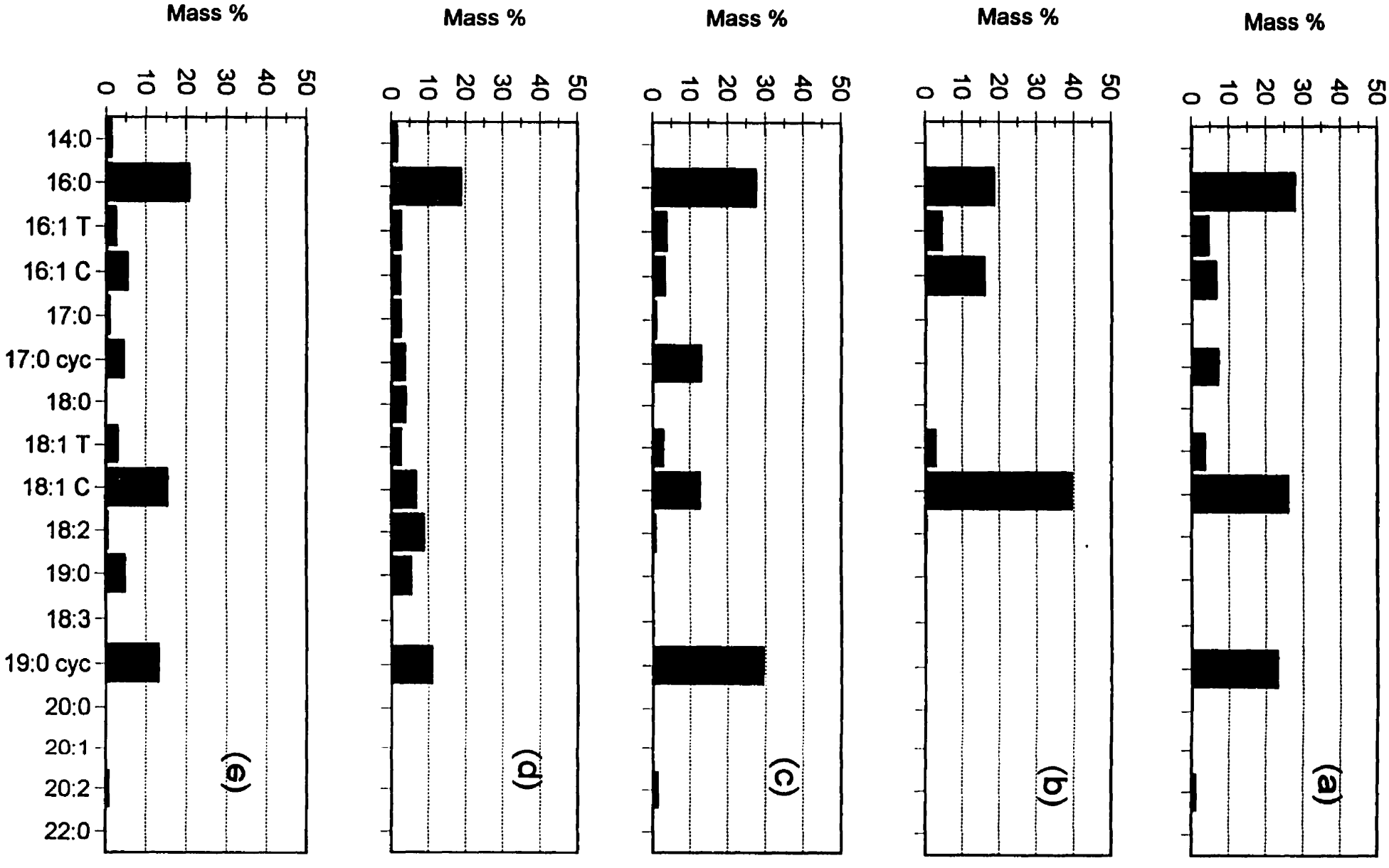
18:1 T
18:1 C
18:2
19:0
18:3
19:0 cyc
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

Figure A-7. Unknown #2 lipid fatty acid profiles of early-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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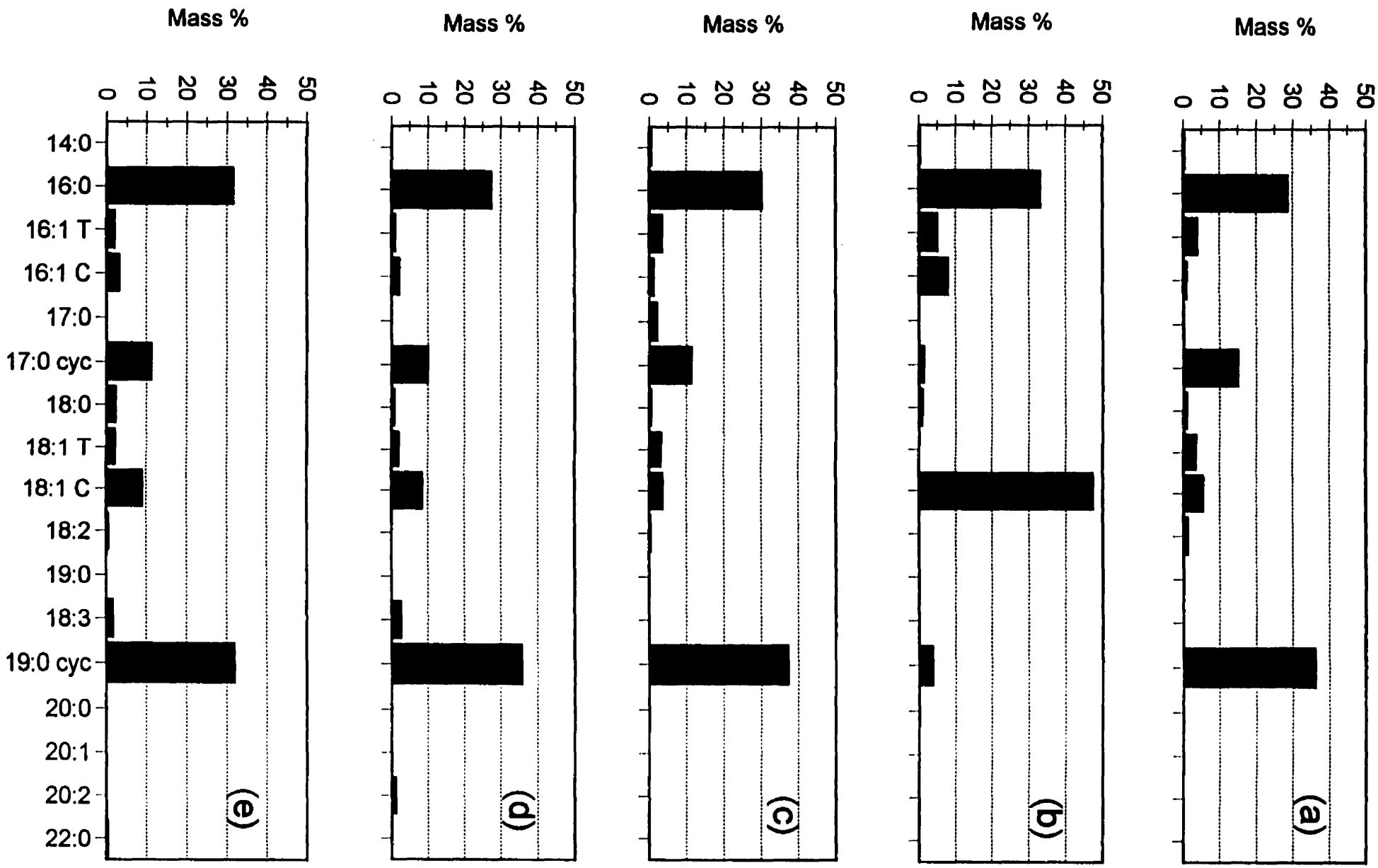
18:2
19:0
18:3
19:0 cyc
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

Appendix B: Late-log phase lipids of *P. aeruginosa* grown on various hydrocarbons.

