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THE LECTIN-LIKE PROPERTIES OF THE EXTRACELLULAR PROTEIN PRODUCED BY *PSEUDOMONAS AERUGINOSA* DURING HEXADECANE DEGRADATION

A

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Richard L. Smith, B.S.

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THE LECTIN-LIKE PROPERTIES OF THE EXTRACELLULAR PROTEIN PRODUCED DURING HEXADECANE DEGRADATION BY *PSEUDOMONAS AERUGINOSA*

By

Richard L. Smith

RECOMMENDED:

Advisory Committee Chair

Department Head

APPROVED:

Dean, College of Natural Sciences

Dean of the Graduate School

4-24-96

Date

ABSTRACT

Numerous microorganisms can degrade hydrocarbons and many produce extracellular compounds. These compounds are generally thought to emulsify hydrocarbons making them more available to the microorganism and stimulating growth. *Pseudomonas aeruginosa* produces both a rhamnolipid surfactant and an extracellular protein during growth on n-hexadecane ($C_{16}H_{34}$). The protein has been hypothesized to stimulate growth by emulsifying hexadecane. However, it has never been shown that the protein has hydrophobic properties characteristic of emulsifiers.

An isolation procedure was developed in this study that produces 20-30 mg of very pure protein per 500 ml culture of strain ATCC 17423. The protein is monomeric with a molecular weight of approximately 14,500 determined by SDS-PAGE. Structurally the protein is similar to two other proteins from strains S7B1 and PG210. The proteins from strain S7B1 and 17423 stimulate growth of *P. aeruginosa* on hexadecane.

The hydropathic index of the protein from strain PG201 shows no strong hydrophobic regions in the amino acid sequence. Also the isolated protein from strain 17423 will not bind during hydrophobic chromatography and always acts as a monomer in solution even at concentrations which should

cause hydrophobic proteins to aggregate. These results indicate that the protein is not hydrophobic and therefore does not have surfactant-like properties.

A surprising result from this investigation is that the protein from strain 17423 has lectin-like (carbohydrate binding) qualities and agglutinates *P. aeruginosa, Escherichia coli,* human type O, and horse red blood cells. The agglutination is inhibited by EDTA, glucose, mannose and rhamnose. The exact carbohydrate(s) the protein binds has not been determined but evidence suggests that it may bind the carbohydrate portion of the rhamnolipid surfactant.

A new model is presented describing the function of the protein. In this model the extracellular protein functions by binding the emulsified hydrocarbon to the outer membrane of the bacterium by both the carbohydrate on the glycolipid surfactant and the lipopolysaccharide of the bacterium. This binding of the hydrocarbon stimulates growth of the bacterium on hexadecane.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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INTRODUCTION

Researchers working with hydrocarbon-degrading microorganisms have known for over 50 years that strains of *Pseudomonas aeruginosa* have the ability to degrade straight chain hydrocarbons (n-alkanes). During alkane degradation the bacterium produces both a glycolipid surfactant and an extracellular protein [Jarvis and Johnson, 1949; Hisatsuka et al., 1972; 1977; Hardegger et al., 1994]. Both compounds stimulate growth of the bacterium when added to media containing alkanes as the sole carbon source. The increased growth is thought to be caused by emulsification of the alkane by the extracellular compounds. Emulsification of hydrocarbon by agitation or addition of surfactants has been shown to stimulate growth of hydrocarbon-degrading microorganisms [Erickson and Nakahara, 1975; Singer and Finnerty, 1984]. Emulsification disperses the hydrocarbon throughout the medium increasing the available surface area to the microorganisms.

The glycolipid surfactant produced by *P. aeruginosa* is a potent emulsifier, explaining its stimulatory effect upon the growth of the bacterium [Hisatsuka et al., 1972]. It has been hypothesized that the extracellular protein acts as a co-surfactant or an emulsion stabilizer and cooperates with the glycolipid surfactant in emulsification of alkanes

[Hisatsuka et al., 1972; 1977]. Cooperation between the two extracellular compounds is thought to lead to formation of pseudosolubilized hydrocarbon (less than 1 μm diameter) [Singer and Finnerty, 1984]. However, no studies have shown that the extracellular protein can bind to alkanes or that the protein has any hydrophobic characteristics that would give it surfactant-like properties. Furthermore, pseudosolubilization explains emulsification and transport of alkane but does not explain alkane uptake by the bacterium. How uptake occurs across the lipopolysaccharide envelope of gram negative bacteria is not known. The hydrophilic lipopolysaccharide envelope of *P. aeruginosa* is a barrier to uptake of hydrophobic compounds conferring resistance to many hydrophobic antibiotics [Siehnel et al., 1990].

The interactions between the emulsified hydrocarbon and the bacterium have not been described adequately. Many questions still remain concerning the function of the extracellular protein and its role in alkane transport and uptake by *P. aeruginosa*.

The first objective of this study was to determine if the extracellular protein has a structure that would give it surfactant-like qualities. The second objective was to use microbiological and biochemical techniques to understand how

the protein interacts with the hydrocarbon, surfactant, and bacterium. These associations could then be related to transport and uptake of the alkane by the bacterium.

The results of this study show that the extracellular protein is similar to two other extracellular proteins from strains S7B1 and PG201. The deduced amino acid sequence from PG201 shows no strong hydrophobic regions. Additionally, the protein from 17423 does not show any hydrophobic characteristic nor does it bind to hydrophobic columns. From observations such as these it appears that the protein is not strongly hydrophobic and therefore does not have surfactant-like qualities. The protein does, however, have a similar amino acid composition to two lectins (carbohydrate binding proteins) from a pathogenic strain of *P. aeruginosa* [Gilboa-Garber, 1986]. The extracellular protein from strain 17423 has lectin-like qualities and will agglutinate *P. aeruginosa*, *Escherichia coli*, human type O red blood cells and horse red blood cells. The agglutination is inhibited by EDTA, glucose, mannose and rhamnose. This study also provides evidence that the extracellular protein binds to the carbohydrate region of the glycolipid surfactant.

A model is proposed in which the extracellular protein cooperates with the glycolipid surfactant to emulsify

hexadecane, then binds the emulsified hydrocarbon to the bacterium. In this model the extracellular protein binds to the carbohydrates on the glycolipid surfactant and the bacterial lipopolysaccharide. In contrast to the prior hypothesis for the function of the protein, the lectin-like ability of the extracellular protein stimulates growth by allowing the emulsified hydrocarbon to adhere to *P. aeruginosa*.

LITERATURE REVIEW

Pioneering work with hydrocarbon-degrading microorganisms

The field of petroleum microbiology began at the turn of the century with the isolation of a methane-utilizing bacterium [Söhngen, 1906]. Another early study found that *Bacillus hexcarbovorum* could degrade hydrocarbons such as toluene, xylol and methane [Stormer, 1908]. The first description of microorganisms that degrade large insoluble hydrocarbons was again by Söhngen [1913] who isolated and characterized microbes from garden soil, compost and ditch water that could mineralize gasoline, kerosene, paraffin oil and paraffin wax. The major genera described in this study were *Mycobacterium* and *Pseudomonas*. An early indication that individual microorganisms are only able to degrade a limited range of hydrocarbons came from Tausz and Peter [1919] who isolated three hydrocarbon-degrading bacteria, two of which Bushnell and Haas [1941] later decided belonged to the genus *Pseudomonas*. These two bacteria could not degrade cyclic hydrocarbons or hexylene but could degrade many straight chain and branched alkanes.

From 1920 until 1940 about 30 papers were published describing new hydrocarbon-degrading isolates and environments in which they were found. The first review

paper concerning hydrocarbon-degrading microorganisms also summarized procedures for isolating these microorganisms [Bushnell and Haas, 1941]. The authors described a modified mineral salts medium from Söhngen [1913] that is used to this day for hydrocarbon-degrading bacteria. Since that time many hydrocarbon-degrading microorganisms have been isolated. A modern review article listed the top eight genera of hydrocarbon-degrading bacteria reported in the literature. These genera are *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Flavobacterium*, *Nocardia* and *Pseudomonas* [Leahy and Colwell, 1990].

The search for metabolic pathways used by microorganisms for hydrocarbon degradation began in the early 1940's. Hydrocarbon-degrading microorganisms were being found almost anywhere; in fact common garden soil was used for many enrichments [Stone et al., 1941]. These authors also found that the light to medium weight hydrocarbon fractions were being degraded first and that the paraffinic fractions (alkanes) were degraded faster than the aromatic hydrocarbons. Two other very important observations were made: 1) the degradation process is highly oxidative and 2) hydrocarbons are emulsified during degradation. These observations led to research into the transport, uptake and metabolism of hydrocarbons by

microorganisms [Stone et al., 1941].

Transport and uptake of hydrocarbons by microorganisms

Transport of hydrocarbons has been defined by Singer and Finnerty [1984] as the process of microbial interaction with hydrocarbons in the medium. Although transport and uptake may occur simultaneously, uptake of hydrocarbons has been defined as the movement of hydrocarbons across microbial membrane(s) to the sites of oxidation [Singer and Finnerty, 1984]. Transport of substrate to the microorganism is usually not considered important for solubilized substrates but is extremely important for large insoluble hydrocarbons. Transport of insoluble hydrocarbon may be facilitated by the bacterium actively seeking and adhering to large droplets or interacting with emulsified oil droplets [Erickson and Nakahara, 1975]. The bioavailablity of insoluble hydrocarbons to bacteria is enhanced by dispersing the hydrocarbon throughout the medium as small droplets [Erickson and Nakahara, 1975]. This process increases the available surface area of the hydrocarbon and increases the probability that the hydrocarbon droplets will contact the microorganisms. Mechanical agitation of hydrocarbon-containing cultures produces fine droplets of hydrocarbon that are easily

degraded and has been shown to enhance growth of hydrocarbon-degrading microorganisms [Erickson and Nakahara, 1975].

Microorganisms can also induce dispersion of hydrocarbons into the medium. It has been known for over 50 years that emulsification of hydrocarbons can occur during growth of the microorganisms [Stone et al., 1941]. Since then it has been shown that some microorganisms induce emulsification by adhering to droplets of hydrocarbon while other microorganisms secrete emulsification factors which have been named "biosurfactants" [Singer and Finnerty, 1984; Van Dyke et al., 1991].

Biosurfactants

Many biosurfactants have been isolated from bacteria, yeast, and fungi. General categories have been used to describe these biosurfactants as lipids, glycolipids, lipopeptides, and proteins [Singer and Finnerty, 1984; Van Dyke et al., 1991]. The function of these extracellular compounds is thought to be emulsification of the insoluble hydrocarbons. One of the first biosurfactants studied was a rhamnose biosurfactant from *Pseudomonas aeruginosa* [Jarvis and Johnson, 1949]. Many surfactants have been discovered and new biosurfactants show up routinely in the literature.

Table 1 shows a few representative hydrocarbon-degrading microorganisms and their corresponding biosurfactants.

One physical property of hydrocarbons is that, as they become chemically larger and more hydrophobic, they become harder to disperse. It is possible for hydrocarbon-degrading bacteria to degrade larger hydrocarbons than they are acclimated to if the bio-availability can be increased. For example, *P. aeruginosa* can degrade hexatricontane (C_{36}), an extremely insoluble hydrocarbon if it is first encapsulated into a liposome [Miller and Bartha, 1989]. *P. aeruginosa* ATCC 9027 which can normally only degrade hexadecane ($C_{16}H_{34}$) could easily degrade octadecane ($C_{18}H_{38}$) when the hydrocarbon was first emulsified with the rhamnolipid surfactant produced by the same bacterium [Zhang and Miller, 1992]. Studies such as these have shown that emulsification of insoluble hydrocarbon in the medium is extremely important for hydrocarbon-degrading microorganisms.

Uptake of alkanes by microorganisms

Many microorganisms undergo morphological changes when grown on hydrocarbons that may be related to uptake [Singer and Finnerty, 1984]. These changes include extra intracellular membranes and/or cytoplasmic hydrocarbon

Table 1. Hydrocarbon degrading microorganisms and their associated biosurfactants.

Microorganism	Emulsifier	Reference
Pseudomonas aeruginosa	Rhamnolipid	Jarvis and Johnson [1949]
Mycobacterium rhodochrous	Emulsifying factor (undetermined)	Holdom and Turner [1969]
Corynebacterium	Emulsifier, composed of protein, lipid and carbohydrate	Zajic et al. [1977]
Acinetobacter calcoaceticus	Emulsifier, "Emulsan", composed of protein, lipid and carbohydrate.	Rosenberg et al. [1979]
Nocardia erythropolis	Emulsifier composed of polar and neutral lipid.	MacDonald et al. [1981]
Arthrobacter paraffineus	Emulsifying factor (undetermined)	Duvnjak et al., [1982]
Arthrobacter paraffineus	Glycolipid	Li et al. [1984]
Rhodococcus H13A	Glycolipid and Protein	Bryant [1990]
Arthrobacter MIS38	Lipopeptide "Arthrofactin"	Morikawa et al. [1993]
Candida lipolytica	Glycoprotein "Liposan"	Cirigliano and Carman [1985]
Torulopsis petrophilum	Glycolipid surfactant or protein emulsifier	Cooper and Paddock [1983]

inclusion bodies which store unmodified hydrocarbon. Compared to hydrocarbon transport, the uptake of hydrocarbons has been largely ignored. It is generally believed that uptake is a passive process where hydrocarbons diffuse into the hydrophobic portions of the cell membrane [Singer and Finnerty, 1984; Munk et al., 1969].

A transport mechanism has been proposed for more soluble types of hydrocarbon called vectorial partitioning. In this model the lipophilic substrate has a greater affinity for the cell membrane than the medium. Thus as the hydrocarbon becomes oxidized it becomes trapped in the cell [Button, 1985].

Methane-utilizing bacteria (methanotrophs) grow on dissolved methane. Methanotrophs have an extensive intracytoplasmic membrane system contiguous with the cell plasma membrane which is related to their methane-oxidizing ability [Singer and Finnerty, 1984]. It has been suggested that methane diffuses into the cell through the membrane system where it can be metabolized [Vestal, 1984]. The membrane system differs between two groups of methanotrophs. Type 1 methanotrophs have extensive bundles of vascular disks while type 2 have paired membranes around the inside of the cell [Vestal, 1984].

Extra intracellular membranes have also been found

associated with microorganisms that degrade larger hydrocarbons. *Acinetobacter* species HO1-N has no intracytoplasmic membrane when grown on non-hydrocarbon substrate. However, when this bacterium is grown on hexadecane, intracytoplasmic membranes that stretch across the length of the cell can be seen with electron microscopy [Singer and Finnerty, 1984]. It is believed that these membranes are involved with hydrocarbon metabolism [Kennedy et al., 1975]. Four possible roles for the extra membrane in *Acinetobacter* have been hypothesized.

> 1) the enzymes responsible for initial oxidation of the alkane molecule, and/or for its subsequent oxidation to fatty acid, may be associated with the membrane system as integral or peripheral proteins, 2) the intracytoplasmic membrane may provide a suitable lipid-rich hydrophobic environment necessary for the oxidation of alkane, 3) the intracytoplasmic membrane may provide a continuous channel for alkane transfer from the cytoplasmic membrane to the intracellular site of alkane oxidation or to the hydrocarbon inclusions, or 4) the intracytoplasmic membrane may be the site of increased respiratory activity, providing additional membrane for the increased total cytochrome content of hexadecane-grown *Acinetobacter* (Singer and Finnerty, 1984).

Another unique feature to some hydrocarbon-degrading microorganisms is the presence of hydrocarbon inclusion bodies. These inclusion bodies are cytoplasmic bodies associated with the cell membrane and composed of non-

modified hydrocarbon surrounded by a monolayer membrane [Singer and Finnerty, 1984]. The production of hydrocarbon inclusion bodies is very closely linked to uptake of hydrocarbon. Hydrocarbon inclusions have been found in *Arthrobacter* sp., *Corynebacterium* sp., *Mycobacterium album lipolytica*, *Candida tropicalis* and naphthalene-grown *Pseudomonas* species [Scott and Finnerty, 1976].

Acinetobacter HO1-N seems to have a unique system for transport and uptake of hydrocarbons. This microorganism produces an emulsification factor that has been identified as an extracellular membrane vesicle [Kappeli and Finnerty, 1979]. This membrane vesicle is very similar to the outer membrane of the bacterium and is composed of protein, phospholipid, lipopolysaccharide and hexadecane. In an uptake experiment, ^{14}C labeled hexadecane solubilized with these vesicles was found 30 minutes later inside hydrocarbon inclusion bodies in the bacterium. This uptake of hexadecane by *Acinetobacter* was sensitive to metabolic inhibitors and uncouplers and therefore may be associated with an active transport process [Ventullo, 1978].

Evidence that uptake can occur by hydrocarbon diffusion comes from studies of hydrocarbon-degrading yeasts [Munk et al., 1969]. Electron microscopy was used to show that hydrocarbon is first adsorbed onto the cell surface. The

hydrocarbon then penetrates the cell wall and accumulates in the cytoplasmic membrane. During the study radiolabeled hydrocarbons were used to show that hydrocarbons were rapidly incorporated into the cell from the medium.

Microbial metabolism of alkanes

The general oxidative processes that occur during hydrocarbon degradation have been elucidated. The first microorganisms studied were methane-degrading bacteria. A methane-utilizing bacterium similar to the one isolated by Söhngen in 1906 was found to oxidize methane, methanol, formaldehyde and formate [Dworkin and Foster, 1956]. From these data the authors speculated that the bacterium oxidized methane step-wise to carbon dioxide via methanol, formaldehyde and formic acid (Fig. 1A). This work was supported when formaldehyde and formic acid were identified as intermediates of metabolism by *Methanomonas methanooxidans* [Brown and Strawinski, 1958].

Molecular oxygen has been shown to be essential for oxidation of larger hydrocarbons. In studies using $[^{18}O]O_2$, oxygen was shown to be essential for oxidation of hexadecane to palmitic acid by a gram negative cocci. The oxidation was shown to occur at the number one carbon of hexadecane [Stewart et al., 1959]. Similarly, octane is oxidized to

A

$CH_4 \rightarrow CH_3OH \rightarrow CH_2O \rightarrow CHO_2 \rightarrow CO_2$

Methane Methanol Formaldehyde Formic acid Carbon dioxide

B

$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_3$

Hexadecane

↓

$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2OH$

Hexadecanol

↓

$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CHO$

Hexadecanal

↓

$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CHO_2$

Hexadecanoic acid (Palmitic acid)

Fig. 1. Proposed mechanism of microbial oxidation of methane to carbon dioxide (A). Proposed mechanism of microbial oxidation of hexadecane to palmitic acid (B).

octanol and octaldehyde by a cell-free soluble enzyme preparation from a *Pseudomonas* species [Baptist, 1963]. Hexadecane has also been shown to be oxidized by a non-membrane bound three component enzyme system to hexadecanol-1 by *P. aeruginosa* [Matsuyama and Nakahara, 1981].

A general mechanism for hydrocarbon oxidation involving two intermediate chemical species has emerged from these studies. First the hydrocarbons are oxidized to alcohols, aldehydes and then carboxylic acids (fatty acids) (Fig. 1B). The fatty acids can then either be degraded using conventional β oxidation enzymes or used to produce membrane lipids [Singer and Finnerty, 1984].

Evidence to support this general model of hydrocarbon oxidation also comes from the study of microbial membranes. When *Micrococcus cerificans* is grown on non-hydrocarbon substrates the fatty acids found in the membranes have an even number of carbons [Makula and Finnerty, 1970]. When grown on hydrocarbons there is a direct relationship between the number of carbons in the hydrocarbon skeleton of the substrate and the number of carbons found in the membrane fatty acids. When the same bacterium was grown on odd number carbon substrates, there appeared both odd numbered and even numbered fatty acids in the membrane. Another study showed that alkane grown *Micrococcus cerificans*

contained 50% more lipid phosphate as phospholipids compared to cells grown on non-hydrocarbon substrates [Makula and Finnerty, 1970]. Additionally, the authors found that the activity of an enzyme used in phospholipid synthesis, phosphatic acid cytidyl transferase, is 50% greater for alkane grown cells then cells grown on non-hydrocarbon substrates.

Genetics of alkane oxidation

Very little is known about the genetics of alkane oxidation by microorganisms. *P. aeruginosa* ATCC 17423 (the microorganism used in this study) could be classified into two groups by mutations; those that could oxidize alkanes of chain length C_6-C_{11} and those that could oxidize alkanes of chain length C_{12}-C_{16} [Nieder and Shapiro, 1975]. The authors concluded that two separate alkane-oxidizing systems were used by the bacterium. *Acinetobacter* HO1-N can degrade alkanes of carbon length C_{12}-C_{18}. No plasmids have been observed for this organism and it is believed that the genes for alkane degradation are chromosomal [Singer and Finnerty, 1984]. Compared to *P. aeruginosa* which has two oxidative systems only one could be found in *Acinetobactor* HO1-N for growth on alkanes. Because it has only one oxidative system, studies of 120 mutants of *Acinetobactor* HO1-N found

that none could grow on alkanes [Singer and Finnerty, 1984].

The most extensively studied genetic system is that of *Pseudomonas putida*. This bacterium can degrade short (C_6-C_{10}) alkanes. The genes encoding the oxidation enzymes are found both on a plasmid and on the chromosome. Degradation of octane by *P. putida* is alkane inducible [Chakrabarty, 1973]. Three proteins are responsible for the initial hydroxylation of octane and the genes for at least two of these proteins are found on the "Oct" plasmid, at loci called *alkA* and *alkB* [Benson and Shapiro, 1975; Benson et al., 1977]. Three loci are responsible for the oxidation of the corresponding alcohol, called *alcA*, *alcB* and *alcC* and are also found on the Oct plasmid [Benson and Shapiro, 1975]. The gene products, however, that oxidize the corresponding aldehydes to fatty acids are constitutively expressed and chromosomally encoded. These loci are called *aldA* and *aldB* [Grund et al., 1975]. An Oct plasmid regulatory locus near *alk*B that regulates *alk*A, B, and C has been found and probably produces a protein for inducer recognition [Fennewald and Shapiro, 1977]. Information about the molecular size of the plasmid or the loci was not available.

Alkane transport and uptake by *P. aeruginosa*

P. aeruginosa has been shown to grow on alkanes with carbon chains ranging from C_6-C_{16}. Growth of this microorganism is inhibited by EDTA but the inhibition can be reversed by adding additional Ca^{2+} to the medium [Hisatsuka et al., 1975; Goswami and Singh, 1990]. *P. aeruginosa* produces both a rhamnose surfactant and an extracellular protein that are thought to participate in transport of alkanes to the bacteria [Hisatsuka et al., 1977]. Chemical analysis of the rhamnose surfactant showed that it was composed of two units of L-rhamnose and two units of hydroxydecanoic acid which are also common components of the lipopolysaccharide cell envelope. The structure of this molecule has been deduced to be 2-O-α-rhamno-pyranosyl-β-hydroxy decanoate (Fig. 2) [Edwards and Hayashi, 1965]. *P. aeruginosa* produces two rhamnolipid surfactants while growing on n-paraffin. The first called R1 has two rhamnose units similar to the one isolated by Jarvis and Johnson [1949] while the second, R2, is composed of only one unit of rhamnose and is thought to be the precursor of R1 [Itoh et al., 1971]. When the purified rhamnose surfactant was added to cultures of *P. aeruginosa* growing on hexadecane it dramatically stimulated growth [Hisatsuka et al., 1971]. However, in the same study it was found that purified rhamnose surfactant had no effect on the growth of other

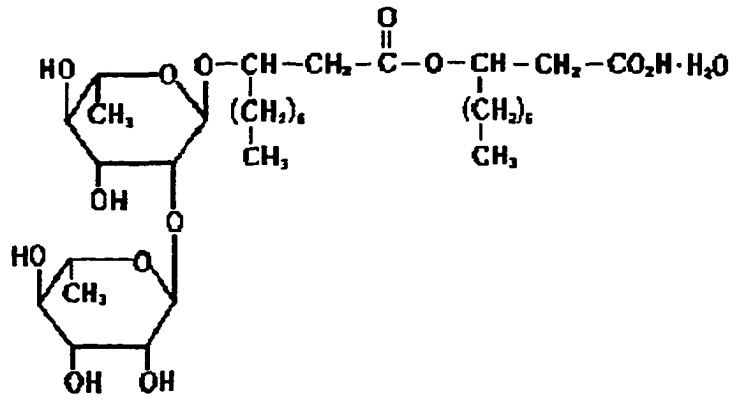


Fig. 2. Rhamnolipid surfactant from *P. aeruginosa*.

hexadecane degraders including *Corynebacterium hydrocarboclastus, Achromobacter cycloclastus, Bacillus* or two genera of *Micrococcus*. Evidence that the rhamnolipid surfactant participates in the transport process but not uptake of alkane comes from studies with [^{14}C]-rhamnolipid. It was found that the rhamnolipid was not incorporated into *P. aeruginosa* even after incubating cells with radioactive rhamnolipid surfactant for 48 hr [Zhang and Miller, 1994]. Extracellular proteins produced by microorganisms that are involved in hydrocarbon transport have also been discovered [Bryant, 1990; Cirigliano and Carman, 1985; Rosenberg et al., 1979; Singer and Finnerty, 1984; Zajic, 1977], but the function of these proteins is not clearly understood. The best characterized protein is produced by *P. aeruginosa* S7B1 when grown on hexadecane [Hisatsuka and Nakahara, 1972]. The extracellular protein stimulates growth of *P. aeruginosa* on hexadecane. Growth was greatly stimulated when both the protein and the rhamnolipid surfactant were combined in the medium. The authors speculated that the protein had a high affinity for hexadecane and cooperated with the rhamnolipid surfactant in emulsification.

