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MOLECULAR CHARACTERIZATION OF THE GROUNDWATER BACTERIUM ABS10

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Alexander W. Breen

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ABSTRACT

A Gram-negative pleomorphic bacterium, designated ABS10, predominating in a pristine aquifer in Ada, Oklahoma, was selected for characterization and potential as a candidate for the introduction of catabolic genes into a groundwater microbial population. This organism was originally identified as an aberrant Arthrobacter sp. and extensive characterization of the isolate was undertaken to further develop the strain for groundwater microbiological research. The fatty acid methylester profile of ABS10 revealed a high percentage similarity value with Pseudomonas putida. Ketodeoxyoctonic acid (KDO) extraction of ABS10 lipopolysaccharide indicates that the KDO of ABS10 is present and modified, possibly due to phosphorylation. DNA:DNA colony hybridization, dot blot and Southern blot analysis showed ABS10 to share a great deal of genetic homology with P. putida. The 16S rRNA sequencing indicated that ABS10 groups with the Gamma division of the purple photosynthetic bacteria. The broad host range plasmid RP4 was introduced into ABS10 and stably maintained indicating that RP4 may serve as a vehicle for introducing catabolic genes into this organism and, hence, into the groundwater microbial community.

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CHAPTER I

INTRODUCTION

This study focuses on the characterization of an indigenous, Gram negative bacterial isolate, predominating in an uncontaminated groundwater environment and the attempt to develop a gene delivery system for the isolate. Therefore, the isolate was characterized using not only standard taxonomic techniques but also genetic methods and cell membrane analysis. Although standard taxonomic methods are often sufficient for characterization of clinical isolates, these methods are often inconclusive when applied to environmental bacteria. For this reason extensive genetic studies in concert with cell membrane analysis were carried out.

Industrial and municipal wastes pose a serious contamination threat to the quality of groundwater resources and characterization of the microbial members of this ecosystem will aid in assessing the impact of contaminants as well as the development of biological remediation alternatives. Very little characterization of groundwater microbial populations has been done and much of the work has yielded ambiguous or inconclusive findings. Characterization of the groundwater microbial populations would greatly aid investigations of the potential for _in situ_ microbial decontamination of groundwater and in predicting community response to environmental contamination as well as the effects of some strategies of _in situ_ biological decontamination.

One strategy for *in situ* biological decontamination involves stimulation of the growth and activity of indigenous biodegradative microorganisms by nutrient addition. This approach has met with mixed results (48). If the contaminants are recalcitrant to degradation by indigenous populations, biodegradation would not be expected. It would be unreasonable to expect microorganisms in a recently contaminated or pristine, aquifer to remove anthropogenic or xenobiotic compounds in a realistic time frame simply by stimulation of the indigenous population. Many anthropogenic compounds have not been in the biosphere long enough for the evolution of catabolic pathways and selection to occur (31). Therefore, characterization of the indigenous groundwater populations is also essential for planning and conducting experiments designed to broaden the degradative capabilities of organisms via genetic manipulation techniques.

An alternative to the stimulation of groundwater populations is the inoculation with bacterial strains of known degradative capabilities, into a contaminated aquifer, to promote decontamination. Detoxification of 2,4,5-trichlorophenoxyacetate contaminated soil by a laboratory strain of *Pseudomonas cepacia* has been demonstrated for a soil system (43). More recently, the introduction of a *P. putida* strain bearing the toluene (TOL) catabolic plasmid, pWW0 (73), has been shown to promote toluene removal from artificially contaminated groundwater microcosms (70). Although the *P. putida* TOL bearing strain is not a laboratory construct, it appears to be alien to the groundwater environment. Inoculation also has its drawbacks:

(1) the groundwater environment is oligotrophic; most of the organic carbon is in the form of humic-type material which is not easily biodegraded (7) and (2) The metabolic activity of nonindigenous inoculum may be minimal if it can, in fact, survive at all.