Figure B-1. Phosphatidylethanolamine fatty acid profiles of late-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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16:0
16:1 T
16:1 C
17:0
17:0 cyc
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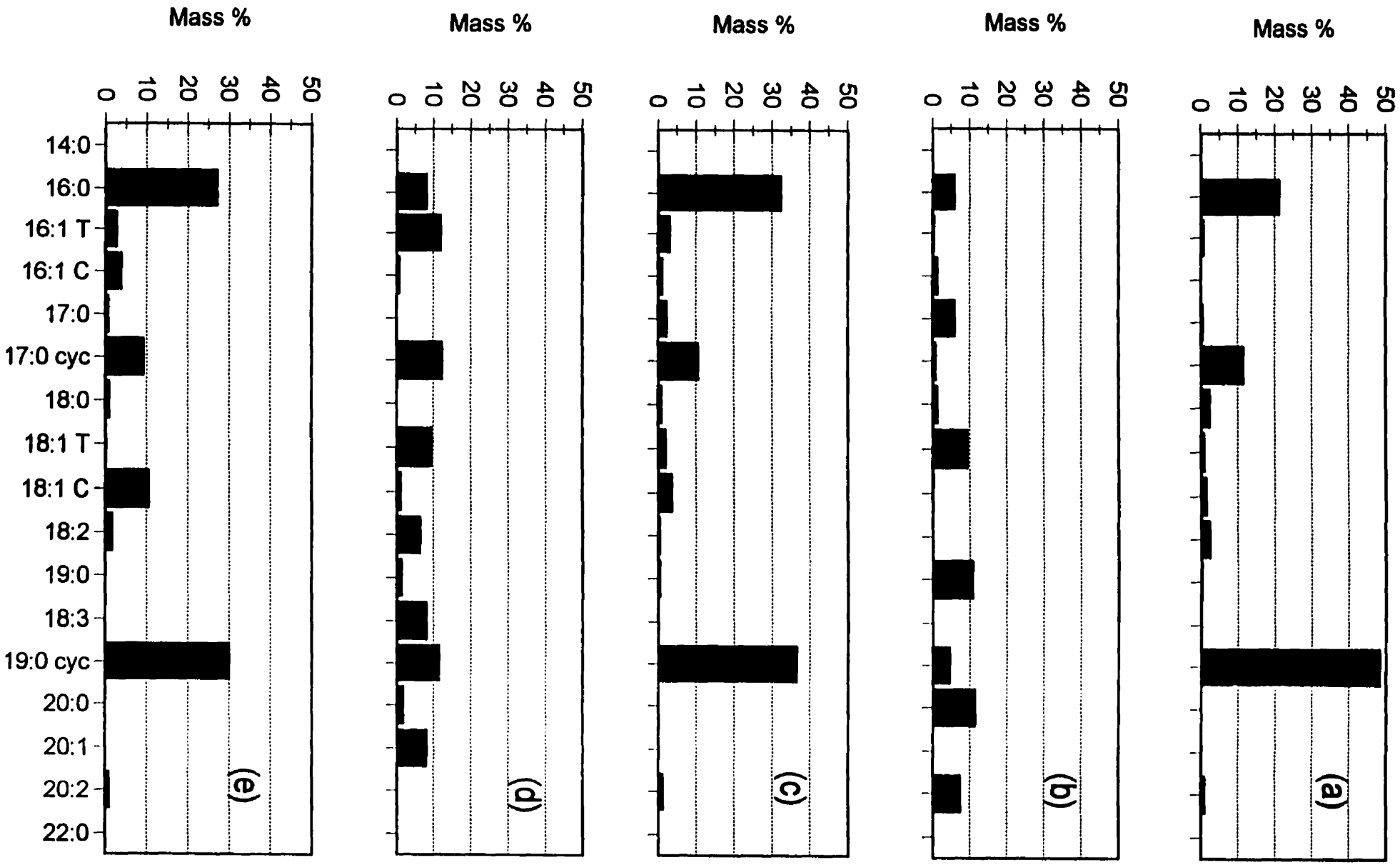
18:1 T
18:1 C
18:2
19:0
18:3
19:0 cyc
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