Using gel filtration and electrophoresis, the extracellular protein was shown to be a monomer with a molecular weight of 14,300. The activity of the protein had

a high resistance to heat and pH [Hisatsuka et al., 1977]. The protein is composed of 147 amino acids and is rich in acidic and hydroxy residues. No histidine or arginine residues were detected. The same study also showed that as the carbon substrate became larger and more hydrophobic, the bacterial culture produced more protein. The extracellular protein was not found when *P. aeruginosa* was grown on glucose, glycerol or palmitic acid. These observations led to the conclusions that the function of the protein might be to help form very small droplets of solubilized hydrocarbon.

Very little is known about alkane uptake by *P. aeruginosa*. It is thought alkanes diffuse directly into cell membranes rather than by being taken up by active transport [Singer and Finnerty, 1984; Munk et al., 1969]. No intracytoplasmic membranes or inclusion bodies have been observed in *P. aeruginosa*. One barrier to alkane uptake is the very hydrophilic lipopolysaccharide layer.

P. aeruginosa is a typical gram negative rod-shaped bacterium with a cytoplasmic membrane, a cell wall and an outer membrane that is an asymmetric bilayer composed of an outer lipopolysaccharide layer and an inner lipid layer. The lipopolysaccharide molecule has three regions; the first being the inner "lipid A" region. The second region is the "core" polysaccharide region and the third is the outer or

"O antigen" region that can be composed of 60-80 monosaccharides in non-hydrocarbon grown cells [Horton, 1983]. Interestingly, many serotypes of this bacterium have up to 50% rhamnose in the "O antigen" region while one of the principal components of the "lipid A" region is hydroxy decanoate. These molecules are also the major components of the rhamnolipid surfactant this bacterium produces when growing on alkanes [Jarvis and Johnson, 1949]. One way for the bacterium to overcome the lipopolysaccharide barrier in alkane uptake might be to change the structure of the lipopolysaccharide. *P. aeruginosa* ATCC 9027 has been shown to have 30 to 40% less carbohydrate in its lipopolysaccharide when grown on hydrocarbon than when grown on glucose [Miguez et al., 1986]. The authors hypothesized that this change would make the bacterium more hydrophobic yet they never observed the bacterium adhering to hydrocarbon.

It is possible that different strains of *P. aeruginosa* have different alkane transport processes. For example two different hexadecane transport processes have been observed in two pseudomonads, M1 and N1 [Goswami and Singh, 1990]. *Pseudomonas* M1 adhered to large hexadecane droplets, produced no detectable emulsifier and was not affected by EDTA. *Pseudomonas* N1, however, did not adhere to large

hydrocarbon droplets but instead interacted with small droplets of emulsified hydrocarbon. N1 also produced a possible glycoprotein and bioemulsifier and was inhibited by EDTA.

Adherence seems to be very important for hydrocarbon degradation in general, either by microbial adherence to large droplets, or the adherence of small droplets to the microorganism [Singer and Finnerty, 1984; Erickson and Nakahara, 1975].

Lectins and microbial adherence to cell surfaces

A lectin, as defined by Goldstein and Poretz [1986], is a "sugar-binding protein or glycoprotein of nonimmune origin that agglutinates cells and/or precipitates glycoconjugates." The first lectins to be discovered were from plant seed extracts and two examples of these are Ricin and Concanavalin A.

Ricin, the toxin from the castor bean (*Ricinus communis*) is a lectin first extracted in 1887 and assumed to be a protein [Goldstein and Poretz, 1986]. This assumption was later confirmed. More recent research has shown that Ricin agglutinates human red blood cells (rbc) and specifically binds the carbohydrates N-acetylgalactosamine and galactose [Goldstein and Poretz, 1986]. Concanavalin A

is a plant lectin extracted from the Jack bean (*Canavalia ensiformis*). Concanavalin A agglutinates red blood cells and specifically binds polysaccharides of mannose, monomers of mannose, and glucose [Goldstein and Poretz, 1986].

Lectins have also been discovered in association with microorganisms. Approximately 100 microbial species have been shown to produce lectins; 90% of these microorganisms are pathogenic for higher organisms [Mirelman and Ofek, 1986]. Microbial lectins are frequently found on the bacterial cell surface and are used for adherence to other cell surfaces. Many adherent bacteria have from 100 to 400 hairy appendages called "fimbriae".

Six groups of fimbriae have been defined: group 1 is the adherent fimbriae, group 2 are the sex pili, group 3 is from *Agrobacterium sp.* with an unknown function, group 4 is from *Pseudomonas* and *Vibrio* and contribute to twitching motility and possibly adherence, group 5 allows soil microorganisms to form clusters with themselves promoting conjugation, and group 6 is from *Corynebacterium renale* and the function is unknown [Ottow, 1975].

Group 1 fimbriae from *E. coli* are composed of identical subunits of a protein called "pilin" which has 163 amino acids (MW 16,600). These subunits are arranged in a right hand helix with a hollow core [Brinton, 1965]. It has been

known for many years that adding *E. coli* to red blood cells can cause agglutination and that this agglutination is inhibited by mannose [Sharon and Ofek, 1986]. Isolated fimbriae from *E. coli* can agglutinate erythrocytes and monkey kidney cells [Rivier and Darekar, 1975; Salit and Gotschlich, 1977]. Agglutination of epithelial cells was shown to be inhibited by adding mannose which lead to the conclusion that *E. coli* pilin is mannose specific [Sharon and Ofek, 1986].

Fimbriae have been found associated with pathogenic strains of *P. aeruginosa* [Martin et al., 1993]. These fimbriae are from group 4 and are also found associated with *Neisseria gonorrhoeae, Neisseria meningitides, Moraxella bovis* and *Dichelobacter nodosus*. The fimbriae from *P. aeruginosa* are found on the polar regions of the cell and contribute to "twitching" motility and adhesion to epithelial cells. Species of *P. aeruginosa* with fimbriae are easily identified on agar plates because the twitching behavior causes colonies to spread out and have serrated edges compared to the smooth dome shaped colonies of non-fimbriated species [Martin et al., 1993]. *P. aeruginosa* ATCC 17423 used in this study is a non-fimbriae species.

Other lectins produced by bacteria are not attached to the cell membrane. One interesting example is a lectin

produced by *Streptomyces* sp. The lectin from this bacterium is a monomer of approximately 5,000 molecular weight that can agglutinate human group B red blood cells. The protein also has a strong affinity for D-galactose and L-rhamnose [Fujita et al., 1973], however, agglutination of *Streptomyces* by the lectin was not reported. Two other non-membrane bound lectins called PA-I and PA-II have been reported from a hospital strain of *P. aeruginosa* (ATCC 33347) [Gilboa-Garber, 1986]. PA-I and PA-II have been found to agglutinate erythrocytes from many kinds of animals, yeast, and some strains of *E. coli* and *Chlamydomonas reinhardi*. PA-I and PA-II, however, cannot agglutinate *P. aeruginosa*. Agglutination by PA-I and PA-II is dependent upon divalent cations and is inhibited by EDTA. PA-I has been shown to principally bind galactose while PA-II is specific for mannose [Gilboa-Garber, 1986]. The function of these *Pseudomonas* lectins has not yet been determined. PA-I and PA-II are very similar to many plant lectins which have approximately the same molecular weight and are also dependent upon divalent cations for binding to cells. Agglutination of red blood cells by many plant lectins is inhibited by EDTA [Goldstein and Poretz, 1986].

The extracellular protein from the alkane-degrading strain of *P. aeruginosa* (S7B1) is similar to the two

lectins, PA-I and PA-II, in that: they are all very heat resistant, they have similar molecular weights, (14,300 for S7B1 compared to approximately 13,000 for PA-I and PA-II), they are rich in acidic and hydroxy amino acids, and the binding activities of PA-I and PA-II are inhibited by EDTA. Alkane mineralization by *P. aeruginosa* S7B1 is also dependent upon divalent cations and is inhibited by EDTA [Hisatsuka et al., 1975; Goswami and Singh, 1990]. Although it may be coincidental, EDTA added to cultures of *P. aeruginosa* may also interfere with the function of the extracellular protein from S7B1.

Summary

Since the early 1900's when hydrocarbon-degrading microorganisms were first discovered, many studies have focused on isolating new strains and examining their metabolism. Other studies have focused on biosurfactants produced by hydrocarbon-degrading microorganisms. Fewer studies have focused on the process of transport and uptake of hydrocarbon by these microorganisms. Major questions remain about how cells interact with hydrocarbons and how hydrocarbons enter the cell.

Many of the studies concerning transport of hydrocarbon were conducted with the purpose of maximizing cell growth in

laboratory bioreactors [Erickson and Nakahara, 1975] or increasing cell growth by emulsifying hydrocarbon with biosurfactants [Singer and Finnerty, 1984; Van Dyke et al., 1991]. It has been hypothesized from these studies that agitation and emulsification stimulate growth by dispersing hydrocarbon into small droplets which interact with the microorganisms. But how the droplets interact with the microorganisms has not yet been described.

Studies concerning uptake of hydrocarbon, or the translocation of hydrocarbon across microbial membranes, have been limited to a few hydrocarbon-degrading bacteria [Kappeli and Finnerty, 1979; Ventullo, 1978] and electron microscopy of hydrocarbon-degrading yeasts [Munk et al., 1969]. Major questions remain unanswered concerning the mechanism of uptake by hydrocarbon-degrading microorganisms.

It is evident from studies with biosurfactants that the interactions that occur with hydrocarbon during transport and uptake are very important. A multitude of microorganisms degrade hydrocarbons and each species seems to use its own unique biosurfactant [Singer and Finnerty, 1984]. Biosurfactants produced by one species may have no effect upon another species [Hisatsuka, et al., 1971; Van Dyke et al., 1991]. In addition, synthetic surfactants can stimulate, have no effect, or even inhibit hydrocarbon

degradation [Nakahara and Hisatsuka, 1981; Foght and Westlake, 1982; Laha and Luthy, 1992]. These observations would indicate that mechanisms of hydrocarbon transport and uptake are more complex than the simple emulsification of hydrocarbon.

One method to study the interactions that occur during transport and uptake of hydrocarbon is to study the biosurfactants and other extracellular products that the microorganisms produce. These extracellular products are custom tailored for the microorganism that produced them and can provide valuable information about how a particular microorganism interacts with hydrocarbon. My research focuses on an extracellular protein produced during hydrocarbon degradation by *P. aeruginosa* and on how the protein affects transport and uptake of the linear alkane, hexadecane, by the bacterium.

MATERIALS AND METHODS

Bacterial strain and growth conditions.

The strain of *P. aeruginosa* used in this study can grow on alkanes from carbon length C_6-C_{16} [Nieder and Shapiro, 1975]. The strain (#17423) was obtained from the American Type Culture Collection (ATCC). The bacterium was maintained in 20 ml culture tubes with 10 ml Bushnell-Haas (BH) medium [Bushnell and Haas, 1941] modified with 1/10 ferric chloride and supplemented with 1% v/v n-hexadecane (Sigma) as a sole carbon source. The medium consists of 1 g NaCl, 1 g KH_2PO_4, 1 g K_2HPO_4, 1 g NH_4NO_3, 0.4 g $MgSO_4$, 0.02 g $CaCl_2$, 0.05 g $FeCl_2$, made to 1 L with distilled water (DI), then adjusted to pH 7.0 with 10 N NaOH. The culture tubes were kept at ~25°C and sub cultured every seven days. Stock cultures were stored on nutrient agar slants at 4°C.

Purification of the extracellular protein

Initial extracellular protein purification. The procedure of Hisatsuka et al., [1972] was initially followed. Fractions were collected at intervals and tested for stimulation of hexadecane degradation using the radiorespiormetric technique of Brown et al., [1991](Fig.3). Briefly, a 400 ml culture of *P. aeruginosa* in BH medium with

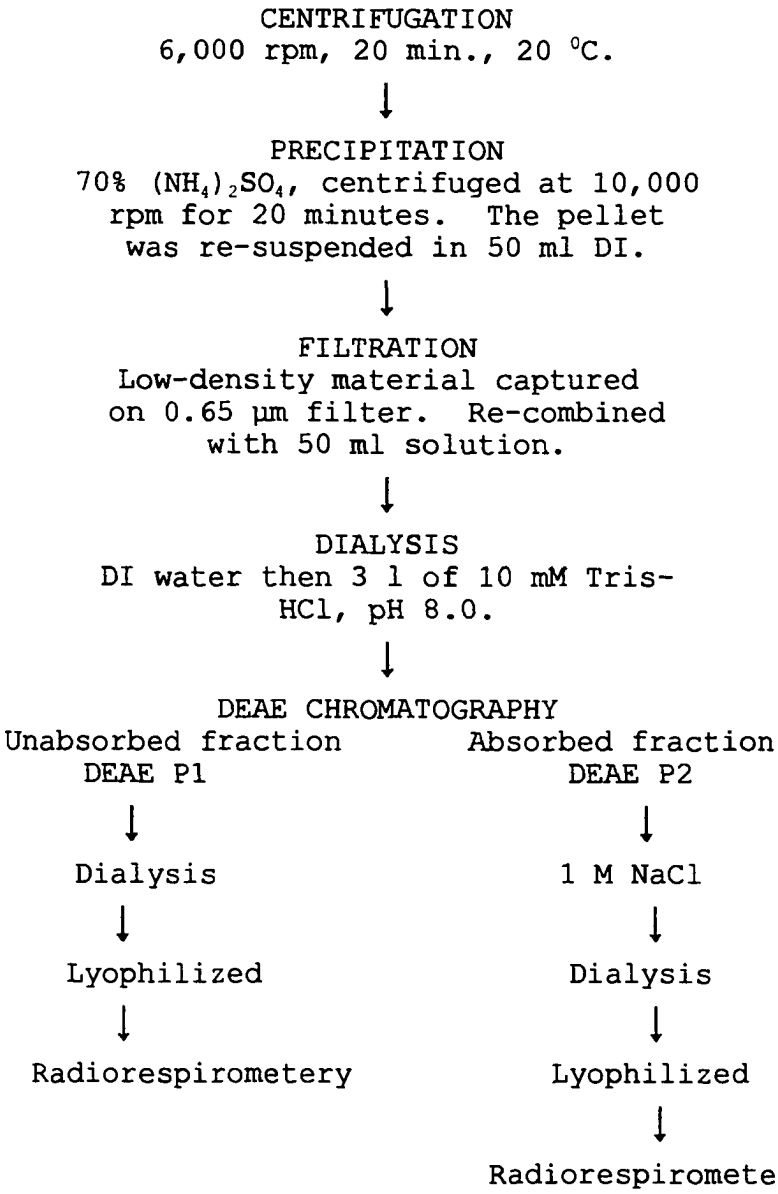


Fig. 3. Initial isolation of two solutions from a three day, 400 ml culture (1% v/v hexadecane) of *P. aeruginosa*.

4 ml of hexadecane (1% v/v, final concentration) was allowed to grow for approximately three days. The bacteria were then removed by centrifugation for 20 minutes at 10,000 rpm with a Sorvall RC2-B automatic refrigerated centrifuge. Solid ammonium sulfate was added to the supernatant to make a 70% solution which was allowed to settle overnight at 4°C. The solution was centrifuged the following day at 10,000 rpm for 20 minutes. Low-density material that would not pellet was captured by filtration through a 0.65 μm filter (Millipore, Bedford MA). The filtered material was combined with the pellet which had been dissolved in 50 ml DI water. This solution was dialyzed against DI water then 10 mM tris buffer, pH 8.0, overnight at 4°C. The dialyzed solution was passed through a diethylaminoethyl (DEAE) anion exchange column and 5 ml fractions of material that did not bind to the column were collected without elution. Material bound to the column was eluted with 1 M NaCl in the same buffer and 5 ml fractions were again collected. Fractions with absorbance at 280 nm (A_{280}) were pooled to produce two solutions, DEAE P1 (material that did not bind to the column) and DEAE P2 (material that did bind to the column). Both solutions were dialyzed overnight against DI water. The protein concentrations of the solutions were determined using a modified version of the Lowry method [Markwell et

al., 1978]. Two mg aliquots were lyophilized and stored at -20°C (Fig. 3).

Stimulation of hexadecane degradation by P. aeruginosa using DEAE P1 and P2. Both DEAE 1 and 2 were tested for their ability to stimulate growth of *P. aeruginosa* on [1-^{14}C]-hexadecane using radiorespirometry according to the method of Brown et al. [1991]. Four hundred ml of an overnight culture of *P. aeruginosa* grown on nutrient broth (Difco) was centrifuged at 5,000 rpm for 20 minutes. The pellets were washed twice and re-suspended in BH medium. One ml of this suspension was added to 40 ml vials (I-Chem Research, Hayward, CA) previously filled with 9 ml of BH medium (controls) or 8 ml of BH medium and 1 ml of solution to be tested for activity (800 µg/ml, Lowry protein assay). Radioactive [1-^{14}C] hexadecane (Sigma, St. Louis, MO) was prepared by diluting in acetone and adding unlabeled hexadecane to yield a final concentration of 2 g/l. During the experiments 50 µl of this solution was injected into each vial for a final concentration of 44.2 nM which had an activity of approximately 60,000 disintegrations per minute. The samples were allowed to incubate for 48 hr after which the activity of the bacteria was stopped and the CO_2 trapped by injecting each vial with 1 ml 10 N NaOH.

The ^{14}C-CO_2 was captured and quantified according to

the method of Brown et. al [1991]. The samples were acidified with 1.5 ml 12 N HCl to release CO_2, then purged for 15 minutes with N_2 gas. Volatile hydrocarbon products were captured with in-line Harvey traps containing acidified toluene (Harvey Biological supplies, Hillsdale, NJ). The ^{14}C-CO_2 was collected in 10 ml CO_2-sorbing phenethylamine cocktail (1 L toluene, 1 L methanol, 1 L phenethylamine, and 8 g omnifluor). The radioactivity in each vial was counted with a Beckman model LSC 1800 liquid scintillation counter (Beckman Instruments, Irvine, CA).

Non heat-treated protein purification (first protocol). From the results of the initial protein purification, a new protocol was devised to isolate the protein. The new protocol added a step to remove unused hexadecane from the culture supernatant, and more steps to further purify the protein. A 2 L shaker flask containing 400 ml of BH medium with 1% hexadecane was inoculated with a 10 ml culture tube of *P. aeruginosa*. The culture was incubated at 25°C on a rotary shaker at 1000 rpm and was allowed to grow to an optical density of 1 at A_{540} (approximately five days). The bacteria were removed by centrifugation at 6,000 rpm at 20°C. The supernatant was placed in a -20°C freezer for 30 minutes to solidify any residual hexadecane, which was then removed by filtration through a 0.65 µm filter (Millipore,

Bedford MA). The supernatant was transferred to a 2 L separatory funnel and solid ammonium sulfate was added to make a 70% saturated solution that was allowed to settle overnight at 4°C. The solution separated into two phases, a lower clear solution with precipitate and a thick low-density layer at the top of the solution. The solution was shaken and transferred to four-250 ml centrifuge bottles and centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant, which still contained some low-density material, was kept and the pellets were re-suspended in 25 ml of DI water. The procedure was repeated with the supernatant to collect any residual low-density material from the first centrifugation and the residue was re-suspended in 25 ml DI. The two fractions were added together (50 ml) and dialyzed against DI water overnight then again with 10 mM tris-HCL, pH 8.0 overnight at 4°C. The dialyzed solution was applied to a 2.5x10 cm DEAE-Sepharose anion exchange column (C-50, Sigma, St. Louis, MO) and 5 ml fractions were collected. The unadsorbed fractions (DEAE P1) at A_{280} were pooled.

The pooled fractions containing the protein were adjusted to pH 6.0 using MES (2-[N-morpholino]ethane sulfonic acid) and the solution applied to a 2.5x10 cm carboxy methyl (CM) Sepharose cation exchange column (CL-6B,

Sigma, St. Louis MO). Bound protein was eluted using a NaCl gradient from 0 to 1 M and collected in 5 ml fractions. The fractions with substantial absorbance at A_{280} were pooled and dialyzed against DI water. The dialyzed protein was separated into 2 mg aliquots in plastic centrifuge tubes, frozen and lyophilized. The lyophilized tubes were frozen at -20°C.

Heat-treated protein purification (final protocol). It was found that two additional steps significantly improved the quality and quantity of the isolated protein. The first was to heat the supernatant to 70°C after removal of the bacteria. The second was added to the precipitation process. After adding $(NH_4)_2SO_4$ to the supernatant and allowing the solution to settle overnight, the clear fluid was carefully removed from the separatory funnel, leaving the low-density material, and centrifuged. The low density material was then added back to the re-suspended pellets and the rest of the isolation procedure proceeded as before (Fig. 4).

Determination of protein concentration

Three protein assays, a modified Lowry procedure, the Bradford method and dry weight determination were tested with the extracellular protein. The first method tested was

FINAL PROTEIN PURIFICATION PROTOCOL

CENTRIFUGATION
Bacteria removed at 6,000 rpm, 20 min, 20°C

↓

HEATING
Supernatant heated to 70°C for 15 minutes.

↓

FILTRATION
One hour at -20°C to solidify residual hexadecane.
Filtration through 0.65 μm filter.

↓

PRECIPITATION
Supernatant, without low-density material, adjusted to 70% $(NH_4)_2SO_4$ and left overnight at 4°C. The solution was centrifuged at 10,000 rpm and the pellets were re-suspended in 50 ml DI water.

↓

DIALYSIS
Low-density material added back to 50 ml solution. Then dialysis against DI water and again against 10 mM Tris-HCl, pH 8.0.

↓

DEAE CHROMATOGRAPHY
Unadsorbed protein fractions collected and adjusted to pH 6.0 with MES buffer.

↓

CM CHROMATOGRAPHY
Protein eluted with NaCl gradient.

↓

DIALYSIS AND STORAGE
The protein was dialyzed against deionized water, stored lyophilized.

Fig. 4. Final protein purification flow chart.
the modified method of the Lowry protein assay [Markwell et

al., 1978]. The modified method is used for membrane proteins solubilized with detergents and was therefore appropriate for working with cultures containing glycolipid surfactant and hexadecane. Two stock solutions were prepared in DI water: Solution A contained 2.0% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate and 1.0% SDS (w/v). Solution B contained 4.0% $CuSO_4$ (w/v) in DI water. One hundred parts solution A was added to 1 part solution B to make solution C. Three ml of solution C was added to 1 ml of protein solution and was allowed to react for 10 minutes. Three hundred µl of Folin & Ciocalteu's Phenol reagent (Sigma, St. Louis MO) which had been diluted 1:1 with DI water was then added and the tubes were allowed to react for 45 minutes. The A_{660} of the tubes was measured using a spectrophotometer.

The Bradford protein assay was also used for this study [Bradford, 1976]. Bradford protein assay concentrate (Bio-Rad, Dye Reagent Concentrate; Richmond, CA) was diluted with four volumes of DI water. A 100 µl aliquot of protein solution was added to 5 ml of the diluted reagent and the A_{595} of the samples was measured using a spectrophotometer.