A strategy which may overcome both the problem posed by the contaminants (resistance to biodegradation by the native micro-organisms), as well as the problem posed by the oligotrophic nature of the aquifer, is to introduce catabolic genes into a native organism from the groundwater environment. Ideally these genes should be expressed in this organism and the organism should be able to survive in the groundwater environment. In this study the broad host range plasmid RP4 (12,19) was introduced into ABS10, a native groundwater isolate, as a model to demonstrate that groundwater organisms can accept and maintain introduced plasmid DNA.

CHAPTER II

LITERATURE REVIEW

Background

Public reliance on groundwater as a potable water source cannot be understated. Groundwater provides 50% of the United States population with drinking water (in the state of Florida 92% of the population relies on groundwater for drinking water) (5,63). One million new wells are drilled annually (5,63). Mismanagement of this resource could result in biological or chemical contamination of groundwater. Waste lagoons and landfill sites are threats to aquifers, as are many agricultural fertilizers and pesticides (5).

The subterranean environment consists of a vadose zone (the region between surface and aquifer) and one or more aquifers above an impermeable bedrock or clay base. The vadose zone is an unsaturated soil zone which links the water table with the aquifer. An unsaturated water flow must be maintained for groundwater recharging. The vadose zone also restricts evaporation from the aquifer. The vadose zone is essential for protecting the aquifer from biological and chemical contaminants on the surface (7).

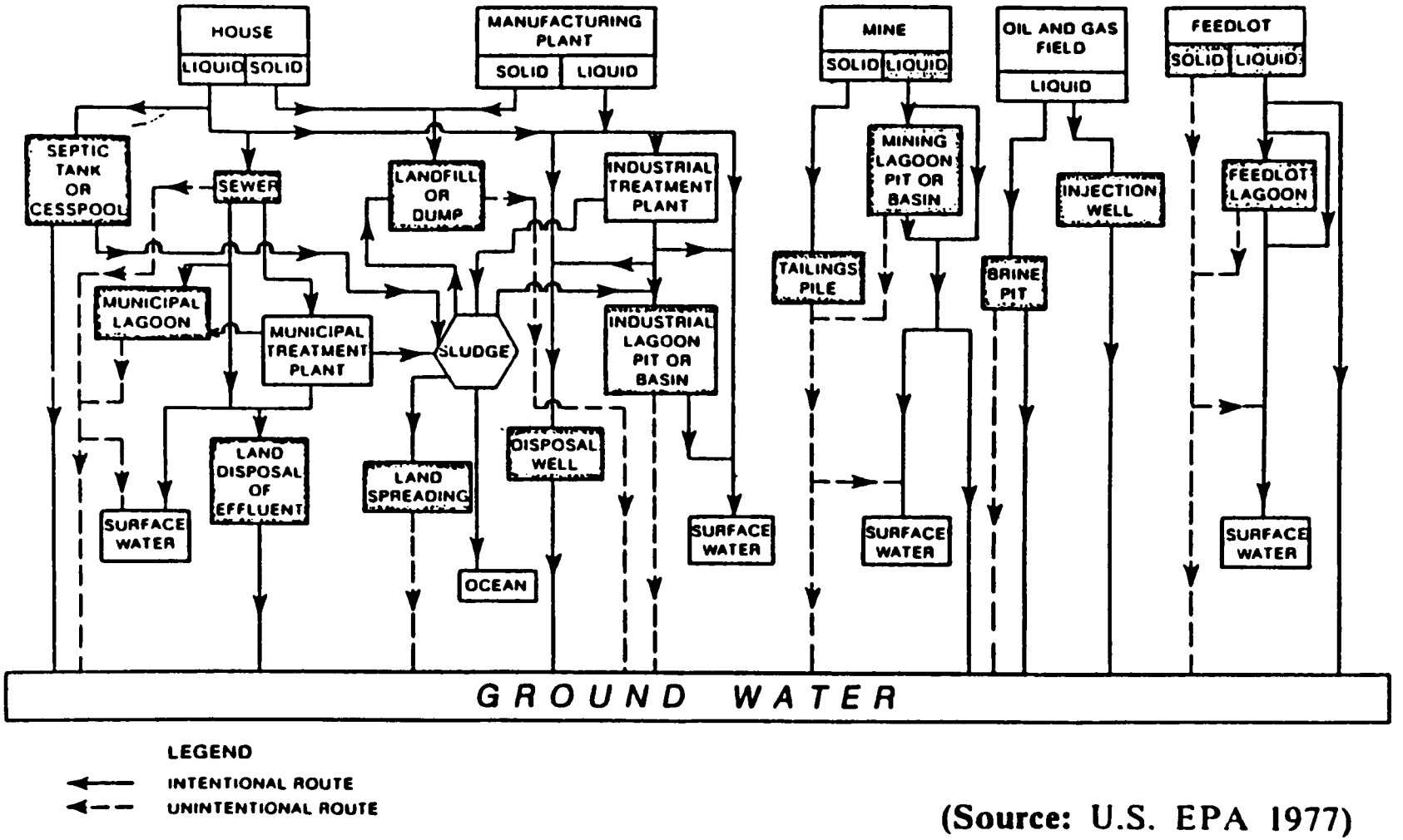
The "groundwater zone" or aquifer is defined by Bouwer as "an area of sufficient lateral and vertical extent and sufficient permeability to produce adequate and sustained flows to wells" (7). Typical groundwater aquifers consist of gravel and sand layers or