Figure B-2. *Lyso*-phosphatidylethanolamine fatty acid profiles of late-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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16:1 T
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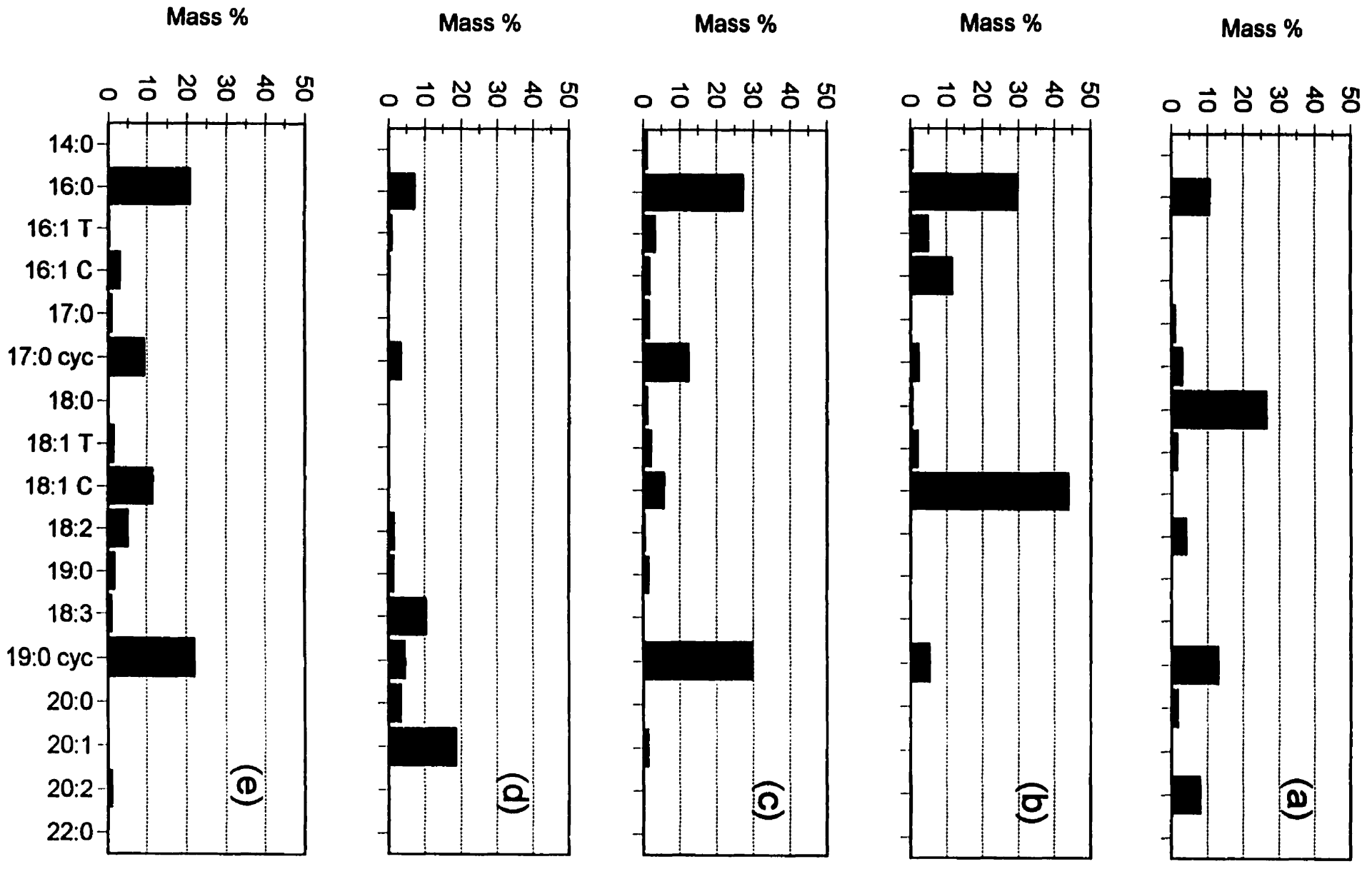
18:1 T
18:1 C
18:2
19:0
18:3
19:0 cyc
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

Figure B-3.. Phosphatidylcholine fatty acid profiles of late-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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19:0
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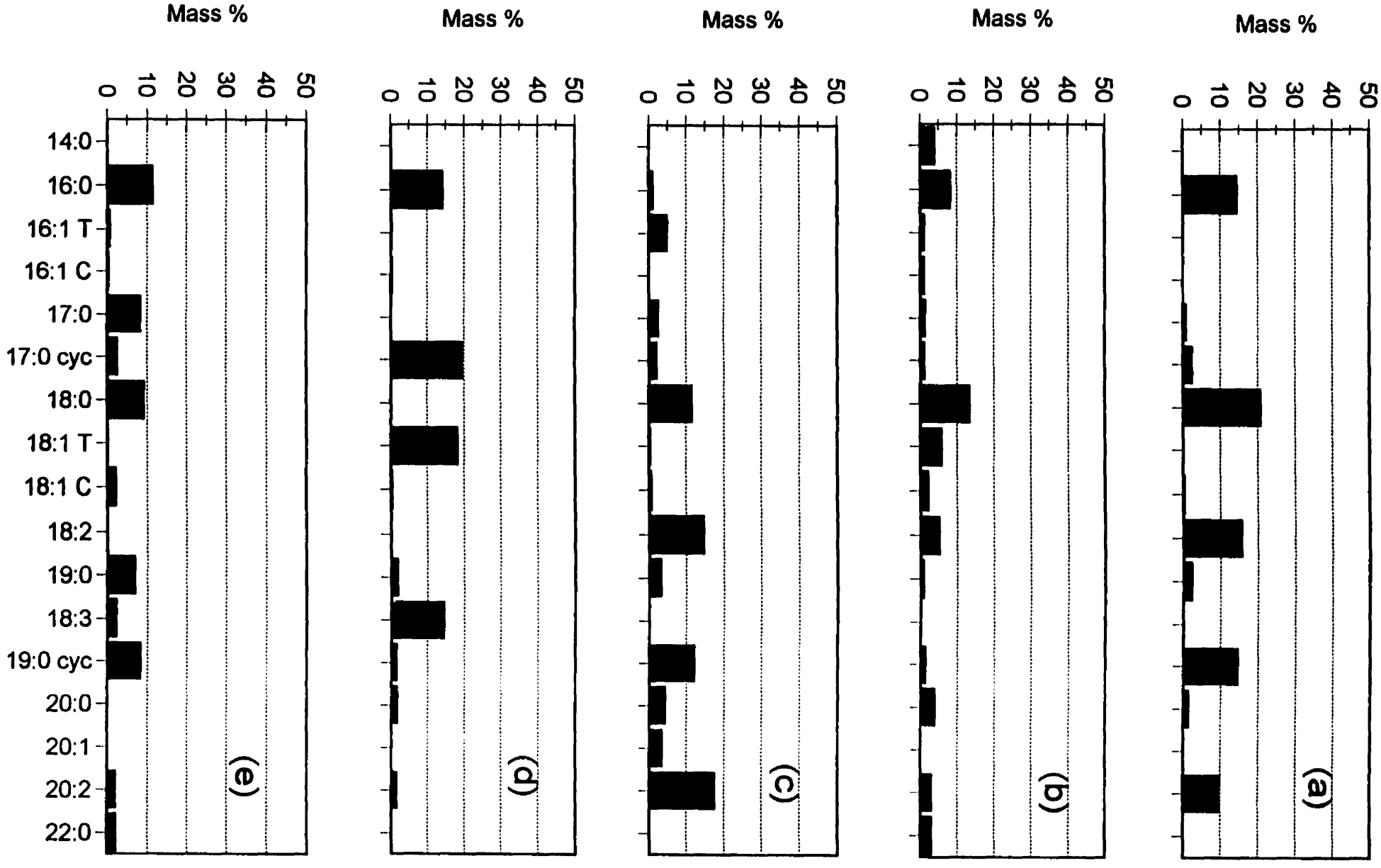
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