Dry weight determination of the protein was carried out using 1.5 ml of a 1 mg/ml (compared to bovine serum albumin, BSA, Lowry method) solution which was dried overnight in pre-weighed aluminum weighing boats at 110°C. The sample

was then allowed to equilibrate at room temperature in a desiccator for four hr before weighing. The weight determination was performed in triplicate

Determination of the molecular weight of the extracellular protein

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was used to determine the purity and molecular weight of the isolated protein. The molecular weight standards that were used for comparison were bovine albumin (MW 66,000), egg albumin (MW 45,000), glyceraldehyde 3-phosphate dehydrogenase (MW 36,000), carbonic anhydrase (MW 29,000), trypsinogen (MW 24,000), trypsin inhibitor (MW 20,100), and α-lactalbumin (MW 14,200) (Low MW standards, Sigma, St. Louis MO). Non heat-treated extracellular protein was loaded into four lanes at concentrations of 20, 60, 100, and 300 µg (Lowry method). The proteins were electrophoresed with a mini-Protean II system (Bio-Rad Laboratories, Richmond CA) using a 13% polyacrylamide gel at 200 volts for one hr. The gel was stained with a 0.1% (w/v) Coomassie blue stain in 40% methanol and 10% acetic acid (v/v). The gel was destained two hr with two 100 ml washes in a solution of 40% methanol and 10% acetic acid (v/v). A Biorad 620 Video Densitometer

was used to scan the gels. The densitometry scan of the standards was used to produce a calibration curve to determine the molecular weight of the extracellular protein.

A second gel was used to observe the effect of heat-treating the culture supernatant during protein isolation. The second gel was prepared exactly as the first. Lane 1 contained 5 µl of low-molecular standards (Bio-Rad Laboratories, Richmond CA). Lane 2 contained 100 µg of heat-treated protein while lane 3 contained 100 µg of non heat-treated protein for comparison.

Determination of molecular weight using gel filtration chromatography. Two ml of tris buffer (10 mM tris, 0.1 M NaCl, pH 7.0) was added to 18 mg of lyophilized extracellular protein (Lowry method). The solution was added to a 1.5x30 cm column of Sephadex G-100 (Sigma, St. Louis MO) and 2 ml fractions were collected using the same buffer. The A_{280} was measured in each fraction using a spectrophotometer. Using the same procedure, the molecular weight of the extracellular protein was determined by comparison to three standards, myoglobin (MW 17,000), cytochrome C (MW 12,400), and cyanocobalamin (MW 1,350).

Determination of carbohydrate associated with the

extracellular protein

Determination of the presence of carbohydrate using electrophoresis. Detection of carbohydrate associated with the extracellular protein was tested according to the method of Gander [1984]. A 13% polyacrylamide gel was prepared as previously described with two lanes for the low molecular weight standards and two lanes of 20 µg extracellular protein. After electrophoresis, the gel was soaked in a solution of isopropanol, acetic acid, and DI water (25:10:25) to fix the protein. The gel was washed for two hr with 0.2% thymol (w/v) in DI water, drained, then soaked in a solution of concentrated H_2SO_4-ethanol (80:20) at 35°C for three hr with shaking. With this stain the gel background becomes yellow and the carbohydrate from the glycoprotein bands stain red [Gander, 1984].

Carbohydrate determination using the anthrone carbohydrate assay. The anthrone test was used to test the isolated protein for the presence of carbohydrate [Scott and Melvin, 1953]. A rhamnose stock solution was prepared in DI water to 400 ppm. From this solution five standards were prepared, 20, 25, 30, 35 and 40 ppm. Anthrone reagent was prepared by adding 0.2 g of anthrone (Sigma, St. Louis MO) to 100 ml of concentrated H_2SO_4. A 2 mg/ml solution of the extracellular protein was prepared by adding 2.5 ml DI water

to 5 mg lyophilized protein. The standards and samples were prepared by first adding 2.5 ml anthrone reagent to test tubes immersed in a running cold water bath (~10°C). A 2.5 ml aliquot of carbohydrate standard or protein solution was added to the tubes and mixed under water until they had cooled to room temperature. The tubes were placed in a 90 °C water bath for 16 minutes, then cooled to room temperature. The A_{625} of the samples was measured using a spectrophotometer.

Structure analysis of the extracellular protein

Amino acid composition. Gas phase acid hydrolysis and spectroscopy were used to determine the amino acid composition of the extracellular protein [Bell and Bell, 1988; Goodwin and Morton, 1946]. A 250 µg aliquot from a solution of the extracellular protein was transferred to a 6X50 mm hydrolysis tube, frozen, and lyophilized. The hydrolysis tube was placed in a 40 ml screw top vial equipped with a Teflon cap with a closable valve. The sample was hydrolyzed by adding 200 µl of 6 N HCl (Sigma, St. Louis MO) and 10 µl of a 0.5% (v/v) phenol solution to the bottom of the screw top vial. The vial was evacuated under vacuum and placed in a 110°C oven for 22 hr. The vial was removed from the oven and dried overnight under vacuum

to remove remaining HCl. The residue from the hydrolysis tube was re-suspended in 500 µl of Beckman HP Amino Acid dilution buffer (Li-s) and filtered through a 10,000 molecular weight cut off microcentrifuge filter (Gelman, Ann Arbor MI) or a microfuge concentrator (10,000 molecular weight cut off, Amicon). The solution was then chromatographed on a Beckman system 6300 High Performance Analyzer. Serine and threonine were analyzed using timed hydrolysis of 22, 48, and 72 hr and correcting for destruction by extrapolating to 0 time. Tyrosine and tryptophan were measured using spectroscopy according to Goodwin and Morton [1946]. Three calculations were used (see below). The first calculation was used to determine any absorbance that is not contributed by the tyrosine and tryptophan residues of the protein. This absorbance is called the irrelevant absorption. The second calculation used the $A_{294.4}$ to determine the total tyrosine and tryptophan residues. Finally, the third calculation at A_{280} was used to calculate the number of tyrosine residues. Subtracting the number of tyrosine residues from the total tyrosine and tryptophan residues yields the number of tryptophan residues.

Three mg of lyophilized protein was dissolved in 2 ml of 0.1 N NaOH to a final concentration of 1.5 mg/ml. The

absorbance of the solution was measured at A_{340}, A_{370}, $A_{294.4}$, and A_{280}. The absorbance at A_{340} and A_{370} was used to measure the irrelevant absorption which was subtracted from the absorbance at A_{280} and $A_{294.4}$. The absorbance values at A_{370} and A_{340} were used to calculate a correction slope using ΔAbs/ΔWavelength. The slope was then multiplied by the absorbance at $A_{294.4}$ and A_{280} to determine the irrelevant absorption. The total concentration of tyrosine and tryptophan (mole/l) was calculated by dividing the absorbance found at $A_{294.4}$ by the extinction coefficient 2375. The concentration of tyrosine (mol/l) was then calculated using: mol/l tyrosine=(A_{280})-(mol/l total)(5225) /(1576)-(5225) where 1576 is the extinction coefficient for tyrosine and 5225 is the extinction coefficient for tryptophan. The concentration of tryptophan (mol/l) was calculated using the following equation: mol/l tryptophan=(mol/l total)-(mol/l tyrosine). The mole% of each residue was calculated by dividing the moles of residue by the moles of protein.

Amino acid sequence. The amino terminal end of the extracellular protein was sequenced to 33 amino acids using automated Edman degradation by the University of Wisconsin Biotechnology Center, 1710 University Avenue., Madison WI 53705. The amino acid sequence was used to search the GENBANK Release 82 data base for comparable sequences from

other proteins.

Spectral properties, approximation of secondary structure, and hydrophilicity predictions

Spectral properties. The absorbance of a 1 mg/ml solution of the protein (Lowry method) was determined by scanning from 190 to 600 nm using a Hewlett Packard 8452A Diode Array Spectrometer and quartz cuvettes.

Approximation of secondary structure. Circular dichroism (CD) was used to estimate the secondary structure of the extracellular protein. A 2 mg/ml protein solution in DI water was scanned from 190-240 nm and the unequal absorption of left vs. right circularly polarized light was measured using a Jasco J-720 Spectropolarimeter. The computerized system subtracted the average of 40 scans of DI water from 40 scans of a 2 mg/ml solution of protein and estimated the secondary structure of the extracellular protein from strain 17423. Secondary structural predictions of the deduced sequence from strain PG201 were calculated using a two-layered neural network that is accessible through the internet at PredictProtein@EMBL-Heidelberg.de [Rost and Sander, 1993].

Hydrophilicity predictions. The local hydrophilicity of the deduced amino acid sequence of strain

PG201 [Hardegger et al., 1994] was predicted according to the method of Kyte and Doolittle [1982]. Using the amino acid hydropathy scale of Kyte and Doolittle [1982], a moving average of seven residues at a time were summed starting from residue 12 at the amino terminal end of the protein and advancing one amino acid residue at a time to the carboxylic end. These sums were plotted at the sequence number of the residue corresponding to the midpoint of the segment which would be the fourth residue in the set.

Protein modification

Reductive methylation. The ε amino group from the side chain of lysine was methylated according to the method of Means and Feeny [1968]. Four ml of 0.2 M sodium borate buffer was added to 16 mg of lyophilized protein. To this solution 2 mg solid sodium borohydride was added. Then five additions of 2 µl 37% formaldehyde were added over a period of 30 minutes. The reaction was stopped by dialysis and the modified protein was re-lyophilized. The results of the modification were determined using amino acid analysis. This is possible because modification of the lysine residues changes their chromatographic properties resulting in an overall loss in the number of residues. Hydrolysis and chromatography of the modified protein were as described

earlier.

Modification of aspartic and glutamic residues. The side chain carboxyl group of aspartic and glutamate were modified in a two step procedure. Solid glycine methyl ester (Sigma, St. Louis MO) was added to 10 ml of a 1 mg/ml solution of the extracellular protein to make the solution 1.0 M glycine methyl ester. The protein solution was adjusted to pH 4.75 with concentrated HCl. Solid carbodiimide (Sigma, St. Louis MO) was added to the protein solution to make it 0.1 M carbodiimide and the reaction was allowed to continue for one hr while maintaining the pH with 1.0 M HCl. The reaction was stopped by adding 20 ml of 1.0 M acetate buffer, pH 4.75 [Carraway and Koshland, 1972]. The protein was dialyzed against DI water with three changes. The protein was further washed and concentrated using ultrafiltration (Centriplus 10,000 MW cutoff, Amicon, Inc., Deverly, MA) and re-lyophilized. The number of modified residues was determined using amino acid analysis. This was neccessary because the modified residues release additional glycine after acid hydrolysis from the glycine methyl ester. The number of additional residues of glycine is related to the extent of modification.

Composition of DEAE P2

SDS-PAGE of DEAE P2. Electrophoresis sample buffer was added to a lyophilized tube of DEAE P2 to make a 20 µg/µl solution. Electrophoresis was carried out as previously described and the proteins were compared to low molecular weight standards (Sigma, St. Louis MO).

Detection and analysis of the rhamnolipid surfactant. DEAE P2 was analyzed for the presence of the glycolipid surfactant according to the method of Itoh et al. [1971]. DEAE P2 was eluted from the DEAE column after a typical protein isolation from the DEAE column using 1 M NaCl. The solution was acidified to pH 2.0 and left at 4^0C for one week to precipitate. The precipitate was collected by filtration through a type A/E glass fiber filter (Gelman, Ann Arbor MI) and re-dissolved in 50 ml of acetone. The acetone was removed by roto-evaporation and re-dissolved in 1 ml of chloroform. Approximately 10 µl of the solution was spotted onto a 25x70 mm silica gel F plate (Sigma, St. Louis MO) and developed using chloroform:methanol (85:15). The spots were visualized using a solution of 0.2 g anthrone in 100 ml concentrated H_2SO_4 which was sprayed on the plate.

Hydrophobic properties of the extracellular protein

Binding of the extracellular protein to hexadecane. The protein was tested for its ability to bind hexadecane

which indirectly also tests the surfactant properties of the protein. Emulsified hexadecane increases the optical density (OD) of an aqueous sample which can be measured easily with a spectrometer [Rosenberg and Rosenberg, 1980]. The emulsifying ability of the extracellular protein was compared to bovine serum albumin (BSA) and DI water (control). A series of 12 microcentrifuge tubes was prepared. The first set of four tubes contained 1.5 ml of 0.2 mg/ml solution of extracellular protein. The second set contained 1.5 ml 0.2 mg/ml BSA, and the third set contained 1.5 ml DI water as controls. To the first tube in each set 10 µl of hexadecane was added, to the second, third, and fourth tubes 15, 20, and 25 µl hexadecane were added. The tubes were vortexed for one minute, allowed to stand for an additional 30 minutes, and the A_{540} was measured with a spectrophotometer.

Hydrophobic chromatography. The extracellular protein was tested for its ability to bind to a hydrophobic column according to the method of Hooper [1992]. A 1x10 cm column was prepared of octyl-Sepharose CL-4B (Sigma, St. Louis MO) and equilibrated with a 10 mM sodium phosphate, 0.1 M NaCl, pH 7.0 buffer. Approximately 5 mg of the extracellular protein was added and 5 ml fractions were collected. Bound protein was eluted with 2% (v/v) Triton X-100 in the same

buffer.

Growth Measurements

Growth of *P. aeruginosa* on hexadecane was determined by measuring the A_{540}. The culture tubes were prepared by adding 1 ml of nutrient broth-grown bacteria, resuspended in BH medium, to 9 ml BH medium as normal growth or 8 ml BH and 1 ml protein solution. The final OD at time 0 was approximately 0.2 at A_{540}. The final concentration of protein was approximately 0.5 mg/ml. To each tube 100 µl of hexadecane was added (1% v/v). The tubes were placed on a 45° angle culture rack mounted on a rotary shaker and rotated at 1,500 rpm. Samples were measured once by vortexing the tubes vigorously for five minutes and measuring the A_{540}. The samples were measured beginning at 19 hr then every 12 hr for 5 days.

Non-specific binding of hexadecane to P. aeruginosa. The affinity of non-acclimated *P. aeruginosa* for hexadecane without the presence of the extracellular protein or glycolipid surfactant was tested. A culture of *P. aeruginosa* was grown on nutrient broth to an A_{540} of 2, then centrifuged at 5,000 rpm and resuspended in BH medium to the same volume. A 20 µl aliquot containing 24,200 DPM [1-^{14}C] hexadecane was added to 2 ml of the bacterial suspension to

give a final concentration of 40 µg hexadecane and 12,200 DPM/ml radioactivity. A 300 µl (3660 DPM) sample of this suspension was added to microcentrifuge tubes containing 1 ml of 58% w/v sucrose. The tubes were centrifuged at 13,000 rpm for 20 minutes, the supernatant was removed and the pellet re-suspended in 300 µl of BH medium. The solution was added to 10 ml of Triton-X 100 scintillation cocktail (1 l Triton-X 100, 2 l toluene and 8 g omnifluor). The radioactivity was counted in a scintillation counter (Beckman Instruments, Irvine, CA).

The effect of having only the extracellular protein as a carbon source was also investigated. One ml of nutrient-broth grown bacterial suspension, resuspended in BH medium, was added to six culture tubes: two tubes contained 9 ml of BH medium, two had 8 ml of medium and 1 ml of a 4.16 mg/ml protein in BH medium, and two tubes had 9 ml medium and 100 µl of hexadecane. The tubes were placed in a 45° angle culture rack mounted on a rotary shaker table and agitated at 1,500 rpm. Optical density (A_{540}) was measured at 12 hr and 66 hr for the two controls and the tubes with protein. The two tubes with hexadecane were measured at 66 hr only.

The effect of EDTA on growth of *P. aeruginosa* on hexadecane was also tested. One ml of nutrient broth-grown bacteria resuspended in BH medium was added to six culture

tubes: tube 1 contained 9 ml of BH medium, tubes 2 and 3 contained 9 ml BH medium with 4 mM EDTA, tube 4 had 8 ml BH medium and 1 ml 0.1 mg/ml protein, and tubes 5 and 6 contained 8 ml BH medium with 4 mM EDTA and 1 ml 0.1 mg/ml protein. Growth was measured at 48 hr by vortexing the tubes vigorously for five minutes and measuring the A_{540}.

Growth of the bacterium on hexadecane and the modified proteins were measured as previously described. Blank controls were prepared by adding 9 ml BH medium, 1 ml of nutrient broth grown bacteria resuspended in BH medium, and 100 µl of hexadecane. The samples were prepared with 8 ml BH medium and 1 ml of approximately 0.6 mg/ml native protein as protein controls or 1 ml of 0.6 mg/ml modified protein. The samples were placed on a 45° angle culture rack and agitated at 1,500 rpm. Samples were measured once by vortexing the tubes vigorously for five minutes and measuring the A_{540}. Enough samples were prepared that measurments could be taken at 18 hr then every 12 hr for five days.

Comparing growth of nutrient broth-acclimated bacteria to hexadecane-acclimated bacteria on hexadecane

Two 500 ml flasks were inoculated with the equivalent of 2 ml of bacterial suspension at 0.3 A_{540} with either

nutrient broth- or hexadecane-acclimated cells. Every 12 hr an aliquot was taken under the organic layer of the culture using a Pasteur pipette. Part of the aliquot was used to determine A_{540} while the remainder was transferred to a 1.5 ml microcentrifuge tube. The microcentrifuge tubes were centrifuged for 20 minutes at 13,000 rpm and the supernatent from the samples were filtered through a Swinnex-25 (Millipore, Bedford MA) filter assembly using Gelman type A/E glass fiber filters. The protein concentration was measured using 750 µl of the supernatant and the modified Lowry protein assay [Markwell et al., 1978].

Agglutination of *P. aeruginosa* and *Escherichia coli*

A 100 ml culture of *P. aeruginosa* was grown on nutrient broth and transferred twice to fresh medium to ensure that the bacteria were not acclimated to hexadecane. The third culture was centrifuged at 5,000 rpm for 20 minutes and the pellet was re-suspended in 100 ml BH medium. Two ml of this suspension diluted to 10 ml with BH medium yielded an A_{540} of 0.35. Five culture tubes were prepared. Two tubes were controls with no protein and were prepared by adding 8 ml BH medium and 2 ml bacterial suspension. Three culture tubes contained 7 ml BH media, 2 ml of bacterial suspension and 1 ml of dilutions of extracellular protein that produced 0.2

mg/ml, 0.3 mg/ml and 0.5 mg/ml final protein concentrations. The culture tubes were placed in a 45° angle rack on a rotary table and were rotated at 1,500 rpm for one hr, then allowed to stand for an additional hr at 25°C. Agglutination was observed visually without magnification and using 1,000x magnification under a light microscope. In a similar procedure a culture of *E. coli* was grown overnight at 37°C in nutrient broth. Four culture tubes were prepared as previously described for *P. aeruginosa* except only two culture tubes with a final concentration of 0.5 mg/ml of extracellular protein were used and two tubes with bacteria but no protein were used as controls.

Final agglutination procedure. A quick agglutination procedure was designed for *P. aeruginosa*. One ml of an overnight nutrient broth-grown culture was added to 1.5 ml microcentrifuge tubes. The tubes were centrifuged for five minutes at 7,000 rpm and the pellets resuspended in 1 ml BH medium. The procedure was repeated again to wash the bacterial pellet. After the last centrifugation the pellets were resuspended in BH medium (controls) or an extracellular protein solution in BH medium. The tubes were then centrifuged and vortexed three times at 7,000 rpm in the same medium for five minutes. Agglutination was observed microscopically at 1,000x.

Agglutination inhibition experiments. Four monosaccharides were tested for their ability to inhibit agglutination of *P. aeruginosa* by the extracellular protein. To five wells of a 24 well cell plate (Corning) 200 µl of extracellular protein solution in BH medium (10 mg/ml) was added and 200 µl BH medium with no protein was added to an additional well as a control. To four of the wells 300 µl of one of four carbohydrate solutions (2 M) was added and 300 µl of BH medium was added to the other two wells. To the six wells 1.5 ml *P. aeruginosa* cell suspension (A_{540} of 0.34) was added. The final concentration per well was 1 mg/ml protein and 0.3 M carbohydrate. The cells were allowed to incubate for two hr then checked for agglutination microscopically at 1,000x.

Agglutination of red blood cells. The ability of the extracellular protein to agglutinate red blood cells was tested. Lyophilized red blood cells from human type A, B, O, and horse were used (Sigma, St. Louis MO). The blood cells were re-hydrated by adding phosphate buffered saline (PBS), pH 7.2, supplemented with 1 mM $MgCl_2$ and $CaCl_2$ to make a 10% hematocrit solution. A portion of the red blood cells was treated with papain, a protease that removes the outer portion of the membrane glycoproteins revealing the carbohydrate portion of the membrane glycolipids. The four

types of blood cells were treated with papain by adding 200 µl of a 1% w/v solution papain in 0.1% cysteine to 1 ml blood in 1.5 ml microcentrifuge tubes. The microcentrifuge tubes were incubated at 37°C for 30 minutes then centrifuged at 2,000 rpm and washed three times in PBS [Gilboa-Garber, 1982]. Agglutination was tested in a 96 well round bottom microtiter plate (Sigma, St. Louis MO). Eight rows were prepared using the four un-treated blood groups and another four rows using the same four groups treated with papain. The microtiter plate was prepared by adding 100 µl of a 3.5 mg/ml solution of the extracellular protein in PBS to the first wells of eight rows from the 96 well microtiter plate and 50 µl of PBS buffer was added to the remaining wells. Protein serial dilutions (1:2) were performed by taking 50 µl of well one and adding it to the next well in the row and repeated with all of the wells in the row. The extra 50 µl from each last well was discarded. The wells were brought to 100 µl by adding 50 µl of 1% solution of blood in PBS to each well. The plate was covered with cellophane and incubated overnight at 25°C. Agglutination was determined by direct observation and microscopy.

Carbohydrate affinity columns

The extracellular protein was introduced to several columns with different carbohydrate matrices to test if the protein would bind. Two of the matrices did not need any modification; Sephadex (for glucose) and Sepharose 4B (for galactose). The buffer used with these columns was 10 mM tris-HCl with 30 mM $MgCl_2$ and 30 mM $CaCl_2$ at pH 7.4. Three different carbohydrates, mannose, cellobiose and rhamnose, were also derivatized to Sepharose 4B according to the method of Uy and Wold [1977]. To a 200 ml flask, 15 g of washed and suction dried Sepharose 4B, 15 ml of 1,4-butanediol diglycidyl ether (Sigma, St. Louis MO) and 15 ml of 0.6 N NaOH containing 30 mg of $NaBH_4$ were added. This solution was placed on a rotary shaker for 10 hr to activate the Sepharose. The activated Sepharose was washed with DI water then suction dried and resuspended in 30 ml 0.1 N NaOH with 1.5 g carbohydrate. The solution was rotated at 37°C for 24 hr at 1,500 rpm. The Sepharose was washed under vacuum through a fritted glass filter assembly (Millipore, Bedford MA) with 500 ml of 0.1 M borate buffer, pH 8.0, then 500 ml DI water. The derivatized Sepharose was then loaded into a 2.5x10 cm column. A solution of the extracellular protein was added and 5 ml fractions of the solution were collected and measured for protein absorbance at A_{280}.

Glycolipid surfactant and interactions between the extracellular protein

Extraction of glycolipid surfactant. A one week old culture tube of *P. aeruginosa* was used to inoculate 400 ml of BH medium with 1% hexadecane. The culture was allowed to grow for one week at 25°C then separated into two-250 ml centrifuge bottles and centrifuged at 10,000 rpm for 30 minutes to pellet bacteria. The supernatant was cooled at 4°C until any remaining hexadecane was solidified. The solid hexadecane was removed by filtration through a type A/E glass fiber filter (Gelman, Ann Arbor MI). The 400 ml of culture supernatant was acidified to pH 2.0 using concentrated HCl. After one week at 4°C the precipitated material was collected by filtration through a type A/E filter and the crude solid extract was dissolved in 50 ml of acetone. The solution was filtered through a Pasteur pipette packed with glass wool to remove cellular debris and other large contaminates. The acetone was removed by roto-evaporation and the residue was resuspended in 95% ethanol [Jarvis and Johnson, 1949]. The solution was further purified according to Itoh et al. [1971]. After roto-evaporation the residue was dissolved in 100 ml of 0.05 M $NaHCO_3$. The pH of this solution was adjusted to 4 with HCl then extracted with 2x25 ml diethyl ether. The diethyl

ether solution was concentrated with roto-evaporation then dissolved in a small amount of chloroform:methanol (85:15). This solution was added to a silica gel column (Baker, 40 μm ave. size) and the glycolipid was eluted in 10 ml fractions using the same solvent.