mixes of the two. Aquifers vary in size from small local aquifers to the massive Ogallala aquifer which underlies several midwestern states (63). Aquifers can be classified as confined or unconfined. The upper limit of an unconfined aquifer is the water table and this will fluctuate depending on the state of the aquifer--charged, depleted or somewhere between. A confined aquifer is situated between layers of little to no permeability such as clay or shale and there is no free water table. In contrast to an unconfined aquifer, compression of the confined aquifer, rather than the displacement of water by air, forces the water to the wells. Unconfined aquifers are more susceptible to contamination as they receive water directly from the vadose zone, while confined aquifers are protected by their relatively impermeable upper boundary (42).

The slow lateral movement of groundwater results in minimal mixing of environmental contaminants which increases persistence, sometimes to the point where groundwater contaminant concentrations exceed those for surface waters. Commonly found chemical pollutants include hydrocarbons as well as heavy metals. Table 1 shows some of the kinds of contaminants and their source (85). Figure 1 shows the pathways of surface contaminants to the groundwater. Sludge disposal, sewage effluent disposal and industrial waste landfills are the major sources of such contamination. Because of their number (32,000-50,000 sites contain hazardous material of which 1,000-2,000 represent a serious health threat) and their potential for leaching, industrial waste landfills pose a most serious danger of contributing contaminants to the groundwater (42,63).

Table 1. Contaminants in Groundwater and Their Source.

Source	Contaminant										
	Arsenic	Cadmium	Chlorinated Hydrocarbons	Chromium	Copper	Cyanides	Lead	Mercury	Organics	Selenium	Zinc
Mining and Metallurgy	x	x		x	x	x	x	x		x	x
Paint and Dye		x		x	x	x	x	x	x	x	
Pesticide	x		x			x	x	x	x		x
Electrical and Electronic			x	x	x	x	x	x		x	
Printing and Duplicating	x			x	x		x		x	x	
Electroplating and Metal Finishing		x		x	x	x					x
Chemical Manufacturing			x		x			x	x		
Explosives	x				x		x	x	x		
Rubber and Plastics			x			x		x	x		x
Battery		x					x	x			x
Pharmaceutical	x								x		
Textile				x	x				x		
Petroleum and Coal	x		x				x				
Pulp and Paper								x	x		
Leather				x					x		



(Source: U.S. EPA 1977)

Figure 1. Pathway of surface contaminants to groundwater.

Low molecular weight chlorinated aliphatic hydrocarbons are the most commonly found organic contaminants in groundwater. Trichloroethylene (TCE) has been a major contaminant in many aquifers and has been linked to human health problems. The TCE problem is widespread and in some cases has been the cause of large scale contamination. In Jackson Township, New Jersey TCE concentration in drinking water wells was 1,000 µg/l. Acute exposure to TCE inhibits the central nervous system and cardiac function (88,18). Chronic exposure causes liver damage and increases in hepatic tumors in mice (88,18). The TCE problem provides a vivid example of the extent of the groundwater contamination dilemma. It is important to note that in most cases only selected compounds have been analyzed. Table 2 shows the panopoly of compounds isolated from the Jackson Township, NJ well drinking water (18).