Figure B-4. Unknown #1 lipid fatty acid profiles of late-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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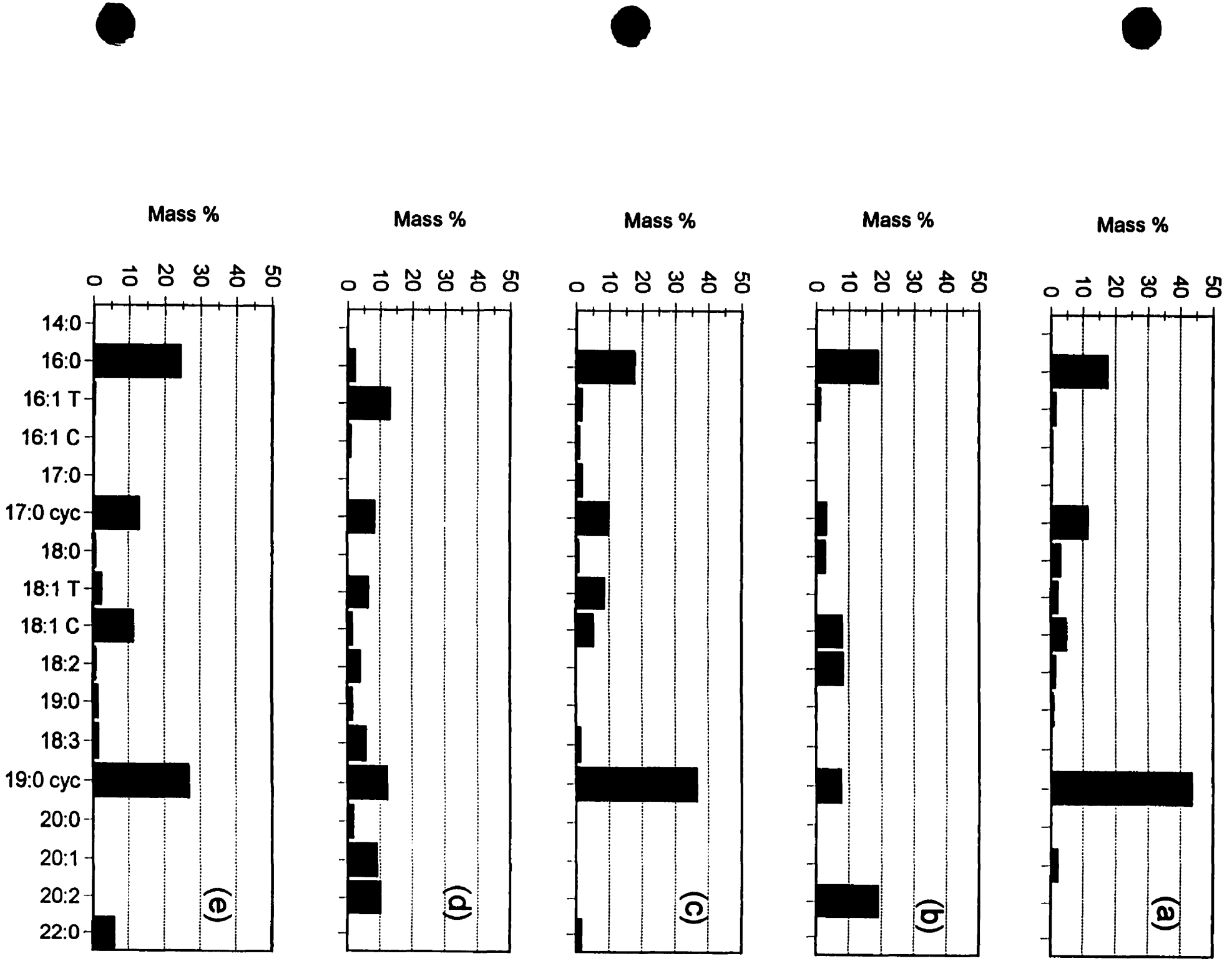
19:0
18:3
19:0 cyc
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

Figure B-5. Cardiolipin fatty acid profiles of late-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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19:0
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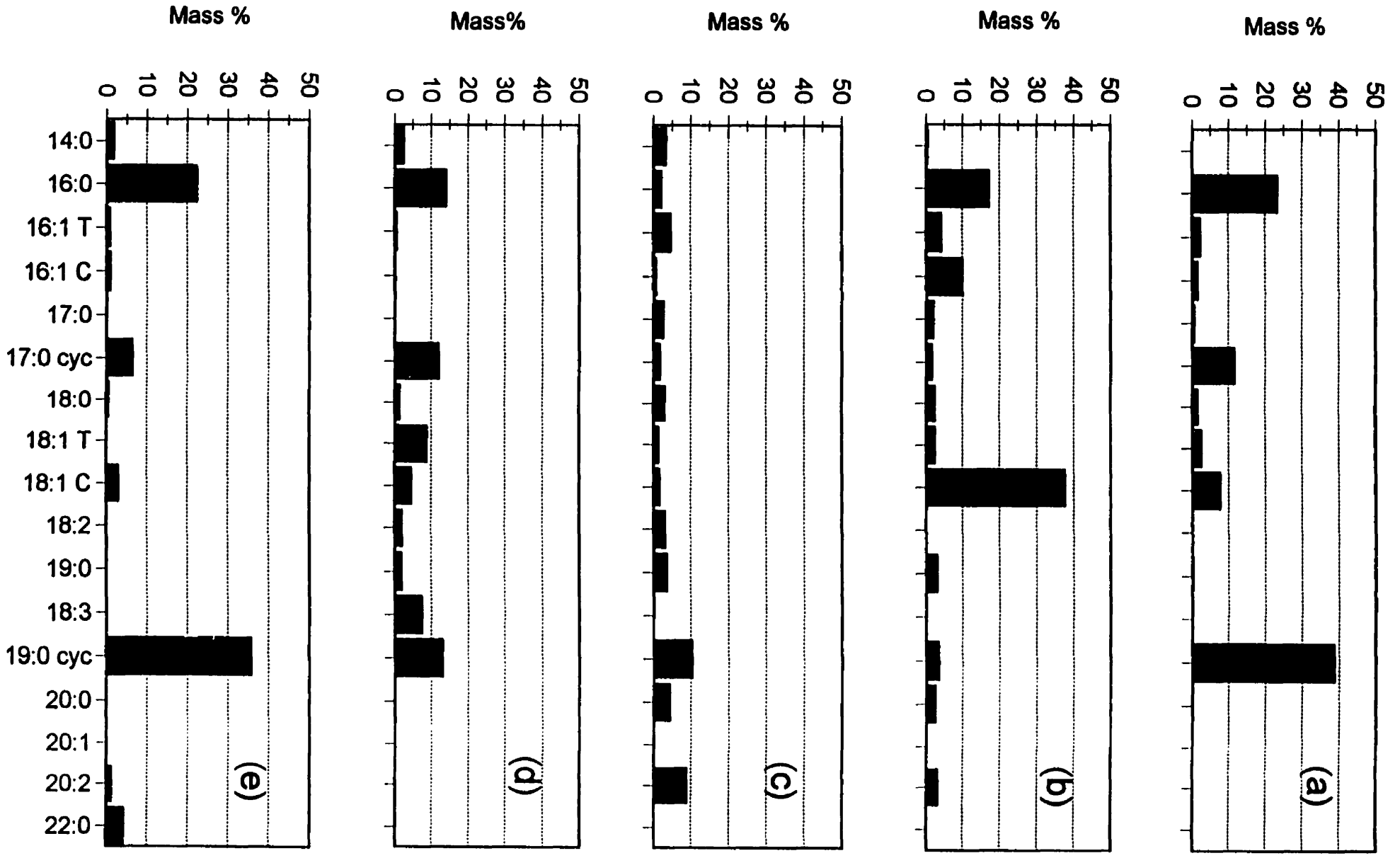
19:0 cyc
20:0
20:1
20:2
22:0
(e)
(d)
(c)
(b)
(a)