After column chromatography thin layer chromatography was used to verify the identity of the glycolipid surfactant from the collected fractions [Itoh et al., 1971]. Two drops of the solution were used for each 25x70 mm silica gel F plate (Sigma, St. Louis MO). The plates were developed with different ratios of chloroform:methanol solutions; 85:15 yielded the best resolution. The spots were visualized using a solution of 0.2 g anthrone in 100 ml concentrated H_2SO_4 which was sprayed on the plate.

Binding of the protein to the glycolipid surfactant. Two general procedures have been used to attach eukaryotic glycolipids, mainly glycosphingolipids, to solid surfaces. These procedures are binding to a plastic surface and binding to thin layer chromatography plates. Protein binding to the glycolipids can then be determined by adding a protein solution to the solid surface, rinsing off the un-bound protein, then detecting the protein using radioactivity or a suitable stain. Both methods were used in this study. In addition a third procedure was developed

to examine binding of protein to the glycolipid surfactant.

For the first procedure ~1 mg isolated glycolipid was dissolved in 5 ml ethanol. A 500 µl aliquot was layered onto polystyrene (plastic) and allowed to evaporate [Leffler and Svanborg-Eden, 1986]. Five ml of a 5 mg/ml extracellular protein solution was layered over the treated plastic for 15 minutes then rinsed.

In the second procedure the extracted glycolipid surfactant was dissolved in ethanol to 0.2 mg/ml and five drops from a Pasteur pipette were applied drop-wise to a mini silica gel 60 thin layer chromatography plate (TLC) (Sigma, St. Louis MO) allowing the ethanol to evaporate between drops. The TLC plates were then coated by immersing the plate in a 50 ml solution of 0.01 M NaH_2PO_4, 0.14 M NaCl, 2% polyvinylpyrrolidone (Sigma, St. Louis MO), pH 7.2 to block unspecific binding of protein [Smith, 1983]. The plate was drained then soaked in 5 ml of a 5 mg/ml extracellular protein solution for 15 minutes. The plates were rinsed and sprayed with ninhydrin.

A third procedure with reversed phase HPLC silica gel was devised to help determine the extent of the binding. The isolated glycolipid surfactant was dissolved in ethanol to 0.2 mg/ml. Two 10 ml test tubes were prepared with approximately 1 g reverse phase silica gel with octadecyl

bonded phase (C_{18}; 40 µm average particle diameter, Baker). Two ml of the glycolipid solution was added to each tube, vortexed and then incubated overnight at 25°C to evaporate the ethanol. An extracellular protein solution was prepared by dissolving 5 mg lyophilized protein in 1 ml BH medium. A nutrient broth-grown culture of *P. aeruginosa* was centrifuged at 5,000 rpm for 20 minutes then resuspended in BH medium. Three microcentrifuge tubes were prepared. Tube 1 contained approximately 1 g of reverse phase beads without bound surfactant, 500 µl of bacterial suspension and 500 µl of BH medium. Tube 2 contained 500 µl of bacterial suspension, approximately 1 g of surfactant-covered reverse phase beads, 300 µl BH medium and 200 µg extracellular protein solution (1 mg/ml final concentration). Tube 3 contained 500 µl of bacterial suspension and 500 µl of BH medium. The tubes were vortexed and allowed to react for 15 minutes. Binding of the bacteria to the silica beads was checked using microscopy at 600x and 1,000x.

RESULTS

Initial protein purification

Two solutions with an A_{280} were obtained from culture supernatant after DEAE anion-exchange chromatography (see Fig. 3). Absorbance was detected at A_{280} in fractions that did not bind to the column (DEAE P1), and in fractions that had been eluted with 1 N NaCl (DEAE P2). Using radiorespirometry, mineralization of hexadecane by *P. aeruginosa* was shown to be enhanced by the addition of freeze-dried material (800 µg, Lowry method) from both fractions. The addition of freeze-dried material from the bound fraction (DEAE P2) increased mineralization of [1-^{14}C] hexadecane to 5365±480 disintegrations per minute (DPM) compared to 3295±92 DPM for normal mineralization (62% increase in mineralization). In another experiment with a different culture, freeze-dried material from the unbound fraction (DEAE P1) increased mineralization to 4726±842 DPM compared to 1020±163 DPM for normal mineralization (82% increase in mineralization).

The quantity of the bound fraction (DEAE P2) produced by the bacterium depended upon the length of time the culture was allowed to grow; the quantity of DEAE P2 decreased with time while DEAE P1 showed no change or

increased (Fig. 5 and 6). Using SDS-PAGE the un-bound fraction (DEAE P1) was later shown to contain the extracellular protein.

Final protein purification protocol

The initial protein isolation procedure was a modification of the one used by Hisatsuka et al. [1972] but produced low yields of degraded protein. The final protein purification protocol consisted of a number of steps including heating the culture supernatant to 75°C, precipitation, dialysis, and purification through two ion exchange columns; diethylaminoethyl anion exchange column (DEAE) and carboxy methyl cation exchange column (CM) (Fig. 7 and 8). Typical protein yields were 20-30 mg per 500 ml culture.

Determination of protein concentration

The concentration of the purified protein was determined by a modified Lowry protein assay [Markwell et al., 1978], the Bradford protein assay [Bradford, 1974], and direct measurements using dry weight. When the three methods were compared it was found that the Bradford assay yielded results inconsistent with the other two assays. This is likely because the extracellular protein binds

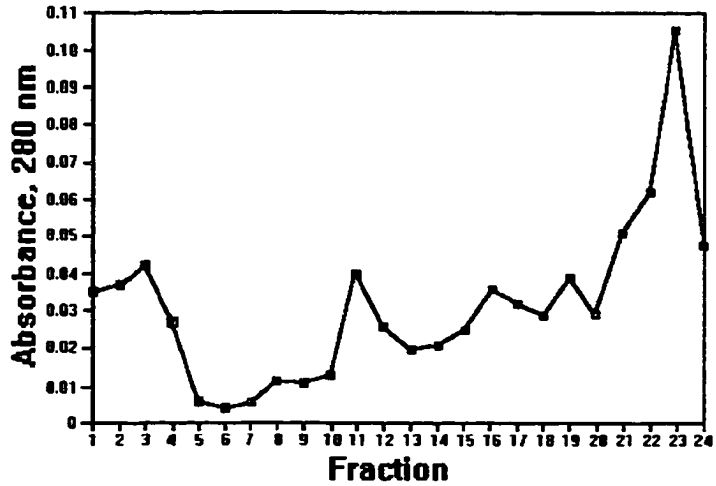


Fig. 5. DEAE column fractions from a three day old culture. Fractions 1-5 are the unbound fraction (DEAE P1). Fractions 6-24 are the bound fractions (DEAE P2) after elution with 1 M NaCl.

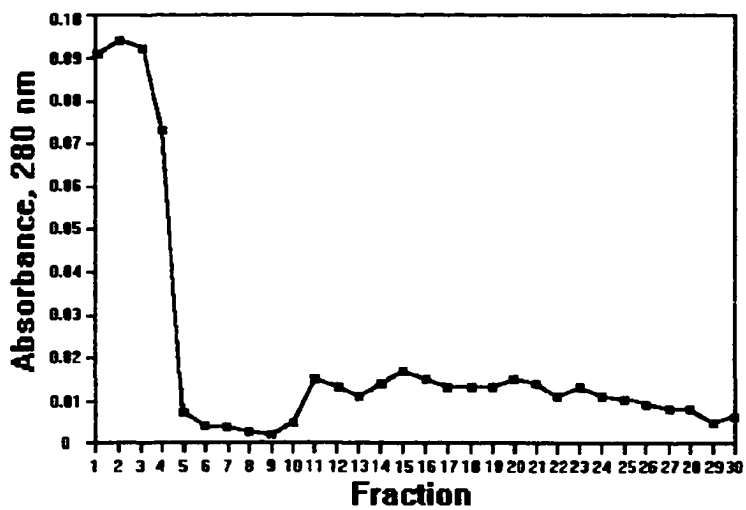


Fig. 6. DEAE column fractions from a seven day old culture. Fractions 1-5 are the unbound fraction (DEAE P1). Fractions 6-30 are the bound fractions (DEAE P2).

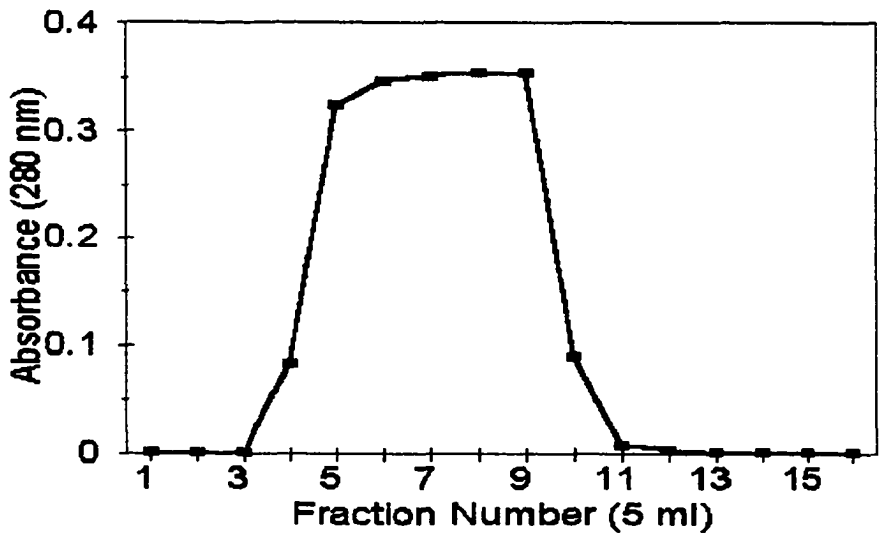


Fig. 7. DEAE anion exchange chromatography. The unadsorbed protein (DEAE P1) was collected in 5 ml fractions.

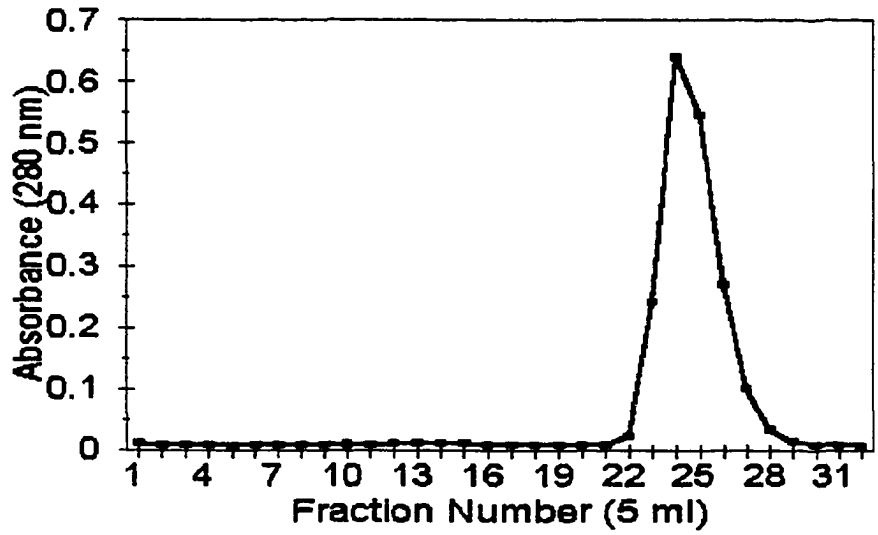


Fig. 8. CM chromatography of the extracellular protein. The adsorbed protein (DEAE P1) was eluted with a NaCl gradient starting at fraction 11 from 0 to 1 M. The protein was collected in 5 ml fractions.

poorly to coomassie blue stain used with the Bradford assay. Dry weight determination of 1.5 ml of a 1 mg/ml extracellular protein solution (Lowry method) gave a final weight of 1.5 ± 0.2 mg. Using the Lowry method there was good correlation between the dry weight determinations but the Bradford method underestimated the protein concentration by a factor of approximately 100. By comparing the Lowry protein assay to the protein A_{280} the absorptivity of the protein was found to be 0.5046 ml/mg•cm. The extracellular protein concentration was measured using A_{280} absorbance and the reciprocal of the absorptivity, 1.9818 mg/ml-A_{280} for the remainder of the study.

Determination of molecular weight

Analysis of the purity and molecular weight of the protein was determined using gel filtration chromatography and SDS-PAGE. When 18 mg of the heat-treated extracellular protein was applied to a Sephadex G-100 gel filtration column, a small amount of contamination was detected in the void volume but most of the protein came off as a single peak (Fig. 9). When the extracellular protein was compared to known molecular weight standards using gel filtration

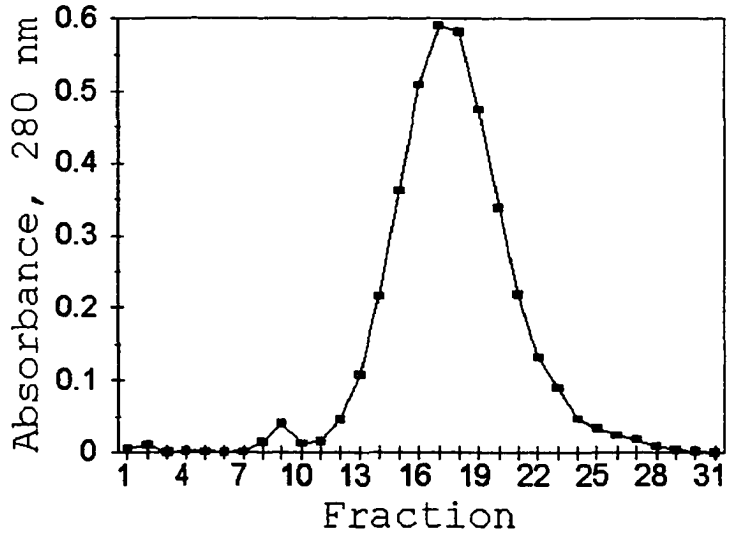


Fig. 9. Gel filtration chromatography of 18 mg of the extracellular protein using Sephadex G-100. The protein was collected in 2 ml fractions.

chromatography the molecular weight could be estimated to be approximately 14,100 (Fig. 10).

Two closely associated bands are visible with SDS-polyacrylamide gels (SDS-PAGE) when the initial protein isolation method (no heating step) was used (Fig. 11). Densitometry was used to scan the gel to determine the relative amounts of protein in each band (Fig. 12). The two bands have approximate molecular weights of 14,500 and 13,100 when compared to molecular weight standards (Fig. 13). The largest detected band is 74.8% of the two major bands while the second band is 25.2% (Fig. 12).

Heat treatment of the culture supernatant during protein purification resulted in a major protein band of approximately 14,500 molecular weight using SDS-PAGE (Fig. 14, lane 2) as determined by comparison to molecular weight markers (Fig. 14, lane 1). The major protein band from the non-heat treated culture supernatant is degraded into five bands with the first two bands having approximate molecular weights of 14,500 and 13,200 (Fig. 14, lane 3).

Hydrophobic chromatography

When 5 mg of the extracellular protein was passed through an octyl-sepharose column, no binding of the protein to the column was observed.

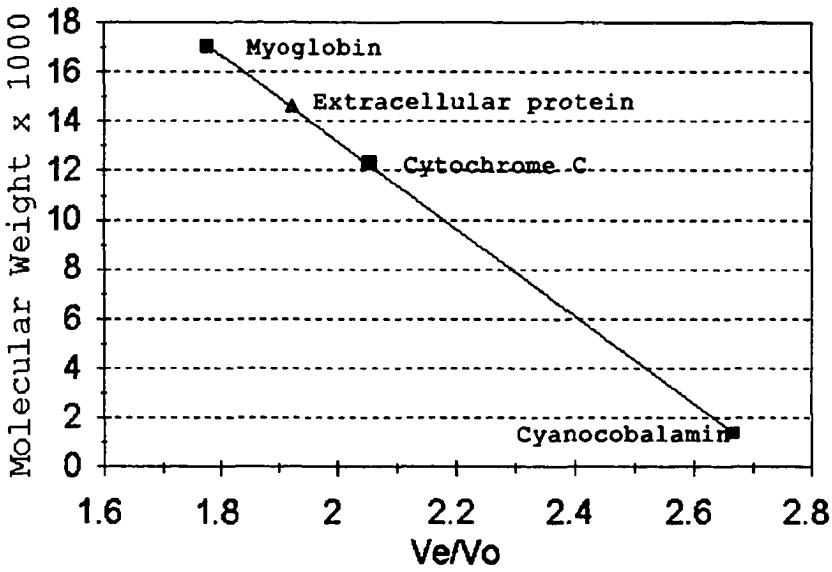


Fig. 10. Determination of the molecular weight of the extracellular protein using gel filtration chromatography. The marker for the extracellular protein (Δ) has been placed on the calibration curve corresponding to its value of Ve/Vo. The protein was found to have a mol wt of approximately 14,100.

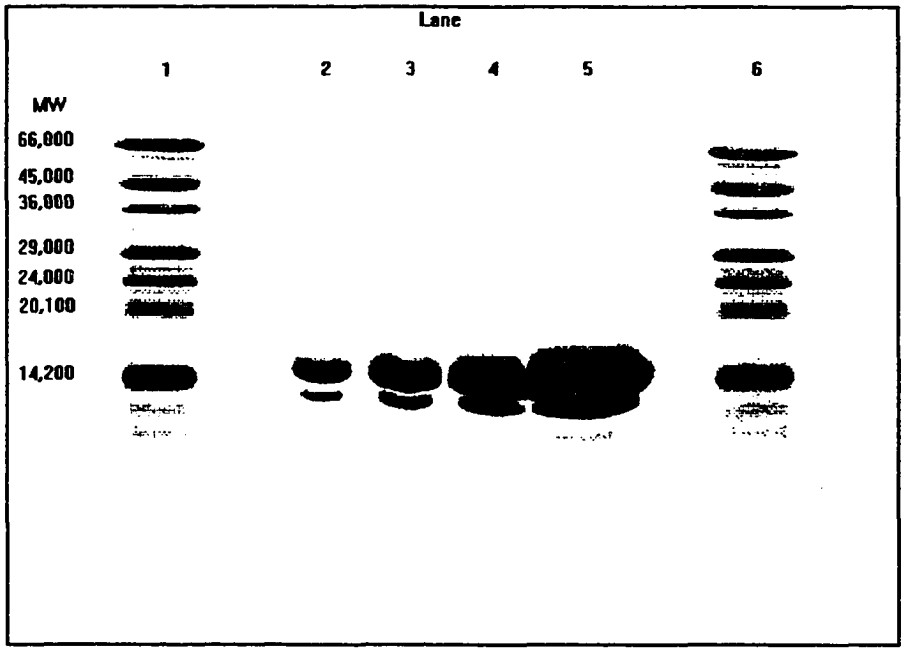


Fig. 11. A 13% polyacrylamide gel showing the banding pattern of the non-heat treated extracellular protein. Lanes one and six are mol wt markers. Lanes two through five are 20, 60, 100 and 300 µg of the extracellular protein.

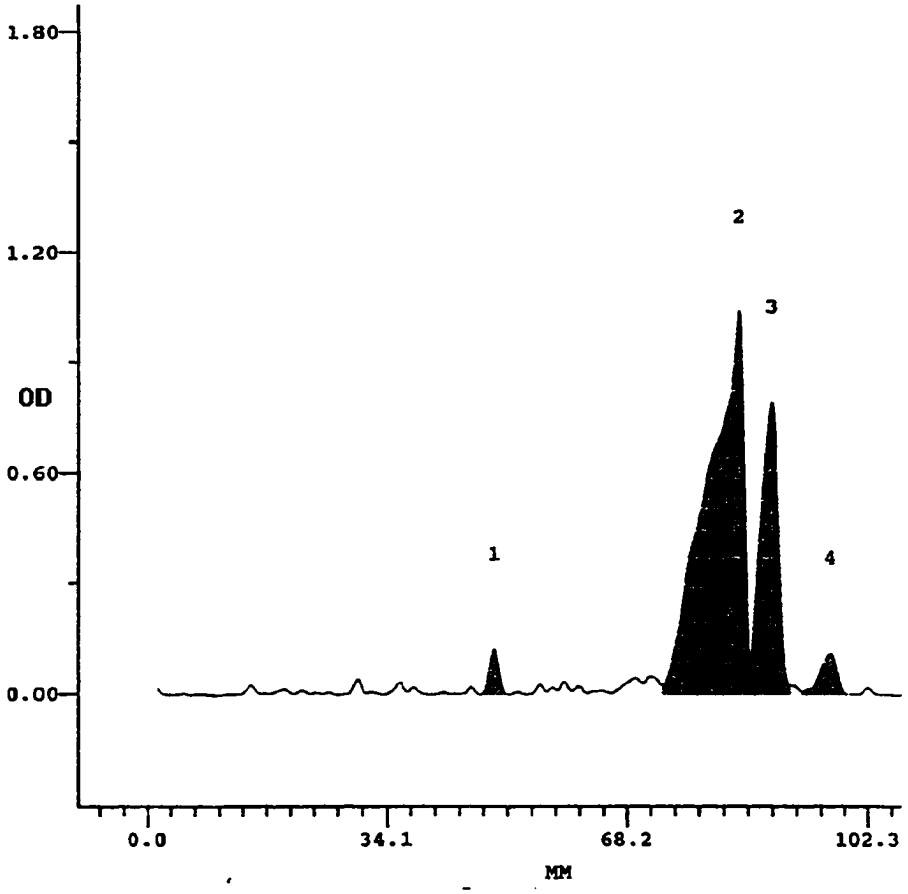


Fig. 12. Densitometry scan of 60 µg of the extracellular protein from lane three of the SDS-PAGE gel (Fig. 11). The two largest bands (2 and 3) were estimated to be 14,460 and 13,080 mol wt based on the calibration curve (Fig. 13).

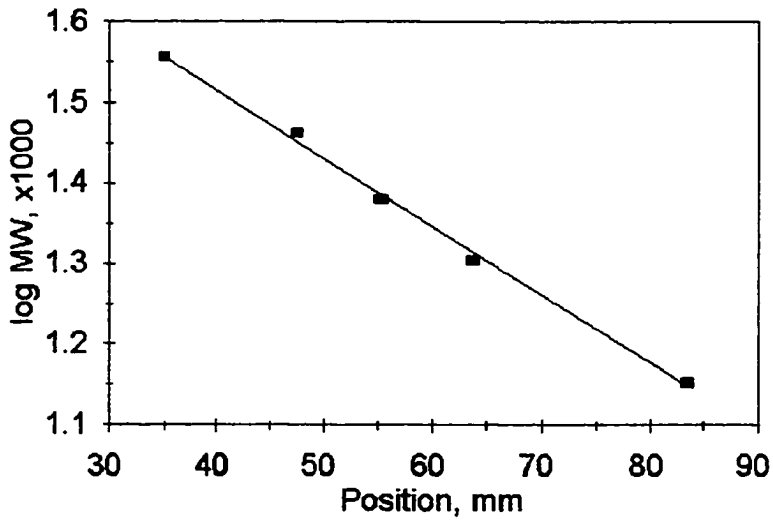


Fig. 13. Calibration of the mol wt markers from the SDS gel (Fig. 11) using densitometry. The two highest mol wt markers (66 and 45 kd) are not included.