Conventional groundwater reclamation techniques involve pumping the water to the surface then treating the water chemically, adsorbing the contaminants onto a resin or activated charcoal or stripping the contaminants out in aeration towers (48,18,63). Decontamination of the groundwater also requires the removal of contaminated soil. Overall these processes which are collectively termed remedial reclamation are uneconomical and notably inefficient. A survey of sites where this type of treatment was used indicated that at best it was only partially successful 54% of the time (48). The transfer of contaminated soils is merely a transfer of the problem and has limited economical and environmental benefits. The drawbacks of

Table 2. Contaminants in Jackson Township, NJ Groundwater.

Contaminant	Concentration
Toluene	6,400 µg/l
Methylene chloride	3,000 µg/l
Acetone	3,000 µg/l
Ethyl benzene	2,000 µg/l
TCE	1,000 µg/l
Benzene	300 µg/l
Chloroform	33 µg/l

physical and chemical groundwater reclamation has forced investigators to look at the feasibility of *in situ* biological decontamination. Biological decontamination methods involve stimulation of the indigenous microflora by nutrient addition or the inoculation of bacterial strains, known to degrade a particular contaminant or contaminants, into the groundwater (48,86,63,21,70). Biological treatment has already demonstrated some success, an understanding of the subsurface environment and its microbial ecology will optimize the effectiveness of the reclamation strategy (26,21,48,88).

Groundwater Microbial Ecology

A great deal of work has been done on the microbiology of the surface soil and rhizosphere but until recently little information was available on the subsurface environment. Investigators, using a number of techniques, have examined the microbial populations of the groundwater and of the vadose zone (84,21,26,4,35). Technical difficulties and the expense involved in obtaining uncontaminated

samples have hampered groundwater microbiology. McNabb and Mallard (54) have reviewed the theory and methods concerning the recent advances in groundwater sampling. As may be expected, the structure of microbial communities varies from aquifer to aquifer. Ghiorse and Balkwill have conducted extensive enumeration studies on a number of shallow water table aquifers (4,26).

Two of the most important parameters to be considered in groundwater microbiology are carbon source availability and O_2 concentration (48,7). Carbonates or other inorganic forms of carbon are the major carbon sources in the subsurface environment. Sedimentary deposits also contain organic carbon in the form of humic-type materials which are only slowly degraded by bacteria (7). Humic substances are described by Atlas and Bartha (2) as that portion of soil organic matter that has undergone sufficient transformation to render the parent material unrecognizable. Humic materials are believed to consist of an aromatic "core" composed of single and condensed aromatic, heterocyclic and quinoidal rings, linked by carbon-carbon, ether, amino and azo bonds. Most of the easily utilized organic carbon is scavenged at the soil surface and in the rhizosphere. This presents groundwater bacteria with starvation conditions with respect to available organic carbon.

Due to enumeration difficulties intrinsic to environmental microbiology, simple aerobic plate counts present an underestimate of bacterial populations in groundwater. Depending on the procedure, anaerobic and autotrophic bacteria as well as those unable to grow

on artificial media may not be enumerated. For these reasons, epifluorescent staining with acridine orange (AODC) has become a useful tool for estimating bacterial numbers in environmental samples (26, 4,47,81). In general, the total cell numbers in aquifers range from $1X10^2$ to $1X10^6$ cells/g dry weight; these values are comparable to the numbers in oligotrophic marine and fresh water environments (21,35,81,4). In general, anaerobic bacteria comprise a considerable portion of the populations (84,21,4,35). Population densities, including anaerobes, decrease as depth increases (4). Eukaryotes are rarely present and are presumed not to play an important role in the ground water environment (26,84).