Figure B-6. Neutral lipids fatty acid profiles of late-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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20:0
20:1
20:2
22:0
(e)
Mass%
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Mass %
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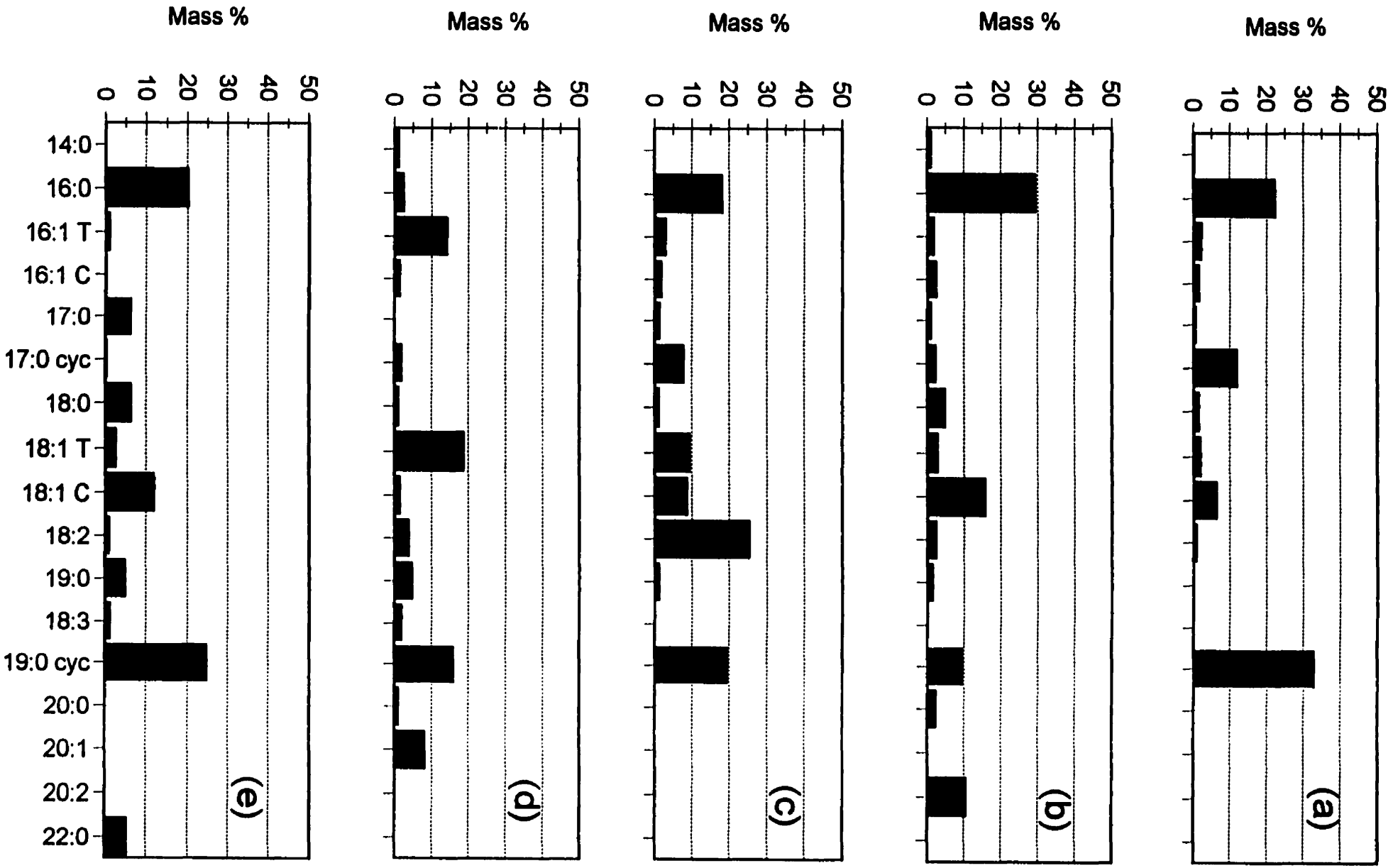
Mass %
(b)
Mass %
(a)

Figure B-7. Unknown #2 lipid fatty acid profiles of late-exponential phase cells grown on various carbon sources: (a) hexadecane; (b) glucose; (c) furnace oil; (d) aviation fuel; and (e) kerosene.

Abbreviations:

cyc, cyclopropane; C, *cis*; T, *trans*.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(e)
(d)
(c)
(b)
(a)

Appendix C: Trans/cis ratios, degree of saturation and total cyclopropane fatty acids characteristics from early- and late-log phase lipids of *P. aeruginosa* grown on various hydrocarbons

Table C-1. The *trans/cis* and saturation characteristics of fatty acids of total lipids isolated from *P. aeruginosa* grown on various hydrocarbon sources.

	CARBON SOURCE				
	HEXADECANE	**GLUCOSE**	**FURNACE OIL**	**AVIATION FUEL**	**KEROSENE**
			Early Exponential Phase		
16:1 *trans cis* (%)[1]	5.99 / 2.82	0.397 / 19.6	2.85 / 1.82	2.48 / 1.54	1.79/1.74
18:1 *trans cis* (%)	3.33 / 14.6	0.281 / 49.0	3.20 / 8.62	2.67 / 8.41	2.08 / 9.60
Total *trans cis* ratio (16:1 + 18:1)	0.0535	0.00989	0.580	0.517	0.341
Degree of Saturation (Sat./Unsat. FA's)	1.47	0.422	2.79	2.24	2.19
			Late Exponential Phase		
16:1 *trans cis* (%)	2.61 / 0.590	3.95 / 5.84	1.52 / 0.770	1.35 / 1.50	N.D / N.D.[2]
18:1 *trans cis* (%)	1.69 / 3.01	3.850 / 31.8	1.63 / 4.29	1.68 / 5.92	N.D. / 1.88
Total *trans cis* ratio (16:1 + 18:1)	1.19	0.207	0.622	0.408	N.A.[3]
Degree of Saturation (Sat./Unsat. FA's)	2.73	0.603	3.39	3.08	3.96

1) (%) = Per Cent
2) N.D.. = None Detected
3) N.A.. = Not Available

Table C- 2. **The *trans/cis* and saturation characteristics of fatty acids of individual lipids isolated from *P. aeruginosa* grown on hexadecane.**