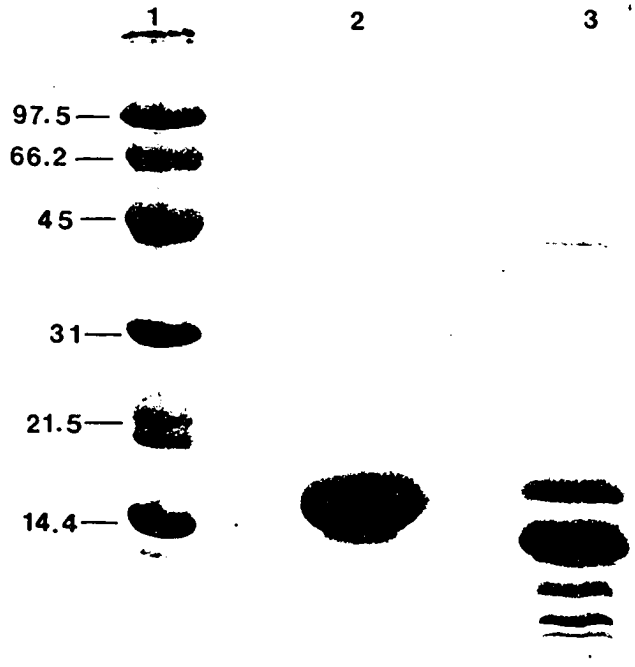


Fig. 14. A 13% SDS-PAGE gel comparing heat treated vs non-heat treated extracellular protein. Lane 1 are the molecular weight markers (x1,000). Lane 2 has 100 µg of the heat-treated protein. Lane 3 has 100 µg of the non-heat treated protein.

Determination of carbohydrate associated with the extracellular protein

The extracellular protein was tested for contamination by the glycolipid surfactant using rhamnose standards and a calorimetric technique (Fig. 15). The absorbance detected with the protein was below the lowest calibration standard (less than 50 µg carbohydrate for 5 mg extracellular protein).

A 13% SDS-PAGE gel was stained to detect the presence of carbohydrate which may indicate that the protein is a glycoprotein [Gander, 1984]. Carbohydrate was not detected.

Analysis of DEAE P2

Using microscopy, the bound fraction (DEAE P2) was shown to be composed of spherical particles with diameters of less than 1 to greater than 10 µm when viewed through a light microscope (Fig. 16). Protein was also detected in fraction DEAE P2 using the Lowry protein assay. When electrophoresed, a number of high molecular weight proteins were visible but not the extracellular protein (Fig. 17). When the fraction was tested for the rhamnose surfactant, two bands were detected with retention factors of 0.4 and 0.73. Bands with similar retention factors have been described as glycolipid with one carbohydrate (Rf 8.0) or

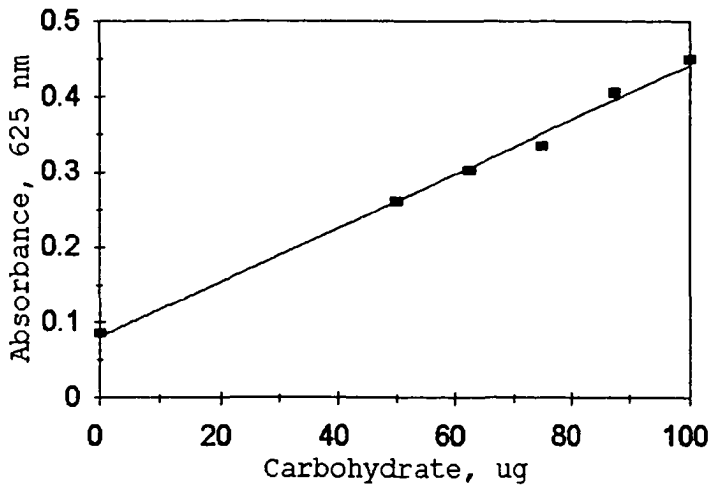


Fig. 15. Calibration curve for the anthrone carbohydrate assay. Rhamnose was used as the standard.

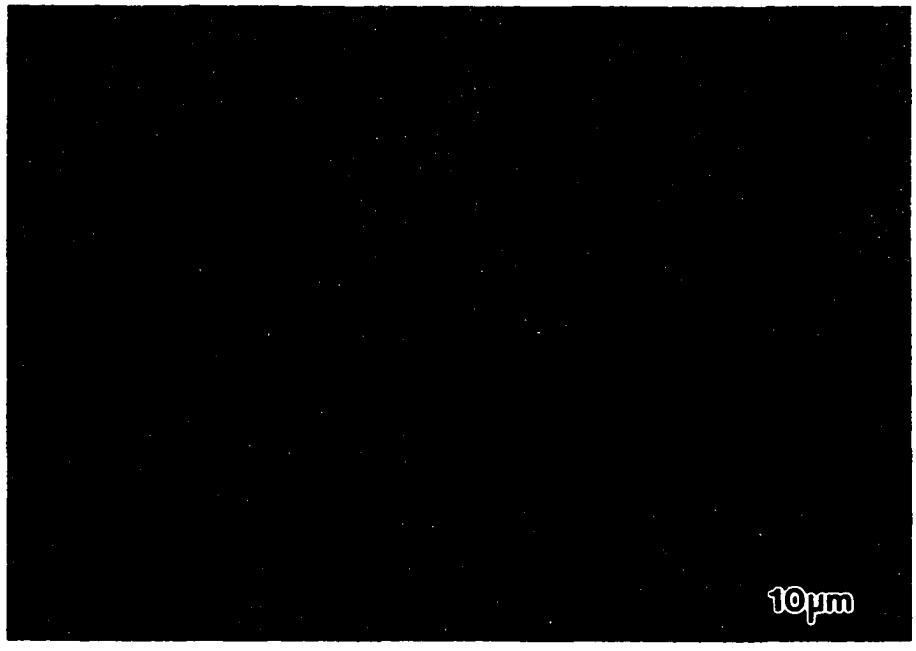


Fig.16. Fraction 1. These spherical particles are the bound DEAE fraction (DEAE P2) after elution using 1 M NaCl. Magnification, x1,000.

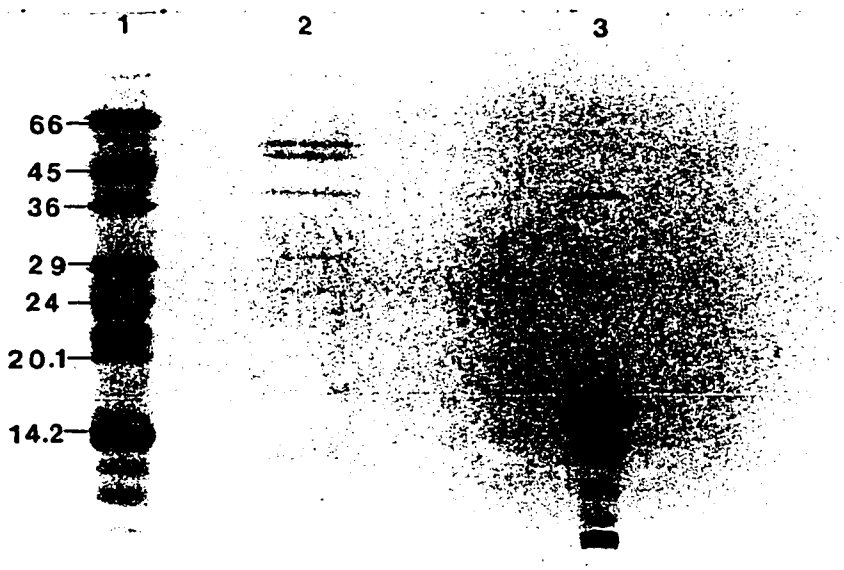


Fig. 17. SDS-PAGE gel comparing the extracellular protein to the proteins associated with the DEAE P2 fraction. Lane one is the mol wt markers. Lane two is 20 µg of the micelle fraction. Lane three is 60 µg of the extracellular protein.

with two (Rf 4.0) [Itoh et al., 1971].

Amino acid composition

The amino acid composition of the extracellular protein was determined using acid hydrolysis and chromatography (Fig. 18). The number of residues of tryptophan (which is destroyed during hydrolysis) and tyrosine in the protein were determined using a spectrophotometric technique. The protein was found to be rich in hydroxy and hydrophobic amino acids. No histidine or arginine were detected (Table 2).

Determination of the N-terminal amino acid sequence

In addition to determining the amino acid composition, thirty three amino acids were sequenced from the amino end of the protein using automated Edman degradation (Fig. 19). This sequence was used to search the GENBANK (Release 82) data base. One perfect match was found from a protein produced from another hydrocarbon degrading strain of *P. aeruginosa* called PG201 [Hardegger et al., 1994, Fig. 20].

UV spectrum of the extracellular protein and determination of secondary structure

Further characterization of the protein involved determining its spectral qualities and an approximation of

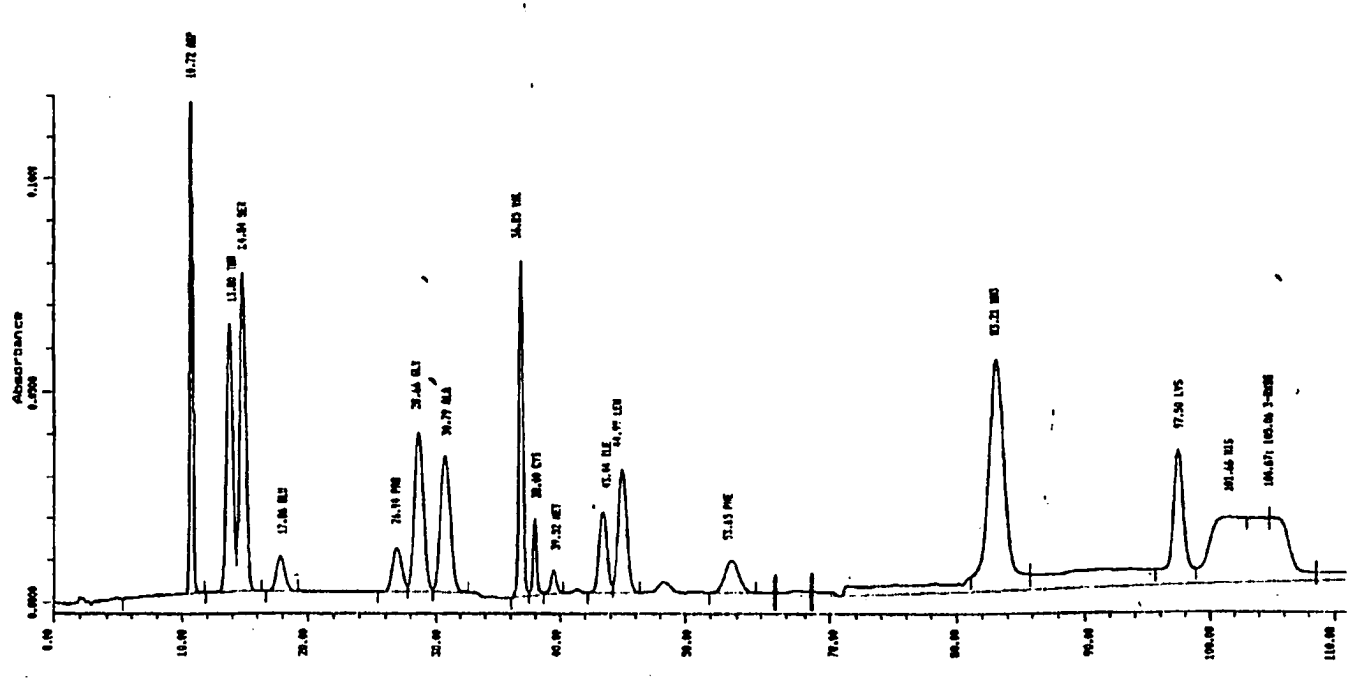


Fig. 18. Amino acid chromatogram of the extracellular protein.

Table 2. Amino acid composition of the extracellular protein.

Amino Acid	Residues
Nonpolar	(39.6%)
Alanine (A)	18
Valine (V)	14
Leucine (L)	9
Isoleucine (I)	5
Proline (P)	10
Phenylalanine (F)	3
Tryptophan (W)	1
Methionine (M)	1
Acidic	(9.7%)
Aspartate[a] (D)	13
Glutamate[a] (E)	2
Basic	(3.9%)
Lysine (K)	6
Arginine (R)	0
Histidine (H)	0
Polar uncharged	(46.8%)
Glycine (G)	21
Serine (S)	26
Threonine (T)	18
Tyrosine (Y)	2
Cysteine (C)	4
Total residues	153
Calculated mol wt	14,530

[a]Asparagine and glutamine are included or assumed to be included.

```
                5                   10
Ala-Thr-Ile-Thr-Pro-Val-Asn-Ser-Ala-Phe-
                15                  20
Thr-Ala-Pro-Gly-Thr-Ile-Ser-Val-Ser-Ser-
                25                  30
Pro-Ala-Ser-leu-Asn-Leu-Pro-Val-Thr-(Thr)-

Asn-(Ile)
```

Fig. 19. Thirty two amino acid sequence from the amino terminal end of the extracellular protein. Parenthesis are tentative identifications.

```
        5    10   15   20   25   30   35   40   45   50   55
NH3-MKSIKSLPSFAALALCLSVSSMASAATITPVNSAFTAPGTISVSSPASLNLPVTCN
                                [ATITPVNSAFTAPGTISVSSPASLNLPVT]
   60   65   70   75   80   85   90   95   100  105  110 115
ITFKGKTAADGSYASIDSVTVSGSNTLCSVPQMTGLPQKLTVSSTTAGKVDGVGFKILSS

   120  125  130  135  140  145  150  155  160
TCGPSTVNGSWSNATNTLSASNQSLAGNCKINSLSVKPTPAFVVNP-COOH
```

Fig. 20. Amino acid sequence deduced from the DNA sequence of the protein like activator of *P. aeruginosa* [Hardegger et al., 1994]. The 29 amino acid sequence from *P. aeruginosa* 17423 is compared (in brackets). The putative signal sequence is underlined.

its secondary structure. The absorbance of the extracellular protein was measured from 190 nm to 600 nm (Fig. 21). The two marked regions on the spectrum are absorbance at A_{214} (peptide bond) and A_{280} (aromatic amino acids). Circular dichroism was used to approximate the secondary structure of the protein (Fig. 22). The results indicate that the protein has 56.6% α helix, 0% β sheet, 43.1% turns, and 0.3% random coil. The CD results are not consistent, however, with a second method of predicting 2° structure. A computerized algorithm was used to predict 2° structure from the deduced pre-protein sequence of strain PG201 (Rost and Sander, 1993). The deduced protein from strain PG201 was estimated to have 16.7% α helix, 20.4% β strand, and 63% loop regions. Two segments of the sequence showed strong α helix characteristics from residues 8-21 and 127-142. Three β sheet regions were predicted from residues 69-78, 98-116, and 145-153 (Fig. 23).

Hydropathic index

Using the hydropathic profile of Kyte and Doolittle [1982] and the deduced amino acid sequence of strain PG201, the sequence showed three hydrophobic and three hydrophilic regions (Fig. 24). The region from residue 15-58 is slightly hydrophobic with interspersed hydrophilic residues.

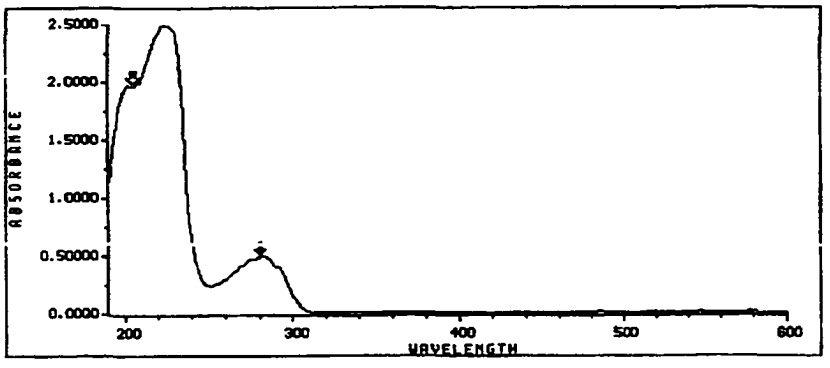


Fig. 21. Absorbance spectrum of a 1 mg/ml solution of the extracellular protein from wavelengths 190 nm to 600 nm. The marked areas are absorbance at 214 nm and 280 nm.

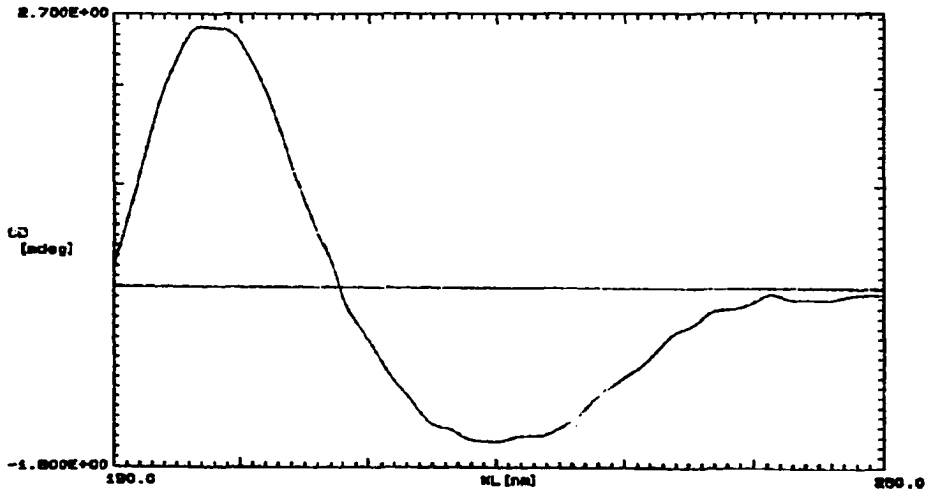


Fig. 22. Circular dichroism spectrum of a solution of the extracellular protein. Forty scans of DI water were subtracted from a 2 mg/ml solution of the protein.

```
        5    10   15   20   25   30   35   40   45   50   55
NH3-MKSIKSLPSFAALALCLSVSSMASAATITPVNSAFTAPGTISVSSPASLNLPVTCN
           αααααααααααααα

   60   65   70   75   80   85   90   95   100  105  110 115
ITFKGKTAADGSYASIDSVTVSGSNTLCSVPQMTGLPQKLTVSSTTAGKVDGVGFKILSS
            βββββββββββ                    βββββββββββββββββββ

   120  125  130  135  140  145  150  155  160
TCGPSTVNGSWSNATNTLSASNQSLAGNCKINSLSVKPTPAFVVNP-COOH
           αααααααααααααααααα  βββββββββ
```

Fig. 23. Secondary structure of the deduced pre-protein amino acid sequence of strain PG201 using a computerized algorithm by Rost and Sander [1993]. The final composition is 16.7% α helix, 20.4% β strand and 63% loop regions.

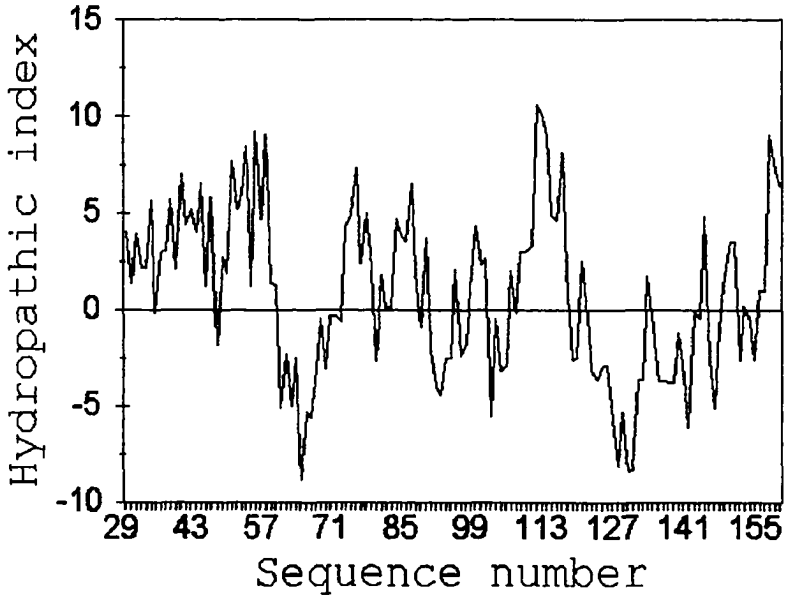


Fig. 24. Hydropathic profile of the deduced amino acid sequence from strain PG201 (Fig. 20). Values above the midpoint represent hydrophobic regions while values below the midpoint represent hydrophilic regions.

Regions 72-87 and 98-117 have similar properties. The regions from residues 59-71, 88-97, and 117-154 are primarily hydrophilic.

Observations of growth

When grown on hexadecane *P. aeruginosa* showed three distinct growth stages: lag, colonization, and turbidity. The lag stage generally lasted four to 12 hours and the culture remained clear. During this stage the bacteria are not found colonizing the hydrocarbon. After approximately 12 hours the colonization phase began and lasted three to four days. During this stage large particles composed of bacteria (confirmed microscopically) and hydrocarbon could be seen floating at the surface of the medium.

The observation that the bacteria were adhering to hydrocarbon led to a hydrocarbon binding experiment. The ability of *P. aeruginosa* to adhere to hexadecane was measured by adding [1-^{14}C] hexadecane to a suspension of nutrient broth-grown bacteria resuspended in BH medium. The final concentration of radioactivity in the culture was 12,200 DPM/ml. Non-specific binding of hexadecane to the bacterium was minimized by increasing the density of the solution with sucrose. When 300 µl was centrifuged, 82% of the radioactivity was removed. When the pellet was

resuspended and centrifuged a second time, 90% of the total radioactivity was removed from the bacterial pellet indicating that binding of hexadecane to the bacterium was minimal (Fig. 25).

After five to six days the growth of the bacterial culture continued in the turbid stage. During this stage it could be seen with a light microscope that the hexadecane had been emulsified into small micelles surrounded by bacteria. To test whether the protein can act as an emulsifier, increased absorbance was used in a separate experiment as an indicator of emulsification of hexadecane. The extracellular protein was compared to a common protein, bovine serum albumin, and was found to be a better emulsifier (Fig. 26).

The colonization and turbidity stages of *P. aeruginosa* can be indirectly observed using growth curves. Growth curves of *P. aeruginosa* on hexadecane are presented in Fig. 27. The growth curve begins with a short growth period, continues with a short stationary phase, then to a more pronounced growth period. The first growth period corresponds to the colonization stage where the bacteria are adhering to the hydrocarbon droplets. Vigorous vortexing releases the bacteria which can then be measured by optical density. During the second growth period, which represents

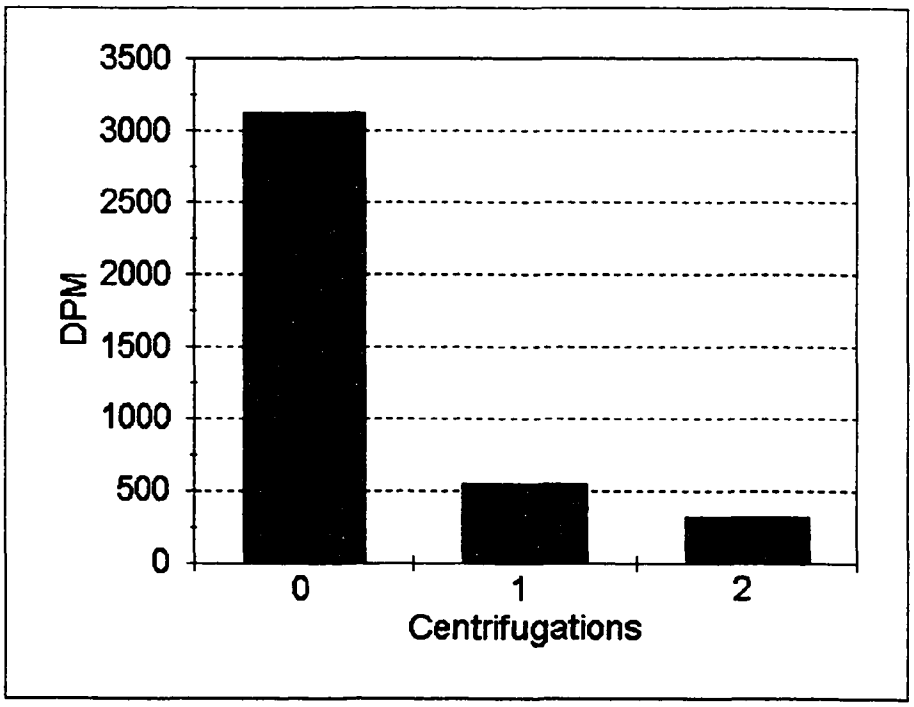


Fig. 25. Non-specific binding of ^{14}C-hexadecane to nutrient broth grown P. aeruginosa. One centrifugation removed 82% of the radioactivity while two centrifugations removed 90%.