Erlich et al. (21) and Ward (82) found iron reducing and denitrifying bacteria occurring in anaerobic regions; interestingly, Erlich found methanogenesis occurring only in a creosoate contaminated aquifer. Ghiorse has noted that a soil extract medium yielded a higher recovery of cells than a rich medium indicating an adaptation to an oligotrophic environment (26,4). Hirsch and Rades-Rohkohl (35) found the bacteria in the groundwater to be very diverse indicating that a number of widely different microhabitats are present in the groundwater environment. Among the over 90 morphotypes they identified were Microcyclus, Gallionella, Caulobacter, Agrobacterium, Hyphomicrobium, Clostridium and Norcardia. Their findings conflict with those of Balkwill and Ghiorse (4) who, using electron microscopy, found 85-90% of the bacteria they encountered in their samples to be Gram-positive Arthrobacter-like organisms. The authors

attribute the conflicting results to the fact that Hirsch and Rades-Rohkohl inoculated enrichment cultures with groundwater from long standing wells while Ghiorse and Balkwill (4) used sedimentary material. Once again it must be emphasized that the nature of the aquifer and the method of sampling greatly influenced the data generated.

Possibly the most innovative method of examining microbial communities is that of White et al. (84) who utilize cell surface chemical analysis (phospholipid, ester-linked fatty acid profiles) as an indicator of community structure. The structure of a community can be described by assaying for particular "signal compounds." Using this method White et al. (84) examined an aquifer near Pensacola, Florida, at a depth of 410 M and found no long chain polyenoic acids were present indicating an absence of microeukaryotes such as algae, fungi and protozoans. Assaying for the ribitol of the techoic acids of Gram-positive bacteria indicated Gram-positive microorganisms to be present at 10^7 bacteria/g dry weight. Assaying for the hydroxy fatty acid from the lipid A of Gram-negative bacteria indicated that they were somewhat less common than the Gram positives but still approached the order of 10^7 bacteria/g dry weight (84).

White et al. (84) also used biochemical techniques to investigate the nutritional status of the subsurface microbial community. High levels of polybeta-hydroxyalkanoic endogenous storage polymers were found which indicate that the organisms are experiencing nutritional stress. When faced with a limiting supply of carbon or energy sources prokaryotic organisms synthesize storage polymers, the most

common of which is polyhydroxybutyrate (36). When the limiting factor is supplied, synthesis of the storage compound is halted (65). Another starvation associated activity discovered in the groundwater microflora by White et al. (84) is the production of extracellular polysaccharide glycocalyx. The postulated function of the glycocalyx is believed to be the concentrating of nutrients or ions around the bacteria. The glycocalyx also facilitates the attachment of bacteria to surfaces which is particularly important in oligotrophic environments since surfaces concentrate nutrients (80). Balkwill and Ghiorse also present evidence indicating starvation conditions in the groundwater environment. Most cells that they observed had a spherical or oval morphology which serves to decrease the surface-area-to-volume ratio resulting in improved transport efficiency (4). The Arthrobacter-like cells that they observed showed signs of starvation, i.e., cytoplasmic depletion and elaborate internal membranes (4). Thus a number of different studies confirm the hypothesis that the groundwater environment is oligotrophic.

Despite the fact that groundwater bacteria are physically isolated from the surface and thus, not exposed to anthropogenic compounds, the indigenous microbial community has demonstrated an ability to metabolize some environmental contaminants (53,48,79,88, 21). Both Ehrlich et al. (21) and Suflita and Miller (79) observed the removal of phenolic compounds from aerobic regions of shallow sandy aquifers. Both groups also noted the need for methanogenic consortia for removal of phenolic compounds from anaerobic

environments. Wilson et al. (87) observed degradation of toluene, styrene and chlorobenzene in artificially contaminated groundwater microcosms. Chlorinated aliphatic hydrocarbons such as TCE are generally recalcitrant to degradation. Wilson et al. (87) found no degradation of 1,1,1-trichloroethane, chloroform or 1,1-dichloroethane. Low molecular weight chlorinated aliphatic hydrocarbons can be transformed in anaerobic environments but the processes are often incomplete and result in products that are as much of a problem as the original contaminant (i.e., vinyl chloride from TCE) (88). As can be seen by these few examples, the degradative potential of groundwater microflora, although certainly not inconsequential, is limited.