LIPIDS	PE [1]	LPE	PC	UNK #1	UNK #2	C	NL
			Early Exponential Phase				
16:1 *trans cis* (%)[2]	6.94 / 3.35	4.90 / 3.32	8.88 / 5.62	8.71 / 5.69	4.40 / 6.36	6.90 / 2.96	3.54 / 2.44
18:1 *trans cis* (%)	4.26 / 19.2	3.22 / 14.4	4.50 / 1.87	4.80 / 21.0	3.37 / 25.8	3.37 / 10.9	4.71 / 10.9
Total *trans cis* ratio (16:1 + 18:1)	0.497	0.458	1.79	0.506	0.241	0.738	0.617
Degree of Saturation (Sat./Unsat. FA's)	1.06	1.36	2.07	0.799	0.682	1.41	1.26
			Late Exponential Phase				
16:1 *trans cis* (%)	3.70 / 0.830	0.666 / N.D.[3]	N.D. / N.D.	N.D. / N.D.	1.99 / 1.40	1.46 / 0.452	2.18 / 1.44
18:1 *trans cis* (%)	3.20 / 5.22	0.773 / 1.32	1.36 / N.D.	N.D. / 0.283	1.95 / 6.17	2.01 / 4.66	2.37 / 7.59
Total *trans cis* ratio (16:1 + 18:1)	1.15	1.03	N.A.	N.A.[4]	0.521	0.679	0.500
Degree of Saturation (Sat./Unsat. FA's)	2.12	4.00	3.03	1.55	1.99	1.78	1.86

1) PE = phosphatidyl ethanolamine; C = cardiolpin; LPE = lyso-phosphatidyl ethanolamine; NL = neutral lipids; PC = phosphatidyl choline; Unk #2= unknown #2 lipid; Unk #1 = unknown #1 lipid
2) (%) = Per Cent;
3) N.D.= None Detected;
4) N.A.= Not Available

Table C-3. The *trans/cis* and saturation characteristics of fatty acids of individual lipids isolated from *P. aeruginosa* grown on glucose.

LIPIDS	PE [1]	LPE	PC	UNK #1	UNK #2	C	NL
			Early Exponential Phase				
16:1 *trans cis* (%)[2]	0.754 / 19.7	0.518 / 19.8	0.445 / 20.0	N.D. / 20.5	4.40 / 15.8	0.645 / 16.1	1.41 / 13.4
18:1 *trans cis* (%)	0.531 / 52.7	0.325 / 50.2	0.223 / 50.0	0.559 / 43.9	2.79 / 39.3	0.740 / 42.1	1.55 / 34.1
Total *trans cis* ratio (16:1 + 18:1)	0.0177	0.0120	0.00954	0.00868	0.130	0.0237	0.0621
Degree of Saturation (Sat./Unsat. FA's)	0.334	0.396	0.409	0.416	0.297	0.420	0.594
			Late Exponential Phase				
16:1 *trans cis* (%)	5.03 / 7.86	0.474 / 1.33	4.77 / 11.4	0.904 / 0.921	1.77 / 2.38	1.92 / N.D.	4.19 / 9.87
18:1 *trans cis* (%)	N.D.[3] / 47.2	0.817 / 9.24	1.75 / 43.7	1.78 / 5.55	2.80 / 15.7	N.D. / N.D.	2.29 / 37.4
Total *trans cis* ratio (16:1 + 18:1)	0.0914	0.122	0.118	0.415	0.252	N.A.[4]	0.137
Degree of Saturation (Sat./Unsat. FA's)	0.570	1.82	0.495	2.08	1.13	0.581	0.476

1) PE = phosphatidyl ethanolamine; C = cardiolpin; LPE = lyso-phosphatidyl ethanolamine; NL = neutral lipids; PC = phosphatidyl choline; Unk #2= unknown #2 lipid; Unk #1 = unknown #1 lipid
2) (%) = Per Cent;
3) N.D.= None Detected;
4) N.A. = Not Available

Table C-4. The *trans/cis* and saturation characteristics of fatty acids of individual lipids isolated from *P. aeruginosa* grown on furnace oil.

LIPIDS	PE [1]	LPE	PC	UNK #1	UNK #2	C	NL
			Early Exponential Phase				
16:1 *trans cis* (%)[2]	3.83 / 2.84	3.39 / 2.01	2.83 / 2.42	2.70 / 3.25	3.59 / 3.05	4.40 / 1.98	2.57 / 1.51
18:1 *trans cis* (%)	4.00 / 11.3	2.13 / 9.42	2.39 / 10.3	3.13 / 8.14	2.69 / 12.6	4.16 / 6.18	2.80 / 6.00
Total *trans cis* ratio (16:1 + 18:1)	0.556	0.483	0.411	0.511	0.403	0.330	0.715
Degree of Saturation (Sat./Unsat. FA's)	1.43	1.02	1.47	1.50	1.18	1.31	1.77
			Late Exponential Phase				
16:1 *trans cis* (%)	3.48 / 1.23	3.03 / 1.03	3.14 / 1.63	4.76 /N.D.[3]	2.77 / 1.66	1.60 / 0.909	4.53 / 0.625
18:1 *trans cis* (%)	3.13 / 3.55	1.98 / 3.53	2.11 / 5.57	0.325 / 0.589	9.31 / 8.59	8.45 / 5.18	1.25 / 1.63
Total *trans cis* ratio (16:1 + 18:1)	1.38	1.10	0.729	8.64	2.56	1.66	0.137
Degree of Saturation (Sat./Unsat. FA's)	2.83	3.22	2.25	0.441	0.451	1.24	0.944

1) PE = phosphatidyl ethanolamine; C = cardiolpin; LPE = lyso-phosphatidyl ethanolamine; NL = neutral lipids; PC = phosphatidyl choline; Unk #2 = unknown #2 lipid; Unk #1 = unknown #1 lipid
2) (%) = Per Cent;
3) N.D.. = None Detected;
4) N.A.. = Not Available

Table C-5. The *trans/cis* and saturation characteristics of fatty acids of individual lipids isolated from *P. aeruginosa* grown on aviation fuel.

LIPIDS	PE [1]	LPE	PC	UNK #1	UNK #2	C	NL
			Early Exponential Phase				
16:1 *trans cis* (%)[2]	2.77 / 2.00	2.89 / 0.769	4.06 / 0.879	4.14 / 0.811	2.43 / 2.27	1.57 / 1.58	2.64 / 10.2
18:1 *trans cis* (%)	4.20 / 10.9	2.62 / 5.08	3.77 / 5.66	3.90 / 5.53	2.43 / 6.66	1.47 / 3.47	3.23 / 31.7
Total *trans cis* ratio (16:1 + 18:1)	0.541	0.941	1.20	1.27	0.544	0.601	0.140
Degree of Saturation (Sat./Unsat. FA's)	1.52	0.959	2.29	2.36	1.40	1.04	0.573
			Late Exponential Phase				
16:1 *trans cis* (%)	1.05 / 2.19	11.8 / 0.658	0.753 / 0.223	0.174 / 0.308	14.0 / 1.40	13.0 / 0.915	0.563 / 0.183
18:1 *trans cis* (%)	1.87 / 8.42	9.38 / 1.02	N.D. / N.D.[3]	18.1 / 0.349	18.6 / 1.41	6.33 / 1.48	8.76 / 4.45
Total *trans cis* ratio (16:1 + 18:1)	0.275	12.7	3.37	27.9	11.6	8.04	2.01
Degree of Saturation (Sat./Unsat. FA's)	1.82	0.241	0.373	0.497	0.208	0.101	0.845

1) PE = phosphatidyl ethanolamine; C = cardiolpin; LPE = lyso-phosphatidyl ethanolamine; NL = neutral lipids; PC = phosphatidyl choline; Unk #2 = unknown #2 lipid; Unk #1 = unknown #1 lipid
2) (%) = Per Cent;
3) N.D. = None Detected;
4) N.A. = Not Available

Table C-6. The *trans/cis* and saturation characteristics of fatty acids of individual lipids isolated from *P. aeruginosa* grown on kerosene.