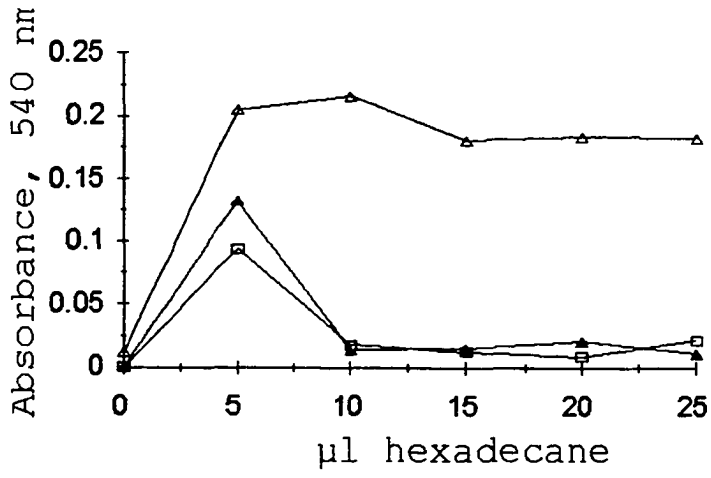


Fig. 26. The absorbance of various hexadecane solutions after agitation with either the extracellular protein, bovine serum albumin or water.

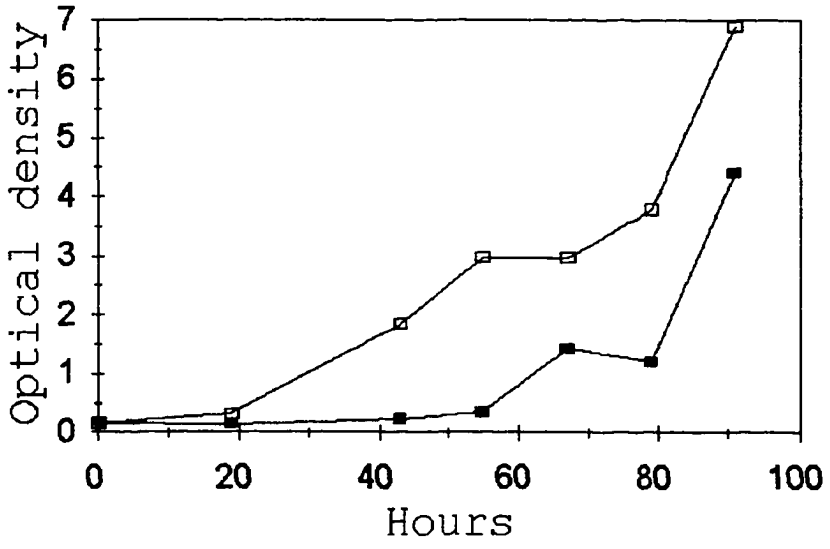


Fig. 27. Stimulation of growth of *P. aeruginosa* on hexadecane by the extracellular protein. Growth with added extracellular protein (0.5 mg/ml, □) is compared to normal growth (■).

the turbidity stage, the bacteria were more free in solution and the optical density was easily measured.

Using optical density (OD) it was shown that the extracellular protein stimulated growth of the bacterium, particularly at the beginning stages of growth (Fig. 27). The added absorbance is not caused by the bacterium using the protein as a substrate. No growth was observed when *P. aeruginosa* was introduced to the extracellular protein as the sole carbon source; even if incubated with the protein for four days at 25°C and agitated at 1,500 rpm.

When EDTA was added to active cultures of *P. aeruginosa* growth was severely curtailed. Part of this inhibition was reversed by adding the extracellular protein (Table 3).

Growth of the bacterium with added protein was studied using chemically modified protein. Approximately five lysine residues of the extracellular protein were modified by methylating ε groups (Table 4). The methylated lysine residues produce an extra peak after hydrolysis and chromatography (Fig. 28). When the modified protein was used in growth experiments and compared to normal growth (no added protein) no inhibition was observed (Fig. 29). Growth with the modified protein was not directly compared to growth with added native protein. However, the growth response to the lysine modified protein was greater than

Table 3. The effect of EDTA upon the growth of *P. aeruginosa* with and without additions of the extracellular protein. The OD of the cultures was measured at 48 hr.

Growth Conditions	Optical density, 540 nm.
Initial (time 0)	0.089
Normal growth (48 hr)	0.86
Growth with 4 mM EDTA (48 hr)	0.155
Growth with 0.1 mg/ml protein (48 hr)	1.2
Growth with 0.1 mg/ml protein and 4 mM EDTA (48 hr)	0.45

Table 4. Amino acid compositon of protein with methylated lysine residues compared to normal protein. For the analysis 25 µg of each protein was used with a 22 hr hydrolysis.

	Mol%, rounded to nearest integer	
Amino acid	Modified	Non-modified
Nonpolar		
Alanine	17	17
Valine	15	15
Leucine	10	9
Isoleucine	6	6
Proline	16	11
Phenylalanine	3	3
Tryptophan[a]	----	----
Methionine	1	1
Acidic		
Aspartic	13	14
Glutamic	2	2
Basic		
Lysine	**1**	**6**
Arginine	0	0
Histidine	0	0
Polar uncharged		
Glycine	22	22
Serine	21	23
Threonine	17	17
Tyrosine	----	----
Cysteine	4	4

[a]Tryptophan is destroyed using hydrolysis by HCl. Values of serine and threonine were not corrected for partial destruction by the HCl.

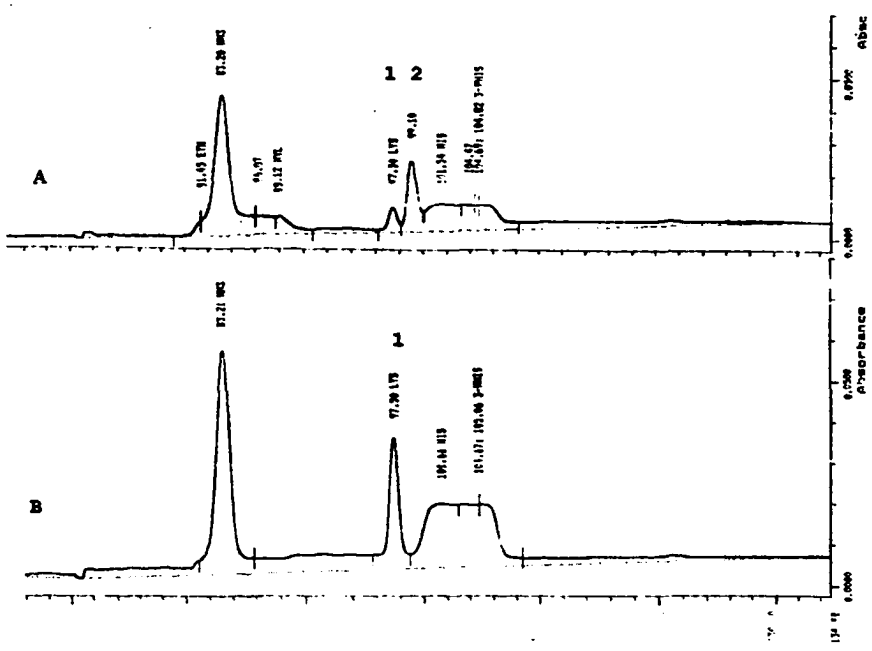


Fig. 28. Effect on amino acid retention after lysine modification of the extracellular protein. The lysine peak for the modified protein (A) is split into two peaks (1 and 2). This is compared to unmodified protein with only one lysine peak (1).

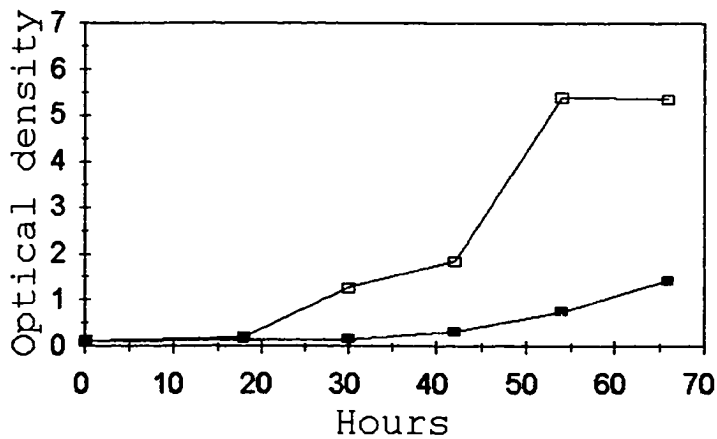


Fig. 29. Effect of protein with modified lysine on growth of *P. aeruginosa*. No inhibition was seen.

Using another procedure, the acidic residues of the protein were modified with glycine methyl ester. After modification it was found that approximately six residues from the protein were modified (Table 5). When this modified protein was used in growth experiments and compared to normal growth, and growth with the native protein, the bacteria were inhibited compared to the native protein but stimulated compared to normal growth (Fig. 30).

Using a different method to compare growth, nutrient broth acclimated bacteria were compared to hexadecane acclimated bacteria. Hexadecane acclimated cells had a much longer colonization phase and produced more total protein than nutrient broth acclimated cells (Fig. 31). In addition after long term exposure to hydrocarbon the bacterium developed a different colony morphology and the cells were 2-3 times larger (Fig. 32-33).

Agglutination of cells and carbohydrate binding by the extracellular protein

A series of experiments was performed to determine the carbohydrate binding ability of the extracellular protein. These experiments included agglutination of cells and

Table 5. Amino acid composition of protein with modified acidic residues compared to normal protein Amino acid composition. For the analysis 25 µg of each protein were used with a 22 hr hydrolysis. Increased glycine indicates modified residues with glycine methyl ester.

	Mol%, rounded to nearest integer	
Amino acid	Modified	Non-modified
Nonpolar		
Alanine	19	19
Valine	13	14
Leucine	10	9
Isoleucine	5	5
Proline	12	12
Phenylalanine	3	3
Tryptophan[a]	----	----
Methionine	1	1
Acidic		
Aspartic	14	14
Glutamic	2	2
Basic		
Lysine	6	6
Arginine	0	0
Histidine	0	0
Polar uncharged		
Glycine	**28**	**22**
Serine	23	23
Threonine	15	18
Tyrosine	----	----
Cystine	4	4

[a]Tryptophan is destroyed using hydrolysis by HCl. Values of serine and threonine were not corrected for partial destruction by the HCl.

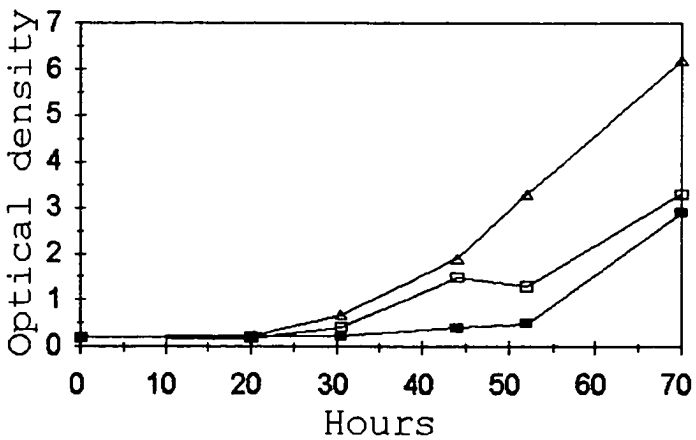
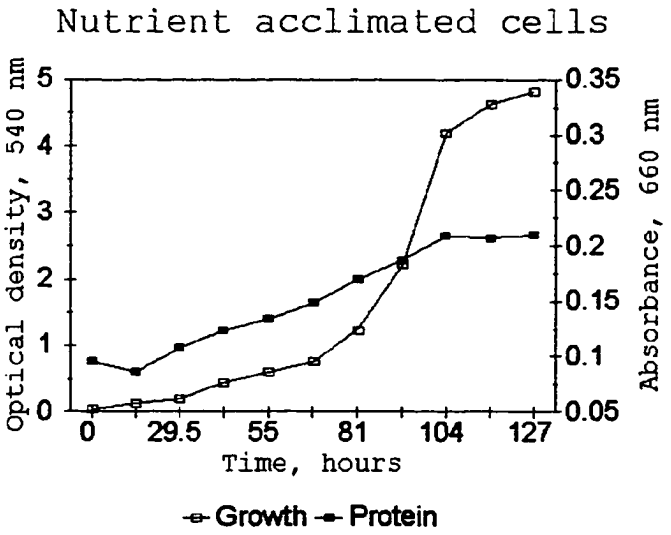


Fig. 30. Growth of *P. aeruginosa* on hexadecane, normal protein, and modified protein. The protein solutions were 0.6 mg/ml and the modifications were to the acidic residues.



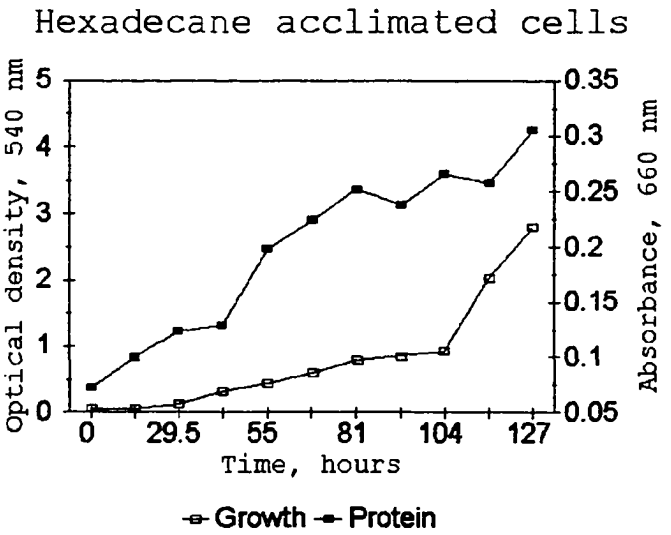


Fig. 31. Growth characteristics and production of extracellular protein by nutrient broth acclimated and hexadecane acclimated cultures of *P. aeruginosa*.

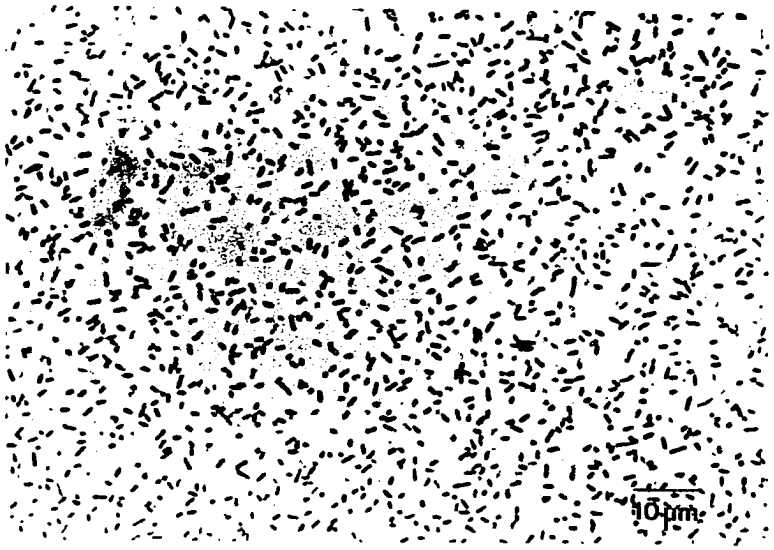


Fig. 32. Nutrient-broth acclimated *P. aeruginosa* 17423 (x1,000).

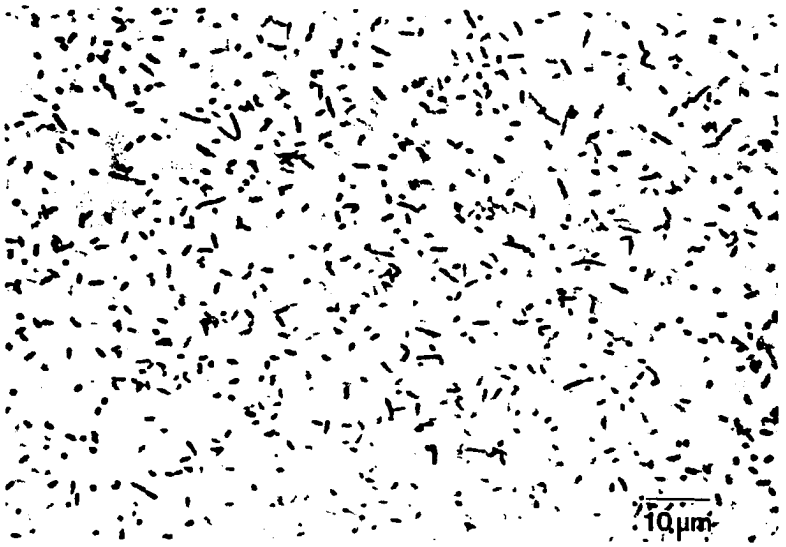


Fig. 33. Hexadecane-acclimated *P. aeruginosa* 17423 (x1,000).

binding to a solid matrix. When agglutination experiments were conducted with just the extracellular protein and nutrient broth-grown bacteria, the solution became cloudy and in some instances the bacterial agglutination caused the bacteria to come out of solution (Fig. 34 and 35). The minimum protein concentration causing noticeable agglutination, for a suspension of *P. aeruginosa* at an optical density of 0.33 at 540 nm, was approximately 7 µg/ml. The protein also agglutinated *E. coli* but the reaction took longer than with *P. aeruginosa* (12 hr compared to one hr) (Fig. 36 and 37).

Agglutination inhibition experiments were used to determine which carbohydrates inhibited binding of the extracellular protein. Inhibition occurred at high concentrations of carbohydrate. Four monosaccharides were tested with *P. aeruginosa:* D-glucose, D-galactose, D-mannose and L-rhamnose at 0.3 M carbohydrate and 1 mg/ml protein. Microscopic examination was used to check for agglutination. Inhibition was detected with glucose, mannose, and rhamnose.

The results from the inhibition test showed that glucose, mannose and rhamnose inhibited agglutination at high concentrations. With this information five columns were tested for binding: 1) Sephadex (for glucose), 2) Sepharose 4B (control, for galactose), 3) Mannose-

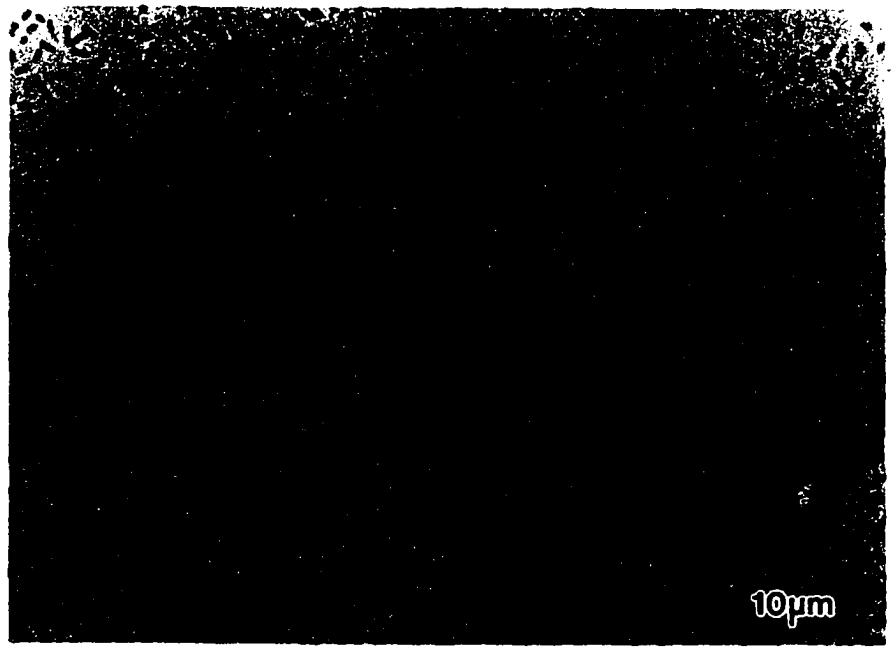


Fig. 34. *P. aeruginosa* without the extracellular protein. Magnification x1,000.

Fig. 35. *P. aeruginosa* in 0.5 mg/ml extracellular protein. Magnification x1,000.

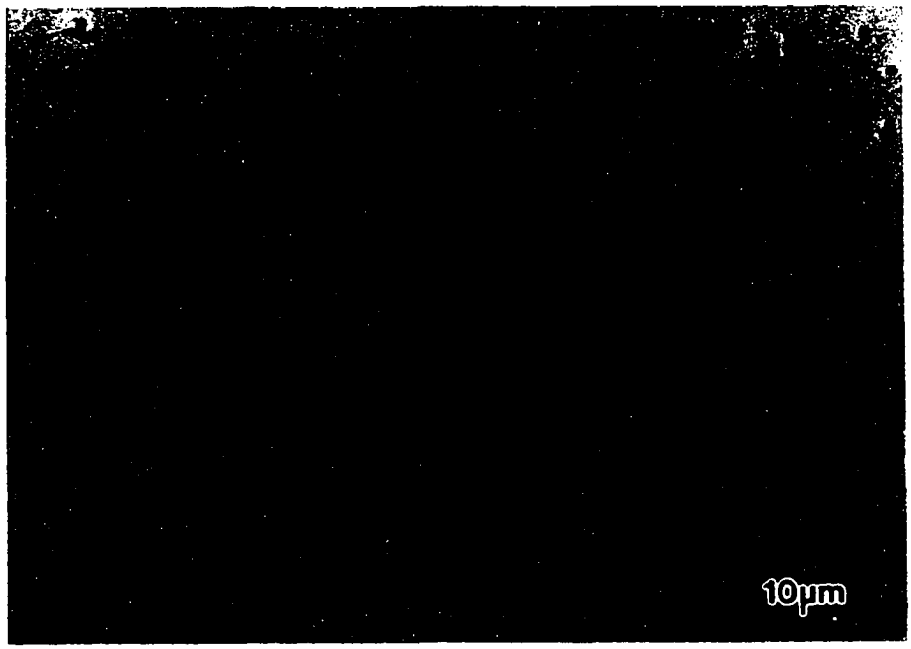


Fig. 36. *E. coli* without the extracellular protein. Magnification x1,000.

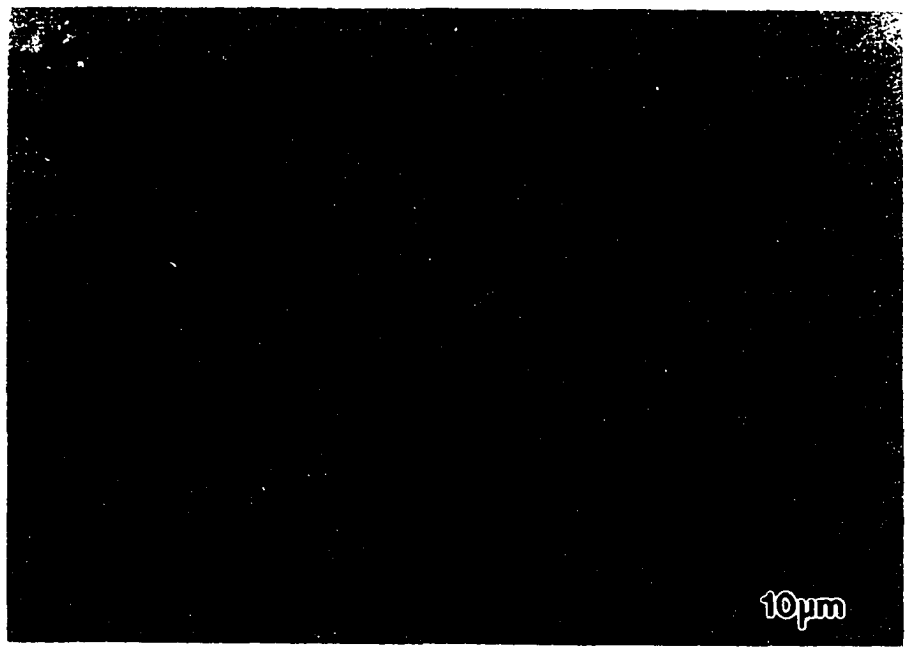


Fig. 37. *E. coli* in 0.5 mg/ml extracellular protein. Magnification x1,000.

derivatized Sepharose 4B, 4) Cellobiose-derivatized Sepharose 4B, and 5) Rhamnose-derivatized Sepharose 4B. The extracellular protein did not adhere to any of these columns.