The success of *in situ* biological decontamination of an aquifer by biostimulation via nutrient injection is dependent on the degradative potential of the indigenous population. Where possible, *in situ* decontamination by biostimulation would be inexpensive and fast. It is environmentally sound relative to alternative decontamination methods, and minimal ecological changes would result since the indigenous population would mediate the process (48). Success would be limited, however, by the degradative capabilities of the indigenous microflora. Wilson and Wilson were able to achieve aerobic TCE degradation in aquifer microcosms by enriching for methanotrophic bacteria (88). This was accomplished by incubating the microcosms in the presence of natural gas. The work of Wilson and Wilson is more sophisticated than simple nutrient addition in that a specific population is targeted for enrichment but its success

is still dependent on the degradative potential of this population. For this reason investigators are examining the feasibility of decontamination by inoculation of strains with known degradative capabilities.

Use of Characterized Microorganisms in Decontamination

A complete review of the use of characterized microorganisms to aid in hazardous waste removal is beyond the scope of this review but a number of studies have particular relevance to the problem of groundwater contamination. A number of investigators have used a characterized inoculum to remove contaminants from various ecosystem models. Chakrabarty's laboratory demonstrated significant removal of 2,4,5-trichlorophenoxyacetic acid in soil when the soil was treated with a degrading strain of Pseudomonas cepacia (43,14). Brunner et al. (11) observed degradation of polychlorinated biphenyls in soil after inoculation with a degradative strain of Acinetobacter. Pignatllo et al. observed pentachlorophenol degradation in artificial freshwater streams inoculated with a degradative Flavobacterium (61). By virtue of the fact that the genes of many degradative phenotypes are located on plasmids and can be manipulated by recombinant DNA technology, a number of genetically improved strains with a wide spectrum of degradative capabilities have been developed (27,28,94,49). Unfortunately, introduction of an organism into an environment often fails to bring about removal of a contaminant (29).

When a nonindigenous organism is introduced into a different environment, it is subject to an array of alien physical-chemical

parameters as well as predation and competition. The ability of a nonindigenous "improved" strain to survive and accomplish the desired task is a problem encountered in a number of areas employing biotechnology such as agriculture and metal ore leaching (74,9). For these reasons the selection of indigenous members of an ecosystem may be the best route to take when looking for candidates for strain improvement and subsequent introduction into a particular environment.

Characterization of Groundwater Bacteria

Standard taxonomic methods are often inadequate for the classification of environmental bacteria. Bacterial taxonomy has, until recently, been plagued by a staunch conservatism resulting in adherence to a taxonomic system that is often ambiguous, contradictory or simply wrong. As a consequence new isolates are often assigned to various "catch all" genera of which Pseudomonas is probably the most notorious. There are many important groups of organisms for which no simple method of species identification is available. For this reason a number of analytical techniques have been used for the identification and classification of environmental isolates. A number of these techniques are summarized as follows:

1. Fatty acid profile analysis using gas-liquid chromatography has been shown to be a useful tool in the identification of soil bacteria. Closely related bacteria exhibit similar profiles and fatty acids are not prone to alteration by moderate genetic mutation (68). A drawback to this technique is the fact that fatty acid

composition is ultimately dependent on the genome of the organism, hence this is an indirect method rather than a direct method of identification.

2. The 2-keto-3-deoxyoctonic acid (KDO) is an integral part of the Gram-negative lippopolysaccharide and can be readily assayed for by the use of a chromogenic indicator (Figure 2) (83,39,62). A simple Gram-stain is often unreliable when studying field isolates due to the fact that many common soil bacteria, particularly Coryneforms, will stain Gram-negative when in fact cell wall analysis shows them to be Gram-positive.

3. DNA-DNA hybridization provides a means of identification at the most basic level. It has the advantages of being highly specific and not at all dependent on gene expression (71).