LIPIDS	PE [1]	LPE	PC	UNK #1	UNK #2	C	NL
			Early Exponential Phase				
16:1 *trans cis* (%)[2]	2.29 / 1.69	1.72 / 2.05	1.58 / 14.6	0.792 / 18.2	2.50 / 5.30	3.18 / 4.54	5.85 / 1.21
18:1 *trans cis* (%)	2.54 / 8.10	1.54 / 7.93	0.612 / 36.1	1.29 / 45.3	2.88 / 15.2	2.96 / 12.3	0.157 / 11.1
Total *trans cis* ratio (16:1 + 18:1)	0.494	0.327	0.0433	0.0327	0.262	0.364	0.487
Degree of Saturation (Sat./Unsat. FA's)	2.18	2.81	0.610	0.446	1.01	0.484	0.710
			Late Exponential Phase				
16:1 *trans cis* (%)	1.92 / 3.04	2.59 / 3.80	0.242 / 2.98	0.585 /0.191	0.832 / 0.0490	0.366 /0.103	0.790 / 0.914
18:1 *trans cis* (%)	2.06 / 8.89	0.167 / 10.4	1.31 / 11.3	0.0503 / 2.04	2.44 / 11.7	2.19 / 11.2	N.D.[3]. / 2.89
Total *trans cis* ratio (16:1 + 18:1)	0.334	0.194	0.109	0.285	0.278	0.225	0.208
Degree of Saturation (Sat./Unsat. FA's)	1.89	1.46	1.02	5.37	2.49	2.01	5.02

1) PE = phosphatidyl ethanolamine; C = cardiolpin; LPE = lyso-phosphatidyl ethanolamine; NL = neutral lipids; Unk #2 = unknown #2 lipid; Unk #1 = unknown #1 lipid; PC = phosphatidyl choline;
2) (%)' = Per Cent;
3) N.D.= None Detected;
4) N.A.= Not Available

Table C-7. Characteristics of cyclopropane fatty acids of *P. aeruginosa* grown on hexadecane

LIPIDS	TOTAL LIPIDS	PE [1]	LPE	PC	UNK #1	UNK #2	C	NL
			Early Exponential Phase					
C17:0 *cyclopropane* (%)[2]	5.12	5.09	6.17	8.60	6.12	7.02	8.31	7.06
C19:0 *cyclopropane* (%)	22.1	22.2	28.8	27.4	21.5	22.9	27.7	35.8
Total % cyclopropanes (C17:0 + C19:0)	27.3	27.3	35.0	36.0	27.7	29.9	36.0	42.9
			Late Exponential Phase					
C17:0 *cyclopropane* (%)	12.7	14.8	11.4	2.77	2.37	11.8	11.3	11.5
C19:0 *cyclopropane* (%)	31.5	35.99	48.2	12.9	14.4	32.7	43.4	38.7
Total % cyclopropanes (C17:0 + C19:0)	44.2	50.8	59.5	15.7	16.8	44.5	54.7	50.2

1) PE = phosphatidyl ethanolamine; C = cardiolpin; LPE = lyso-phosphatidyl ethanolamine; NL = neutral lipids; PC = phosphatidyl choline; Unk #2 = unknown #2 lipid; Unk #1 = unknown #1 lipid

2) (%) = Per Cent;

Table C-8. Characteristics of cyclopropane fatty acids of *P. aeruginosa* grown on glucose

LIPIDS	TOTAL LIPIDS	PE [1]	LPE	PC	UNK #1	UNK #2	C	NL
			Early Exponential Phase					
C17:0 *cyclopropane* (%)[2]	0.285	0.273	0.273	0.277	0.283	N.D.	0.303	N.D.
C19:0 *cyclopropane* (%)	0.579	0.519	0.490	0.527	0.534	N.D.[3]..	0.784	1.27
Total % cyclopropanes (C17:0 +C19:0)	0.864	,0.792	0.763	0.803	0.818	N.A.[4]..	1.09	1.27
			Late Exponential Phase					
C17:0 *cyclopropane* (%)	1.33	1.24	0.739	2.10	0.930	2.29	3.02	1.52
C19:0 *cyclopropane* (%)	3.75	3.55	4.55	5.21	1.20	9.44	7.55	3.47
Total % cyclopropanes (C17:0 +C19:0)	5.08	4.79	5.29	7.31	2.13	11.7	10.6	5.00

1) PE = phosphatidyl ethanolamine; C = cardiolpin; LPE = lyso-phosphatidyl ethanolamine; NL = neutral lipids; PC = phosphatidyl choline; Unk #2= unknown #2 lipid; Unk #1 = unknown #1 lipid
2) (%) = Per Cent;
3) N.D.. = None Detected;
4) N.A. = Not Available

Table C-9. Characteristics of cyclopropane fatty acids of *P. aeruginosa* grown on furnace oil

LIPIDS	TOTAL LIPIDS	PE [1]	LPE	PC	UNK #1	UNK #2	C	NL
			Early Exponential Phase					
C17:0 *cyclopropane* (%)[2]	9.55	10.7	10.3	11.5	10.4	12.8	11.1	9.67
C19:0 *cyclopropane* (%)	26.6	26.8	27.5	32.0	20.7	29.3	35.1	38.7
Total % cyclopropanes (C17:0 + C19:0)	36.1	37.5	37.8	43.4	31.1	42.1	46.1	48.4
			Late Exponential Phase					
C17:0 *cyclopropane* (%)	9.67	11.2	10.6	12.3	2.01	7.69	9.62	1.58
C19:0 *cyclopropane* (%)	33.3	37.2	36.4	29.5	12.1	19.4	36.4	10.2
Total % cyclopropanes (C17:0 + C19:0)	43.0	48.4	47.0	41.8	14.1	27.0	46.1	11.8

1) PE = phosphatidyl ethanolamine; C = cardiolpin; LPE = lyso-phosphatidyl ethanolamine; NL = neutral lipids; PC = phosphatidyl choline; Unk #2= unknown #2 lipid; Unk #1 = unknown #1 lipid

2) (%) = Per Cent;

Table C-10. Characteristics of cyclopropane fatty acids of *P. aeruginosa* grown on aviation fuel