Four red blood cell groups were tested for agglutination by the extracellular protein. The four blood groups were human A, B, O and horse all at 1% hematocrit. The extracellular protein only reacted with human blood type O at 1.7, 0.85, and 0.43 mg/ml protein while horse reacted at 1.7 and 0.85 mg/ml. The agglutination that occurred did not produce the typical positive test seen with agglutination using titer plates and had to be visualized microscopically. The reaction took place over a 24 hour period indicating poor binding.

Binding of the protein to the glycolipid surfactant

The glycolipid surfactant from *P. aeruginosa* 17423 was isolated, and the purity confirmed using thin layer chromatography (TLC) sheets. After the initial isolation two bands were detected using a carbohydrate specific developer that had retention factors (Rf) of 0.39 and 0.76 (Fig. 38). Bands with similar Rf's have been described as glycolipid with one carbohydrate (Rf 8.0) or with two (Rf 4.0) [Itoh et al., 1971]. The solution was further purified

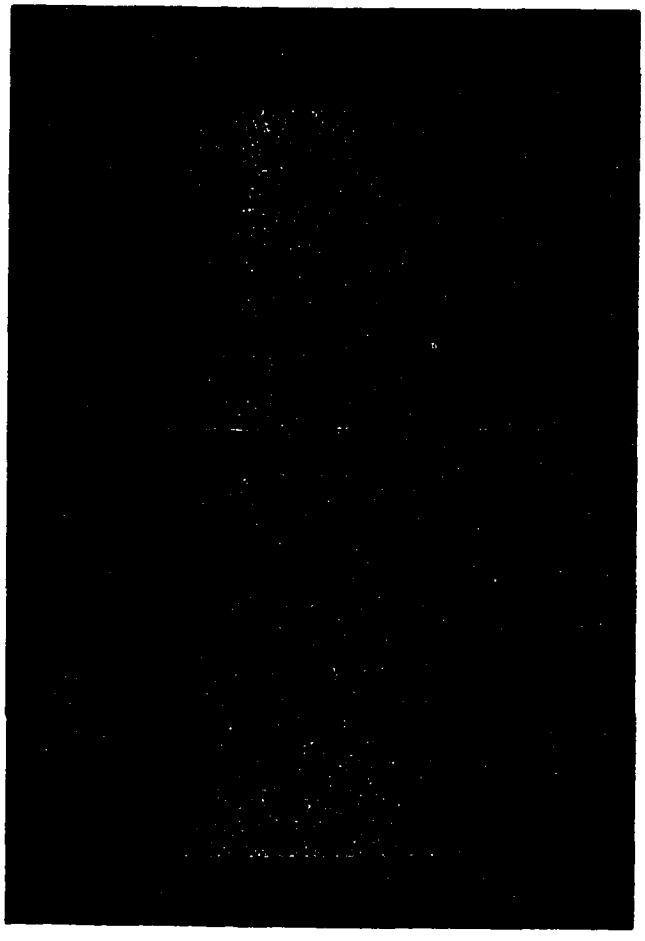


Fig. 38. Thin layer chromatography showing the two bands detected from the glycolipid solution. The lower band has an Rf of 0.39 while the upper band is 0.76.

by column chromatography and when this solution was developed using a TLC plate the band corresponding to an Rf of 0.39 was detected. The purified glycolipid surfactant was immobilized using several different methods to determine if the extracellular protein could be bound.

A series of three glycolipid immobilizing experiments were performed for the protein binding experiments. For the first method the glycolipid surfactant was bound to a plastic surface [Leffler and Svanborg-Eden, 1986]. Before the binding could be measured the surfactant lifted off the plastic when a solution of the extracellular protein was added. For the second method the protein was bound to a thin-layer chromatography (TLC) sheet [Smith, 1983]. Once again the surfactant pulled off from the solid surface when the protein solution was added.

In another attempt to immobilize the glycolipid from *P. aeruginosa*, reverse phase HPLC silica gel with octadecyl bonded phase C_{18} (which are actually chips of glass) was employed. The glycolipid was attached by adding the glycolipid surfactant in ethanol to the gel then allowing the ethanol to evaporate. The C_{18} silica gel treated in this manner was soluble and slowly precipitated over time while un-treated gel was insoluble and stayed at the top of the solution. The treated gel was added to a solution of

the extracellular protein, then a suspension of *P. aeruginosa*. If the bacteria bound to the outside of the bead it was considered positive for binding. Binding of the bacteria occurred within five minutes compared to agglutination which can take an hour (Fig. 39 and 40). While some non-specific binding of bacteria and gel occurred in the absence of protein the degree was substantially less than in the presence of protein.

Fig. 39. *P. aeruginosa* and glycolipid treated HPLC reverse phase silica gel. Magnification x600.

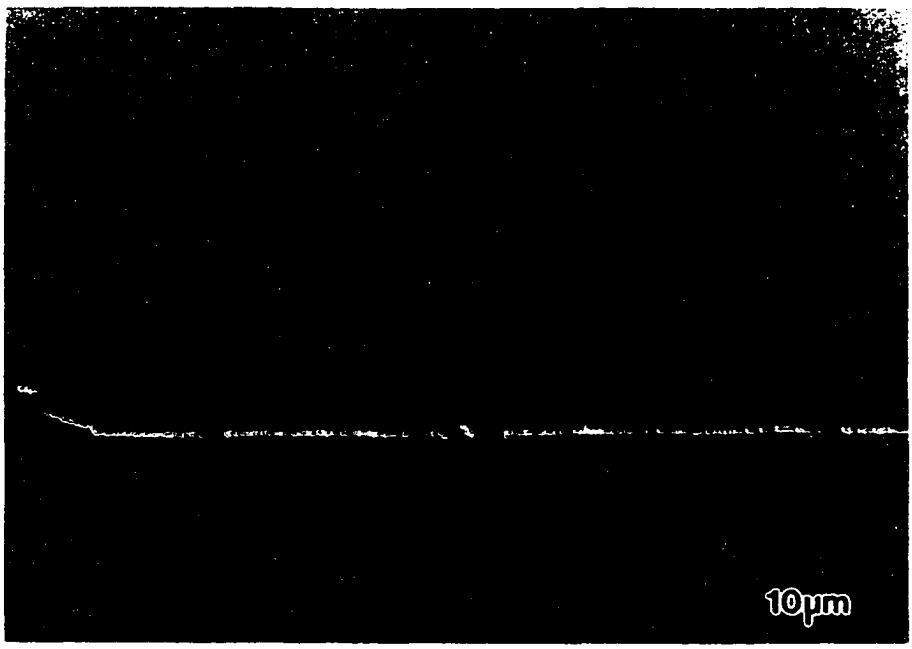


Fig. 40. *P. aeruginosa*, extracellular protein and glycolipid treated HPLC reverse phase silica gel. Magnification x1,000.

DISCUSSION

Introduction

Pseudomonas aeruginosa produces both an extracellular protein and a glycolipid surfactant while growing on hexadecane. Both extracellular compounds have been shown to stimulate growth of the bacterium on hexadecane. While much is known about the glycolipid surfactant [Jarvis and Johnson, 1949; Hisatsuka et al., 1972; 1977; Zhang and Miller, 1992; 1994], little is known about the extracellular protein.

P. aeruginosa S7B1 produces an extracellular protein with a molecular weight of approximately 14,300. When the isolated protein is added to active cultures it stimulates growth of the bacterium. The protein is only found during growth on hydrocarbon and is not found when the bacterium is grown on nutrient broth, glucose, glycerol, or palmitic acid [Hisatsuka et al., 1977]. It has also been shown that production of the protein by *P. aeruginosa* S7B1 increases as the solubility of the hydrocarbon substrate used decreases; the more hydrophobic the substrate the more protein is produced [Hisatsuka et al., 1977]. The authors hypothesized that the protein stimulated growth by acting as an emulsifier or an emulsion stabilizer in conjunction with the

glycolipid surfactant.

P. aeruginosa PG201 produces a similar extracellular protein of approximately 16,000 molecular weight. The gene for this protein, called the *pra* gene, has been sequenced and mutants of strain PG201 with an inactivated *pra* gene are growth impaired during hydrocarbon degradation. It has also been shown that mutants of strain PG201 with both the *pra* gene and the rhamnolipid genes inactivated could still degrade hydrocarbons slowly [Hardegger et al., 1994].

I have isolated an extracellular protein from strain 17423 that shares an identical 29 amino terminal sequence with strain PG201 and has a similar amino acid composition as strains S7B1 and PG201. It has previously been proposed that the protein from S7B1 acts as a surfactant. Evidence from my study suggests that the protein is dissimilar to surfactant-like molecules but instead may be similar to lectins (carbohydrate binding proteins). A model is presented for the function of the protein during growth on linear alkanes by *P. aeruginosa*.

Isolation and purification of the extracellular protein

Purification of the extracellular protein is complicated by its close contact with the emulsified alkane and the glycolipid surfactant. Additionally, degradation of

the protein occurs during the purification process. I have developed a new protein purification method that eliminates protease degradation and produces a very pure product as shown by SDS-PAGE (Fig. 14). The purification procedure yields 20-30 mg of very pure protein per 500 ml culture. A previous purification procedure utilized to isolate the protein from S7B1 used precipitation and column chromatography to yield approximately 100 mg of protein from 2 l of culture medium [Hisatsuka et al., 1972]. This procedure did not work well with *P. aeruginosa* 17423 resulting in small yields of partially degraded protein.

A more recent purification procedure [Hardegger et al., 1994] used recombinant technology to bypass the isolation problems. The gene for the protein produced by strain PG201 was cloned into *E. coli* to produce a fusion protein with six additional histidine residues. The protein was easily purified using a Ni column. While this approach may prove to be a good one, Hardegger et al. (1994) did not establish if the structure of the protein is similar to the structure of the native protein. It was also not established what effect the additional histidine residues might have on the activity of the protein. The authors did not show any experiments where they tested the protein for stimulation of growth with the bacterium. Their isolation procedure only

yields 2 mg of protein for a 500 ml culture.

I was able to obtain high yields of native protein by modifying the technique of Hisatsuka et al. [1972]. Two major steps were added which increased the yield and purity of the protein. The two steps were: 1) collecting the low density material after adding ammonium sulfate, and 2) heat treating the culture supernatant. The first step was added because it was found that the majority of the protein is associated with the low-density material produced when ammonium sulfate is added to the culture supernatant. In contrast to most isolation procedures that use NH_4SO_4, the protein of interest is interacting with the low density material and is eventually found floating at the surface of the solution instead of as a precipitate. The low density material when viewed through a light microscope was composed of spherical particles that appear to be droplets of emulsified hexadecane (Fig. 16). When this material was dialyzed with the resuspended pellets from the centrifugation step, the protein recovery was increased considerably. Interaction of the protein with the low density material may be the reason that the protein would not precipitate with organic solvents [Hisatsuka et al., 1972]. The low density material also bound to the DEAE column and could be eluted using NaCl. The spherical

particles are partially protein (Fig. 17), glycolipid surfactant, and hexadecane. Binding to the DEAE column may be the result of the glycolipid surfactant which has an acidic group with a pKa of 5.6 [Zhang and Miller, 1995].

In the second step the culture supernatant was heat treated to inhibit protease degradation. When the culture supernatant was not heat treated several degradation products of the extracellular protein were observed on polyacrylamide gels. The banding was presumably due to degradation of the protein by a protease that was inhibited by heating the culture solution immediately after removing the bacteria by centrifugation (see Fig. 14). It has been shown that the extracellular protein from strain S7B1 is very heat resistant compared to most proteins [Hisatsuka et al., 1977]. Similarly I found that the heat treatment used while isolating the protein from strain 17423 had no effect on the activity of the extracellular protein.

Structural similarities between the three extracellular proteins

Sequence similarities. When the N terminal sequence of the mature protein from strain PG201 was compared to the deduced sequence it was found that residues 1-25 were missing. The missing residues are assumed to be the signal

sequence for the protein [Hardegger et al., 1994]. When the N terminal sequence from the non-heat treated protein isolated from strain 17423 was compared to the deduced sequence from strain PG201, the two sequences were found to be identical from residue 26 to residue 55 (Fig. 20). Interestingly, cleavage of the deduced sequence from strain PG201 between residues 25 and 26 produces a mature protein with a calculated molecular weight of only 13,700 which is inconsistent with the published SDS-PAGE molecular weight of 16,000 [Hardegger et al., 1994]. Why this discrepancy exists has not been determined.

Amino acid composition. Acid hydrolysis and spectroscopy of the protein from strain 17423 revealed an unusual amino acid composition. The protein has a high percentage of polar amino acids (47%) and hydrophobic residues (40%) while no arginine or histidine was detected. When the amino acid composition of strain 17423 was compared to the published composition of strain S7B1 and to the deduced amino acid composition of strain PG201 they were all very similar (Table 6). All three proteins lack arginine and histidine while having high percentages of hydroxy amino acids. The lack of arginine residues hindered the detection of small amounts (< 10 µg) of the extracellular protein using SDS-PAGE gels. This has been observed with other

proteins that lack arginine and it is believed that arginine residues are important for binding of coomossie blue stain [Scopes, 1988]. This observation may explain why the Bradford protein assay did not yield reliable results in this study. The extracellular protein isolated from strain 17423 and the deduced amino acid sequence from strain PG201 were found to be practically identical by sequence similarities and amino acid composition. The 29 amino acids that were compared to the deduced sequence had perfect correlation (Fig. 20) while the amino acid composition only differed by a few amino acids (Table 6). Because of these similarities the deduced sequence from strain PG201 was used for analysis of structure and function of the extracellular protein.

The structure of lectins and similarities to the extracellular protein

Comparative analysis with the entire sequence from strain PG201 using the GENBANK release 82 data base showed no extensive homology with other sequences. This lack of sequence homology is common for carbohydrate binding proteins (lectins) and lectin-like proteins due to variations in the protein and in the binding site [Schoolnik

Table 6. Amino acid composition of the extracellular protein from *P. aeruginosa* strain ATCC 17423 and S7B1 [Hisatsuka et al., 1977]. The amino acid composition from the deduced amino acid sequence of strain PG201, starting at residue 26, is also given [Hardegger et al., 1994].

Amino Acid	ATCC 17423	PG201	S7B1
Nonpolar	(39.6%)	(42%)	(39%)
Alanine (A)	18	13	12
Valine (V)	14	13	13
Leucine (L)	9	9	9
Isoleucine (I)	5	6	6
Proline (P)	10	10	10
Phenylalanine (F)	3	4	4
Tryptophan (W)	1	2	2
Methionine (M)	1	1	1
Acidic	(9.7%)	(11.7%)	(11%)
Aspartate[a] (D)	13	14	14
Glutamate[a] (E)	2	2	2
Basic	(3.9%)	(5.1%)	(4.8%)
Lysine (K)	6	7	7
Arginine (R)	0	0	0
Histidine (H)	0	0	0
Polar uncharged	(46.8%)	(41.6%)	(46%)
Glycine (G)	21	11	11
Serine (S)	26	23	32
Threonine (T)	18	18	19
Tyrosine (Y)	2	1	1
Cysteine (C)	4	4	4
Total residues	153	137	147
Calculated MW	14,530	13,700	14,580

[a]Asparagine and glutamine are included or assumed to be included.

et al., 1986]. These variations make comparing a new protein to known lectins difficult. One clue that the protein may be a lectin is in its amino acid composition. Lectins typically have high percentages of amino acids such as the hydroxy amino acids and amides (asparagine and glutamine) that participate in hydrogen bonding to carbohydrate. Hydrophobic interactions with carbohydrates occur with alanine, valine and other hydrophobic residues. Also, it is not unusual for a lectin to have a few aspartic acid residues that function by binding to divalent cations [Goldstein and Poretz, 1986].

When the amino acid composition of the extracellular protein from strain 17423 was compared to two known *P. aeruginosa* lectins, the galactose binding protein (PA-1) from strain 33347 and pilin from strain PAK it had similar characteristics (Table 7). Although lectins have similar amino acid compositions, comparing DNA or amino acid sequences can be difficult because the critical binding residues are discontinuous in sequence yet conserved in space. For this reason the critical binding residues of many lectins are compared by their 3D structure derived from x-ray crystallography. Once the critical residues are determined, the sequence can be used as a template to compare closely related lectins from the same group [Young

Table 7. Amino acid composition of the extracellular protein from *P. aeruginosa* sp. 17423. Also, a lectin from a pathogenic strain of *P. aeruginosa* (ATCC 33347), and pilin from strain PAK [Gilboa-Garber and Garber, 1977; Paranchych, 1989].

	Strain		
Amino Acid	17423	PA-I	PAK
Nonpolar	(39.6%)	(41%)	(45%)
Alanine (A)	18	10	19
Valine (V)	14	10	8
Leucine (L)	9	5	12
Isoleucine (I)	5	10	12
Proline (P)	10	7	7
Phenylalanine (F)	3	3	3
Tryptophan (W)	1	4	2
Methionine (M)	1	1	2
Acidic	(9.7%)	(21.5%)	(16%)
Aspartate[b] (D)	13	17	12
Glutamate[b] (E)	2	9	11
Basic	(3.9%)	(6.6%)	(12%)
Lysine (K)	6	4	13
Arginine (R)	0	2	4
Histidine (H)	0	2	0
Polar uncharged	(46.8%)	(30.6%)	(28%)
Glycine (G)	21	17	15
Serine (S)	26	7	7
Threonine (T)	18	7	14
Tyrosine (Y)	2	4	2
Cysteine (C)	4	2	2
Number of residues	153	121	143
Molecular weight	14,530	12,760	15,100

[a]Destroyed with method used or not given.
[b]Asparagine and glutamine are included or assumed to be included.

and Oomen, 1992]. One important conclusion confirmed by analyzing many 3D structures of lectins is that the binding site is always a deep cleft between two domains of the protein [Newcomer et al., 1979; Vyas et al., 1991]. An example of the three dimensional structure of a lectin is given by the arabinose binding protein from *E. coli* (Fig. 41).

Secondary structure and comparison to PA I

The secondary structure of the extracellular protein from strain 17423 determined by circular dichroism was found to have 56.6% α helix, 0% β sheet, 43.1% turns and 0.3% random coil. The CD results are not consistent with a second method of predicting 2° structure using a computerized algorithm. The computerized algorithm predicted a 2° structure from the deduced pre-protein sequence of strain PG201. The protein was estimated to have 16.7% α helix, 20.4% β strand, and 63% loop regions (see Fig. 23). The discrepancy between the secondary structure predictions may be due to unknown errors using circular dichroism at the far end of the UV spectrum or to an unusual structure of the protein. The predicted results from the algorithm agree better with the secondary structure of most lectins which have a large percentage of β sheet secondary

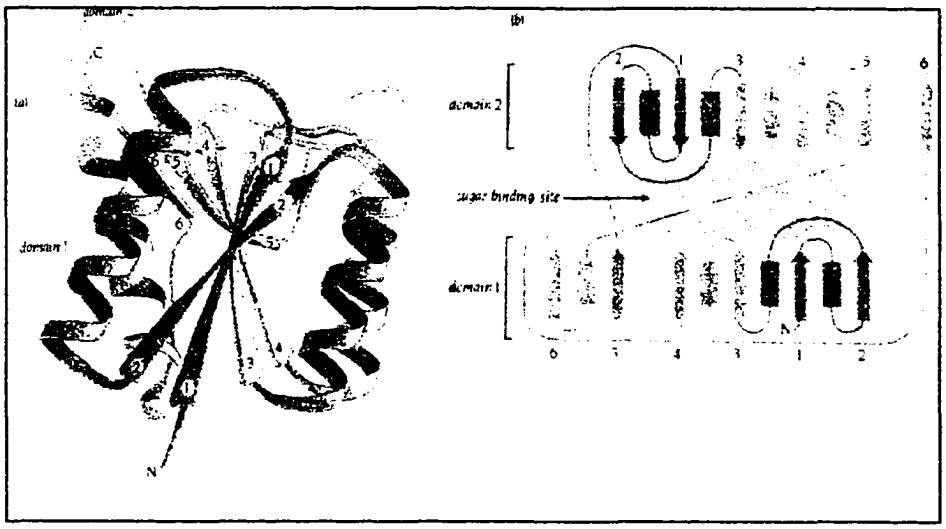


Fig. 41. Structure and binding site of the arabinose binding protein from *E. coli* (Re-drawn from Branden and Tooze, 1991).

structure.

Hydrophobic structure and interactions

The method of Kyte and Doolittle [1982] was applied to the deduced amino acid sequence from strain PG201 to determine whether the extracellular protein has a hydrophobic domain that allows it to bind to hexadecane (see Fig. 24). The results are very similar to those seen with soluble proteins. No outstanding hydrophobic regions were evident although the segment from residues 26 to 60 appear to be slightly hydrophobic. Even though much of the deduced protein is comprised of hydrophobic residues (40%), which might indicate the presence of hydrophobic domains, they are interspersed with hydrophilic polar residues (47%).

During hydrophobic chromatography, using octyl-sepharose, the protein passes through the column without binding. This technique is used for hydrophobic proteins, such as lipoprotein, therefore showing that the protein is not strongly hydrophobic.

The signal sequence from strain PG201

General trends that have been observed with signal sequences are that they have a charged residue within the

first five amino acids, a core of at least nine hydrophobic amino acids to span the membrane, and a helix breaking residue such as glycine or proline four to eight residues before the cleavage site [Watson, 1984]. Another characteristic of signal sequences is that the cleavage site has at least one amino acid with a small uncharged side chain. Out of 40 prokaryotic signal sequences 88% of the cleavage sites end with an alanine or glycine [Watson, 1984]. The signal sequence for the deduced amino acid sequence fits all of these criteria. The secondary structure predictions show that the signal sequence has strong α helix characteristics (Fig. 23). The signal sequence from the deduced sequence from strain PG201 is compared to other microbial signal sequences in Fig. 42.

Growth of *P. aeruginosa* on hexadecane

Nutrient broth acclimated *P. aeruginosa* 17423 changed morphology during long-term acclimation to hexadecane. The bacteria became larger (see Fig. 32, 33) and "sticky", resulting in the bacteria sticking to each other. This was apparent during centrifugation; the bacterial pellets from the hexadecane-acclimated form were more difficult to resuspend. It is possible that the bacteria are more hydrophobic as a result of changes to their

```
A)     MKVKVLSLLVPALLVAGAANA AEVYMKDGNK...
B)      MKKSTLALVVMGIVASAVQA AEIYNKDGNK...
C)   MKKSLVLKASVAVATLVPMLSFA AEGDDPAKAA...
D)    MKIKTAIAIAVALAGFATVAGA APKDNTWYAG...
E)     MKKTAIAIAVALAGFATVAQA APKDNTWYAG...
F)    MKKLALSLSLVLAFSSATAAFA AIPQKIRIGT...
G)    MKKTVLALSLLIGLGATAASYA ALPQTVRIGT...
H)     MKKTAIAITVALAGFATVAGA APKDNNTWYT...
I)      MKKVSTLDLLPVAIMGVSPA AFAADLIDVSK..
J)   MKAQKGFTLIELMIVVAIIGILA/AIAIPQYQNYV..
K)MKSIKSLPSFAALALCLSVSSMASA ATITPVNSAFT...
```

Fig. 42. Signal sequences from varicus microorganisms. A) *Escherichia coli* outer membrane protein C (ompC), and B) ompE. C) Phage M13 major coat protein. D) *Enterobacter aerogenes*, ompA. E) *Salmonella typhimurium* ompA, F) histidine binding protein, and G) lys-arg-orn binding protein. H) *Shigella dysenteriae* ompA [Watson, 1984]. I) *P. aeruginosa* elastase, J) Pilin from strain PAK (does not get cleaved), and K) Extracellular protein [Bever and Iglewski, 1988; Paranchych et al., 1990; Hardegger et al., 1994].

lipopolysaccharide. At least one strain of *P. aeruginosa* has been shown to have 30-40% less carbohydrate in their lipopolysaccharide when grown on hydrocarbons than when grown on glucose [Miguez et al., 1986]. Miguez et al. hypothesized that less carbohydrate would make the bacterium more hydrophobic but they did not observe the bacterium adhering to hydrocarbon. These morphological changes may explain how some growth occurs with mutants of strain PG201 that cannot produce the extracellular components [Hardegger et al., 1994].