LIPIDS	TOTAL LIPIDS	PE [1]	LPE	PC	UNK #1	UNK #2	C	NL
			Early Exponential Phase					
C17:0 *cyclopropane* (%)[2]	11.0	7.13	7.07	9.42	9.25	3.44	1.23	2.34
C19:0 *cyclopropane* (%)	32.5	27.4	27.1	34.2	34.6	10.8	6.05	8.81
Total % cyclopropanes (C17:0 +C19:0)	43.5	34.5	34.2	43.6	43.9	14.3	7.28	11.2
			Late Exponential Phase					
C17:0 *cyclopropane* (%)	9.13	9.86	12.1	3.32	19.4	1.85	8.28	11.8
C19:0 *cyclopropane* (%)	36.0	35.6	11.4	4.38	1.44	15.8	12.2	13.0
Total % cyclopropanes (C17:0 +C19:0)	45.2	45.5	23.5	7.70	20.8	17.7	20.5	24.8

1) PE = phosphatidyl ethanolamine; C = cardiolpin; LPE = lyso-phosphatidyl ethanolamine; NL = neutral lipids; PC = phosphatidyl choline; Unk #2= unknown #2 lipid; Unk #1 = unknown #1 lipid
2) (%) = Per Cent;

Table C-11. Characteristics of cyclopropane fatty acids of *P. aeruginosa* grown on kerosene

LIPIDS	TOTAL LIPIDS	PE [1]	LPE	PC	UNK #1	UNK #2	C	NL
			Early Exponential Phase					
C17:0 *cyclopropane* (%)[2]	11.8	11.8	9.94	3.75	1.22	4.27	1.10	1.15
C19:0 *cyclopropane* (%)	32.0	33.1	29.9	9.62	2.84	13.1	3.28	6.46
Total % cyclopropanes (C17:0 + C19:0)	43.8	45.0	39.8	13.4	4.06	17.4	4.38	7.60
			Late Exponential Phase					
C17:0 *cyclopropane* (%)	8.26	11.1	9.14	9.18	2.41	0.170	12.7	6.26
C19:0 *cyclopropane* (%)	30.9	31.9	29.9	21.9	8.22	24.7	26.8	35.7
Total % cyclopropanes (C17:0 + C19:0)	39.1	43.0	39.1	31.0	10.6	24.9	39.5	41.9

1) PE = phosphatidyl ethanolamine; C = cardiolpin; LPE = lyso-phosphatidyl ethanolamine; NL = neutral lipids; PC = phosphatidyl choline; Unk #2= unknown #2 lipid; Unk #1 = unknown #1 lipid

2) (%) = Per Cent

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IMAGE EVALUATION
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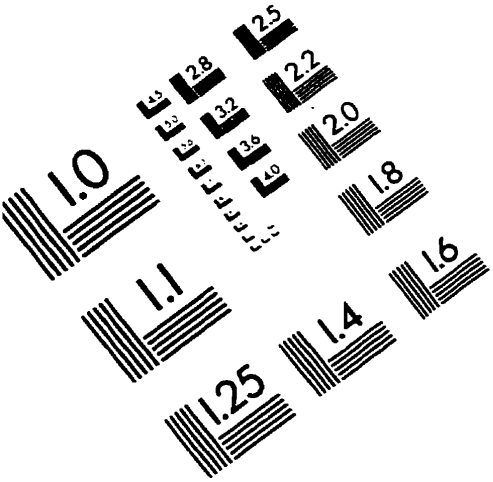

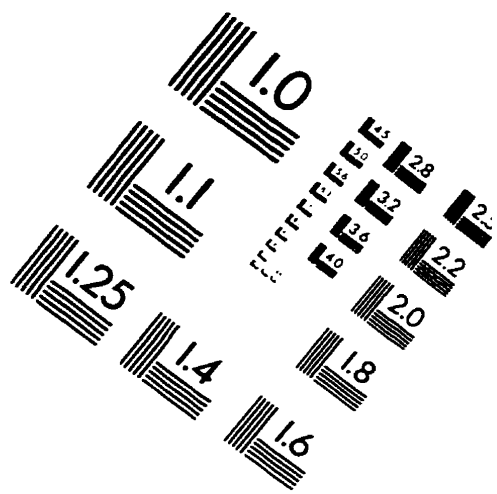

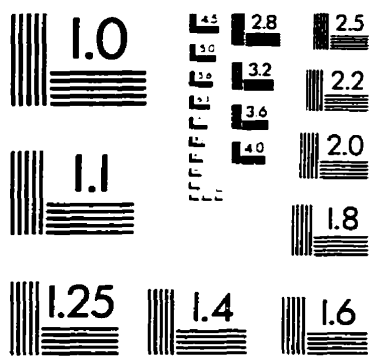

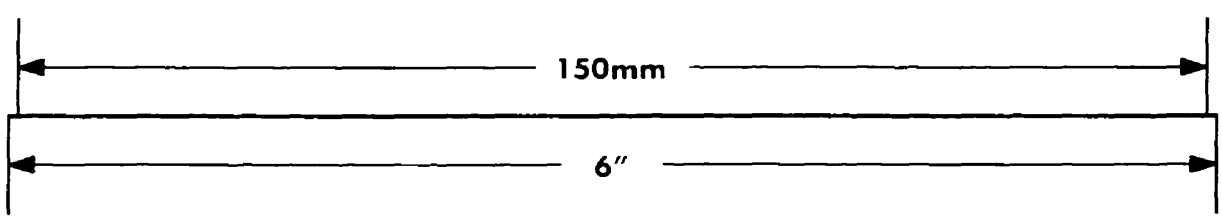

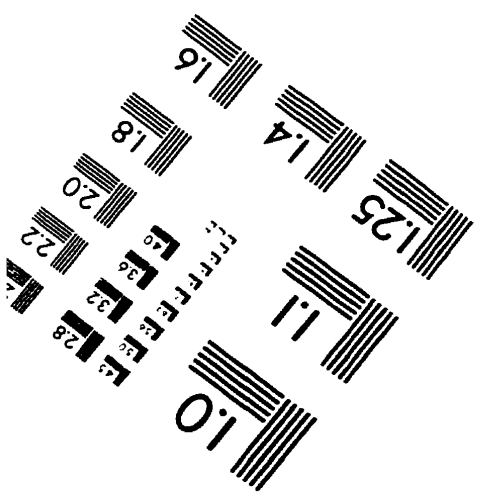

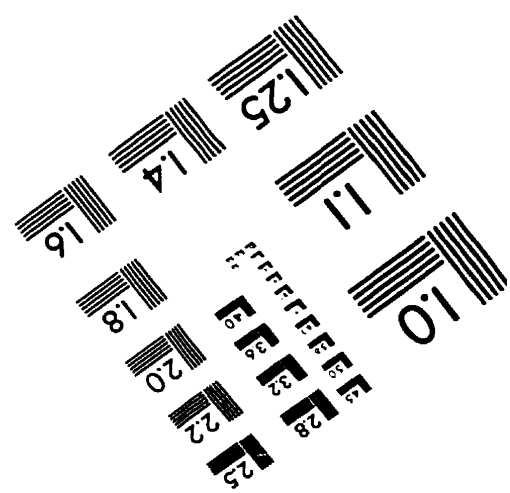

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