The growth of *P. aeruginosa* 17423 on hexadecane has three distinct stages: lag, colonization and turbidity stage. This is in contrast to most hydrocarbon degrading microorganisms which either adhere or emulsify the hydrocarbon. However, another gram negative bacterium, *Acinetobactor* RAG-1, has been shown to also have three growth stages [Rosenberg et. al, 1980]. This bacterium is similar to *P. aeruginosa* in that *Acinetobacter* Rag-1 produces an extracellular protein associated with carbohydrate and lipid called "emulsan". It is believed that *Acinetobactor* obtains hydrocarbon by colonization until the surfactant concentration is high enough for emulsification to occur [Rosenberg and Rosenberg, 1981].

A similar strategy may be used by *P. aeruginosa* when

non-acclimated cells are introduced to hexadecane. During the lag stage the bacterium does not colonize the hexadecane droplets. In addition, the bacterium does not have any affinity for hexadecane when it is not acclimated to the hydrocarbon (Fig. 25). Therefore, during this four to eight hour period, the bacterium may be either changing its outer morphology for colonization and/or producing factors that allow it to adhere to the hydrocarbon droplets. During the colonization phase, bacteria can be seen covering the exterior of large hydrocarbon droplets. The next stage, the turbidity stage, begins when the glycolipid surfactant concentration is high enough to emulsify the hydrocarbon (Fig. 27).

The first increase of growth shown on the growth curve corresponds to the colonization stage where the bacteria are adhering to the hydrocarbon droplets and the surfactant concentration is low. To measure growth at this stage, vigorous vortexing was needed to release the bound bacteria which could then be measured by optical density. The second increase in growth shown on the growth curve corresponds to the turbidity stage where the surfactant concentration is high and the bacteria are free in solution.

Effects of EDTA on growth with hexadecane

EDTA has been reported to inhibit growth of some hydrocarbon-degrading microorganisms including *P. aeruginosa* [Hisatsuka et al., 1975; Goswami and Singh, 1990]. How EDTA affects growth has not been determined although there is some evidence that EDTA inhibits microorganisms that produce emulsification factors but does not inhibit microorganisms that directly adhere to hydrocarbon [Goswami and Singh, 1990; Roy et al., 1979]. EDTA has also been shown to inhibit agglutination of cells by known plant and microbial lectins by depriving the proteins of divalent cations [Goldstein and Poretz, 1986; Gilboa-Garber, 1986]. EDTA severely curtails growth of *P. aeruginosa* while growing on hexadecane (see Table 3).

Protein modification

The addition of the extracellular protein, produced using the isolation procedure developed during this study, to cultures of *P. aeruginosa* stimulated growth of the bacterium on hexadecane (see Fig. 27). This stimulation has also been observed with the extracellular protein from *P. aeruginosa* S7B1 [Hisatsuka et al., 1972; 1977]. One objective of this study was to determine if the protein had more of a role in uptake of hydrocarbon than just

emulsification. One possible role of the protein might be to hydrophobically bind emulsified hydrocarbon, then bind to the negatively charged exterior of the bacterium. This binding to the bacterium might be caused by the positively charged residues of the protein or by the negative residues of the protein by virtue of a divalent cation salt bridge. This second explanation would also explain the inhibition by EDTA. Two methods were used to investigate both possibilities. Both methods involved chemical modification of the charged amino acid residues to determine if they had any role in the activity of the protein.

For the first method a methyl group was added to the ε amino group of lysine. This modification added a steric group to the lysine residues which would interfere with any binding. The modified protein was used in experiments to determine if any change in growth occurred when compared to growth with the native protein. No inhibition was seen and in fact this modification possibly stimulated growth slightly (see Fig. 29).

For the second method the extracellular protein was modified by adding glycine methyl ester to the carboxyl group of aspartic and glutamic acid. When this modified protein was added to growing cultures the results were very different from the modified lysine experiment. The

stimulatory activity of the protein was reduced compared to the native protein (see Fig. 30). Some stimulation of growth still occurred relative to normal growth implying that the protein was not directly binding by a salt bridge. A more plausible explanation for the inhibition is that the modification disrupted the protein's ability to bind Ca^{2+}. Calcium is frequently found with lectins which, by way of its binding, positions residues in the binding site properly to interact with carbohydrate. This same modification with known lectins will inhibit agglutination of cells. For example it has been shown that two residues of aspartic acid are involved in each binding site of the plant lectin concanavalin A. The sugar binding ability of concanavalin A can be eliminated by chemical modification of the carboxylate groups [Goldstein and Poretz, 1986].

Surfactant-like properties of the extracellular protein

It has been hypothesized that the stimulation of growth that occurs when the protein is added to cultures of *P. aeruginosa* is caused by the extracellular protein acting as a co-surfactant, in conjunction with the glycolipid surfactant, to emulsify the hydrocarbons [Hisatsuka, et al., 1977]. However, no mechanism has been proposed for the interaction of the protein with droplets of hexadecane. The

results of this study do not generally support that hypothesis although the protein did act as a better surfactant than bovine serum albumin (see Fig. 26). The protein did not display any hydrophobic characteristics such as binding to plastic or precipitation at high concentrations. Also, the protein does not bind to hydrophobic columns. Another indication the protein lacks hydrophobic qualities is that it has a similar molecular weight using gel filtration chromatography as was found using SDS-polyacrylamide gel electrophoresis (see Fig. 10, 11). This suggests that no self-association of the protein is occurring and that the protein is soluble and monomeric. Although the protein may not be a surfactant, it still may aid emulsification without directly binding to the hexadecane droplet. If the extracellular protein binds to the carbohydrate portion on the glycolipid surfactant, which initially emulsified the hexadecane, the protein would enhance the solubility of the hexadecane droplets and produce a more stable emulsion.

Two activities are associated with the extracellular protein

The purified protein has two activities, stimulation of growth and agglutination of *P. aeruginosa*. Both activities may be related, contributing to the growth of the bacterium

on hexadecane.

Stimulation of growth. The growth curve observed after the addition of extracellular protein to cultures of *P. aeruginosa* 17423 exhibited increased optical density, indicating stimulated growth. But the growth pattern remained unchanged (see Fig. 27). After long term acclimation to hydrocarbon the bacteria are larger and seem to be more hydrophobic. When growth of acclimated and non-acclimated bacteria is compared, the acclimated cells have a longer colonization phase and produce more total extracellular protein (see Fig. 31).

Agglutination of cells. During this study it was observed that adding excess protein to cultures would cause an agglutination of bacteria after a couple of hours. This type of activity is similar to that of carbohydrate binding proteins (lectins) that bind to carbohydrates on the surface of cells and cause agglutination. It was observed that the extracellular protein from *P. aeruginosa* agglutinates *P. aeruginosa*, *E. coli*, human type O and horse red blood cells. The extracellular protein was a poor hemagglutinin.

Similarities between the extracellular protein and microbial adhesions (lectins)

The extracellular protein does not seem to be a common

microbial lectin. Most of the known microbial lectins come from pathogenic strains of bacteria and are called "adhesions". These adhesions are subunits of fimbriae, the hair-like projections found on some bacteria. Fimbriae associated with *P. aeruginosa* are from group 4 and are associated with pathogenic strains of the bacterium but it has not yet been determined which carbohydrates the pilin subunit of the fimbriae bind [Martin et al., 1993].

The extracellular protein has a similar molecular weight as pilin (14,500 compared to 15,000) but it is lacking a conserved amino terminal sequence of approximately 20 hydrophobic amino acids found with pilin that also has an unusual amino acid at the amino terminus, N-mono-methylphenylalanine [Paranchych et al., 1978; 1990]. When the sequenced amino terminal region of the extracellular protein was compared to the amino terminal sequence of pilin there was no similarity (Fig. 43). This hydrophobic region is thought to be essential for the formation of fimbriae [Sharon and Ofek, 1986].

The amino acid composition of the protein is very similar to the amino acid composition of two lectins called PA-I from strain 33347 and the pilin subunit from fimbriae of strain PAK [Gilboa-Garber, 1986; Paranchych et al., 1990]. The amino acid composition from both sets of

```
      1                   5                   10
PAK   MePhe-Thr-Leu-Ile-Glu-Leu-Met-Ile-Val-Val-
17423  Ala-Thr-Ile-Thr-Pro-Val-Asn-Ser-Ala-Phe-

                         15                  20
PAK     Ala-Ile-Ile-Gly-Ile-Leu-Ala-Ala-Ile-Ala-
17423   Thr-Ala-Pro-Gly-Thr-Ile-Ser-Val-Ser-Ser-

                         25                  30
PAK     Ile-Pro-Gln-Tyr-Gln-Asn-Tyr-Val-Ala-Arg-
17423   Pro-Ala-Ser-leu-Asn-Leu-Pro-Val-Thr-Thr-

            32
PAK     Ser-Glu
17423   Asn-Ile
```

Fig. 43. A comparison of the N-terminal amino acid sequences of pilin from strain PAK [Paranchych et al., 1978] and the extracellular protein from strain 17423. The bold amino acids are tentative identifications.

proteins have large percentages of hydroxy and hydrophobic amino acids which are common for lectins [Goldstein and Poretz, 1986]. There are also some similarities when the secondary structure of the extracellular protein from strain PG201 was compared to the secondary structure of PA 1. The extracellular protein has approximately 20.4% β strand and 63% loop regions compared to PA I with 38.5% β strand and 61.5% loop regions (Fig. 44). PA-I can agglutinate red blood cells, yeast and other eukaryotic cells but cannot agglutinate *P. aeruginosa*. Agglutination by PA-I and PA-II is dependent upon divalent cations and binding is inhibited by EDTA [Gilboa-Garber, 1986]. EDTA has been shown to inhibit growth of *P. aeruginosa* on hexadecane [Gaswami and Singh, 1990; Table 3]. EDTA also inhibits agglutination of *P. aeruginosa* 17423 by the extracellular protein.

Binding of the extracellular protein to carbohydrates

The agglutination of *P. aeruginosa* by the extracellular protein is inhibited by glucose, mannose and slightly by rhamnose [see carbohydrate binding experiments]. When these carbohydrates were bound to a solid matrix, however, the protein did not bind. This may indicate that the protein primarily binds a carbohydrate or disaccharide that has characteristics found with all three mono-saccharides. This

A

```
  5    10   15   20   25   30   35   40   45   50   55
NH3-MKSIKSLPSFAALALCLSVSSMASAATITPVNSAFTAPGTISVSSPASLNLPVTCN
           αααααααααααααα

   60   65   70   75   80   85   90   95   100  105  110 115
ITFKGKTAADGSYASIDSVTVSGSNTLCSVPQMTGLPQKLTVSSTTAGKVDGVGFKILSS
            βββββββββββ                  βββββββββββββββββββ

   120  125  130  135  140  145  150  155  160
TCGPSTVNGSWSNATNTLSASNQSLAGNCKINSLSVKPTPAFVVNP-COOH
          αααααααααααααααααα  βββββββββ
```

B

```
        5    10   15   20   25   30   35   40   45   50   55
NH3-MAWKGEVLANNEAGQVTSIIYNPGDVITIVAAGWASYGPTQKWGPQGDRGHPDQGL
        βββββββ     βββββββ    βββββββ                        β
   60   65   70   75   80   85   90   95   100  105  110 115
ICHDAPCGALVMKIGNSGTIPVNTGLPRWVAPNNVQGAITKIYNDVPGTYGNNSGSFSVN
βββ     βββββββ                      βββββββ     βββ  βββββ
   120
IGLDQS-COOH
β
```

Fig. 44. Secondary structure of the deduced pre-protein amino acid sequence of strain PG201 (A) compared to the lectin PA-I from strain 33347 (B) using a computerized algorithm by Rost and Sander (1993). The final composition for strain PG201 is 16.7% α helix, 20.4% β strand and 63% loop regions. The composition for strain 33347 is 38.5% β strand and 61.5% loop regions.

would not be surprising considering the number and complexity of the carbohydrates associated with the lipopolysaccharide of *P. aeruginosa*.

Carbohydrates associated with eukaryotic cells are usually typical monosaccharides such as glucose, mannose etc. Carbohydrates associated with the lipopolysaccharide of *P. aeruginosa* are commonly de-oxy sugars, amino sugars or both [Horton et al., 1983]. Typical examples of these exotic carbohydrates are fucosamine and quinovosamine (2-amino-2,6-dideoxy-glucose). Carbohydrates such as these are unavailable commercially, therefore, testing these carbohydrates was beyond the scope of this project.

Another possibility is that the extracellular protein binds to polysaccharides. This type of binding has been shown with the pilin adhesion from *E. coli*. This pilin is more inhibited by the trisaccharide Manα3Manβ4GlcNac (found with eukaryotic glycolipids) than monomers of mannose [Sharon and Ofek, 1986]. It seems possible that the extracellular protein of *P. aeruginosa* may bind to disaccharides of rhamnose, which is a component of the glycolipid surfactant and the bacterial lipopolysaccharide. This disaccharide is also unavailable commercially.

The isolated glycolipid was tested with three techniques to determine if the extracellular protein bound

to the carbohydrate portion of the glycolipid. One of the methods provides evidence that the extracellular protein binds to the carbohydrate region of the glycolipid surfactant. In procedures where lectins are tested for binding to eukaryotic glycolipids, the glycolipid is first immobilized to a solid surface. For the first two procedures the purified surfactant was added to a plastic surface or thin layer chromatography (TLC) plate. The glycolipid surfactant is insoluble in water, yet it pulled off both the plastic surface and the TLC plate when immersed in a solution of the extracellular protein. One possible reason why the glycolipid detached is that the protein interacted with the surfactant making it more soluble.

For the third technique, isolated glycolipid was immobilized with reverse phase silica gel. When the extracellular protein and *P. aeruginosa* were added to silica gel treated with the glycolipid surfactant the bacteria could be seen covering the silica gel within 5-10 minutes (see Fig. 39-40). Some binding to silica gel from the control with no protein was seen. The evidence from this experiment indicates that the extracellular protein and the glycolipid surfactant interact with each other. This interaction may be crucial for hydrocarbon transport and uptake by *P. aeruginosa*.

Transport and uptake of hydrocarbon

Transport of hydrocarbon to the microorganism is thought to be achieved by one of three ways: 1) cell contact with hydrocarbon that is "dissolved" in the aqueous phase, 2) direct contact of cells with large hydrocarbon droplets, and 3) direct cell contact with pseudosolubilized or accommodated hydrocarbons which are submicron droplets less than 1 µm in size [Käppeli and Finnerty, 1979].

Two transport methods were observed for *P. aeruginosa*, direct contact with large hydrocarbons (colonization stage) and interaction with solubilized hydrocarbon (turbidity stage). The extracellular protein is thought to aid in transport of hexadecane by acting as a co-surfactant in conjunction with the glycolipid surfactant secreted by the bacterium. The two compounds work together to produce very small or "pseudosolubilized" hydrocarbon droplets [Hisatsuka et. al, 1977; Singer and Finnerty, 1987; Gaswami and Singh, 1990]. This pseudo-solubilization has been the explanation for the stimulatory effect by the protein. In this respect it is thought that the protein aids in the transport process.

The uptake process for *P. aeruginosa*, according to the pseudosolubilization hypothesis, is ignored. For uptake to occur, the hexadecane droplets must get close enough to the

outer membrane of *P. aeruginosa* for hexadecane to diffuse into the membrane from the droplet [Singer and Finnerty 1984; Munk et al., 1969]. The droplet must also adhere long enough for this process to take place. Cellular adhesion to droplets of hydrocarbon has been shown to be important for uptake by some bacteria and hydrocarbon degrading yeasts [Kennedy et al., 1975; Miura et al., 1977; Rosenberg and Rosenberg, 1981]. Very little is known, however, about uptake by gram negative bacteria, such as *P. aeruginosa*. Furthermore how *Acinetobacter* and *P. aeruginosa* adhere to hydrocarbon droplets during the colonization stage has not been described.

Repulsive forces are present that *P. aeruginosa* must overcome before it can colonize the droplets. The carbohydrate portion of the bacterial lipopolysaccharide makes the bacterium very hydrophilic. The bacterium then is repelled by hydrophobic substances. In fact this hydrophilic property of *P. aeruginosa* makes pathogenic strains of the bacterium very resistant to hydrophobic antibiotics such as erythromycin and rifampin [Siehnel et al., 1990]. During uptake the bacterium, therefore, must somehow overcome the repulsive forces and adhere to the droplet. In some way the hydrophilic bacterium has found a way to directly interact with the hydrocarbon.

One possible way the bacterium increases its ability to interact with the hydrocarbon is by changing its outer morphology. The bacteria become larger and possibly more hydrophobic during long term acclimation to hexadecane. These changes may allow the bacterium to interact more with the hydrocarbon. Another possible clue to these interactions is the observation that when *P. aeruginosa* is growing on hexadecane the bacterium produces a large surge of the extracellular protein. This surge in protein production correlates well to an increase in growth. The production of the glycolipid surfactant reaches a peak about 10 hr after the protein [Hisatsuka et al., 1977]. Hisatsuka et al. [1977] have speculated that the protein is very important at the initial stages of growth. Therefore, the extracellular protein of *P. aeruginosa* may have a function in hydrocarbon transport but it may also have a function with uptake. The hypothesis for a role of the protein in uptake is supported by its lectin-like properties.

A new hypothesis for the function of the extracellular protein

I propose that the function of the extracellular protein depends upon its lectin-like ability. This function would affect both transport and uptake of hexadecane by *P.*

aeruginosa and the three observed growth stages. During the lag stage of growth *P. aeruginosa* cannot be seen colonizing the hexadecane droplets. During this stage it is hypothesized that the bacterium is producing the oxidative enzymes, glycolipid surfactant and the extracellular protein. As the lag phase continues, the surface of the hydrocarbon becomes covered with the glycolipid surfactant lowering the surface tension and producing large droplets of hexadecane. During this stage, if the agitation speed of the culture is too high, the combination of surfactant and agitation produces very small droplets of hydrocarbon.

Under these conditions the culture appears the same as the turbidity stage. This may account for the fact that the colonization stage has not previously been described in the literature. During the lag stage the bacteria also produces the extracellular protein which eventually allows them to adhere to the hexadecane droplets.

I hypothesize that during the colonization stage the protein acts as an adhesion, binding to the lipopolysaccharide of the bacterium and the carbohydrate portion of the glycolipid surfactant (see carbohydrate binding experiments). Adhesion of the bacterium to the surface of the hexadecane droplet may allow the bacterium to adhere near enough and long enough for hexadecane uptake to

occur (Fig. 45). During the turbidity stage the glycolipid surfactant concentration becomes high enough to emulsify the hexadecane into small droplets that disperse throughout the medium. This correlates well with a study from *P. aeruginosa* S7B1 which showed that a surge of the glycolipid surfactant was produced about 10 hr after the cultures were begun [Hisatsuka et al., 1977]. One possible explanation for this observation is that the bacterium finally has enough carbon by this time to produce the glycolipid surfactant in abundance.

During the turbidity stage the smaller droplets adhere to the bacterium in the same manner as when the bacterium adhered to the large hexadecane droplets. Also during the turbidity stage another process may be occurring. As the hexadecane becomes depleted and the surfactant concentration increases, the glycolipid surfactant begins inhibiting the binding of the protein to the bacterium. This hypothesis is supported by the observation that the number of free bacteria increases (microscopic examination) during the turbidity stage even though the extracellular protein concentration is high and should lead to bacterial agglutination.

This inhibition of agglutination is similar to agglutination inhibition tests used for lectins to determine

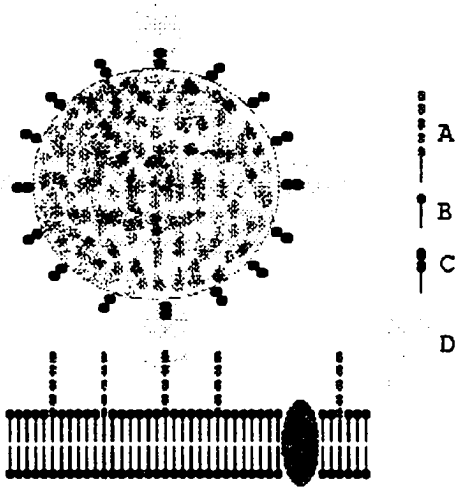


Fig. 45. Hypothetical function of the extracellular protein. The protein (D) is shown binding an emulsified hexadecane droplet to the bacterial outer envelope. The protein is binding the carbohydrate region of the rhamnose surfactant (C) to the carbohydrate region of the lipopolysaccharide (A).

carbohydrate binding. Agglutination inhibition studies have been done using eukaryotic glycolipids [Leffler and Svanborg-Eden, 1986]. Agglutination inhibition experiments with the glycolipid surfactant were complicated because as the micelles were disrupted by isolating the glycolipid, the glycolipid was insoluble in water [Jarvis and Johnson, 1949]. At the end of the turbidity stage, after all of the hydrocarbon has been depleted, free non-agglutinated bacteria remain in the culture with both the glycolipid surfactant and the extracellular protein.

Conclusion

The purpose of this study was to determine the structure and function of an extracellular protein produced by *P. aeruginosa* 17423 and to understand how the protein affects hydrocarbon transport and uptake by the bacterium. *P. aeruginosa* is one of the most extensively studied hydrocarbon-degrading microorganisms. Studies on hydrocarbon degradation by *P. aeruginosa* have focused on the oxidative enzymes [Baptist, 1962; Matsuyama, 1980] and the extracellular compounds the bacterium produces. It has been shown that the bacterium produces a glycolipid surfactant and an extracellular protein while growing on alkanes [Hisatsuka et al., 1972; 1977]. The structure of the

glycolipid surfactant has been previously determined [Jarvis and Johnson, 1949; Edwards and Hayashi, 1965] and it has been found that the glycolipid surfactant stimulates growth of the bacterium when added to cultures of *P. aeruginosa* [Hisatsuka et al., 1972].

The extracellular protein produced by *P. aeruginosa* also stimulates growth of the bacterium. It has been hypothesized that it acts as a co-surfactant with the glycolipid to produce pseudo-solubilized (very small) droplets of alkane which increases the availability of the hydrocarbon to the bacterium [Hisatsuka et al., 1977; Singer and Finnerty, 1984]. It has not been previously shown, however, that the extracellular protein acts like a surfactant by adhering to the droplets of hydrocarbon. It has also not been shown how the bacterium interacts with the emulsified hydrocarbon or how uptake of the hydrocarbon occurs.

The extracellular protein produced by *P. aeruginosa* 17423 is secreted into the medium while degrading hexadecane. Additional protein stimulates growth of the bacteria when supplemented to cultures of *P. aeruginosa* growing on hexadecane. The protein is a soluble monomer of approximately 14,500 molecular weight, by SDS-PAGE, and is not a glycoprotein. The protein has a similar molecular

weight and amino acid composition to two other proteins called the "protein-like activator" from *P. aeruginosa* S7B1 and the protein from strain PG201 [Hisatsuka et al., 1977; Hardegger et al., 1994]. The three proteins appear to be essentially the same protein.

The protein from strain 17423 also has the ability to agglutinate *P. aeruginosa*, *E. coli*, human type O and horse red blood cells. Agglutination of *P. aeruginosa* is inhibited by EDTA, glucose, mannose and rhamnose. These characteristics define it as a lectin-like protein or, in microbial terms, as an adhesion. The extracellular protein may also bind to the carbohydrate portion of the glycolipid surfactant. These lectin-like qualities may enable the bacterium to adhere to hydrocarbon that has been emulsified with the glycolipid surfactant. The extracellular protein then may function as an adhesion to facilitate transport and uptake of alkane by the bacterium.

Based on the evidence provided the extracellular protein is important for both transport and uptake of hexadecane by *P. aeruginosa*. For transport, the protein cooperates with the glycolipid to emulsify the hexadecane by binding to the carbohydrate portion of the glycolipid surfactant. For uptake, the protein acts as an adhesion that allows droplets of hexadecane to bind to the bacterial

outer membrane (see Fig 45). This action facilitates the diffusion of hydrocarbon to the outer membrane. The agglutination properties of the protein, therefore, have a major function in hexadecane transport and uptake by *P. aeruginosa*. This is the first report of an extracellular compound produced by a hydrocarbon-degrading microorganism that stimulates growth but is not a surfactant. These results are important in contributing to a basic understanding of hydrocarbon metabolism by microorganisms.

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