4. RNA sequencing is one of the most powerful tools available for determination of phylogeny among the prokaryotes. Woese and colleagues have used RNA analysis to present not only a new picture of bacterial phylogeny but of the origin of life itself. Attention has focused on the rRNAs for a number of reasons:

i) They are functionally and evolutionarily homologus in all organisms.

ii) They constitute a considerable portion of cell mass and are easily isolated.

iii) They seem to be free from artifacts of lateral transfer and, therefore, reflect an evolutionary relationship among organisms.

iv) They provide enough sequence information to allow statistically significant comparison.

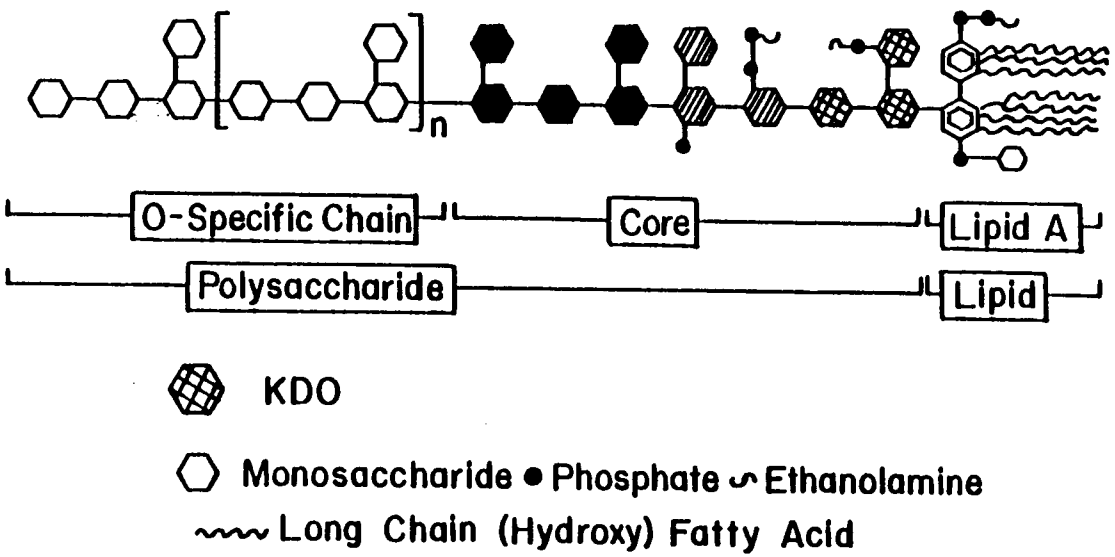


Figure 2. Gram negative lipopolysaccharide structure (as exemplified by Salmonella). The KDO subunits are represented by cross-hatched hexagons which connect the core region to the lipid A region. Taken from Reference 41.

Traditionally RNA analysis was conducted by the oligonucleotide catalog method (24). This method involves digesting 32P-labeled RNA from a particular species with ribonuclease T1 and resolving the oligonucleotides by two dimensional paper electrophoresis. The oligonucleotides are then sequenced thus producing a catalog of sequence characteristic of the organism in question. These catalogs are then compared with other organisms to determine the geneological relationships among the organisms considered (24,60). This method revolutionized bacterial taxonomy and made possible the study of prokaryotic evolution. Advances in sequencing technologies have greatly simplified the collection of RNA sequence data (46). Regions of the 16S rRNA molecule are perfectly, or nearly perfectly, conserved amongst all organisms hence, these regions can serve as priming sites from which to initiate nucleic acid sequencing (see Figure 3). Since the RNA itself is primed directly, there is no need for cloning. The primers (called "universal" or "core" primers due to their ability to prime most rRNAs) are oligonucleotides of about 20 nucleotides in length and are designated by the region of the RNA molecule that they prime; thus the 1400R primer primes at nucleotide number 1400 of the RNA molecule, the 1100R primer primes at nucleotide number 1100, etc. Approximately 300 base pairs of sequence are accessible from each primer. Due to the location of the conserved sites, it is possible to sequence virtually all of the 16S rRNA molecule. Due to the newness of the sequencing technology the bulk of RNA sequence data is still in the oligonucleotide catalog form rather

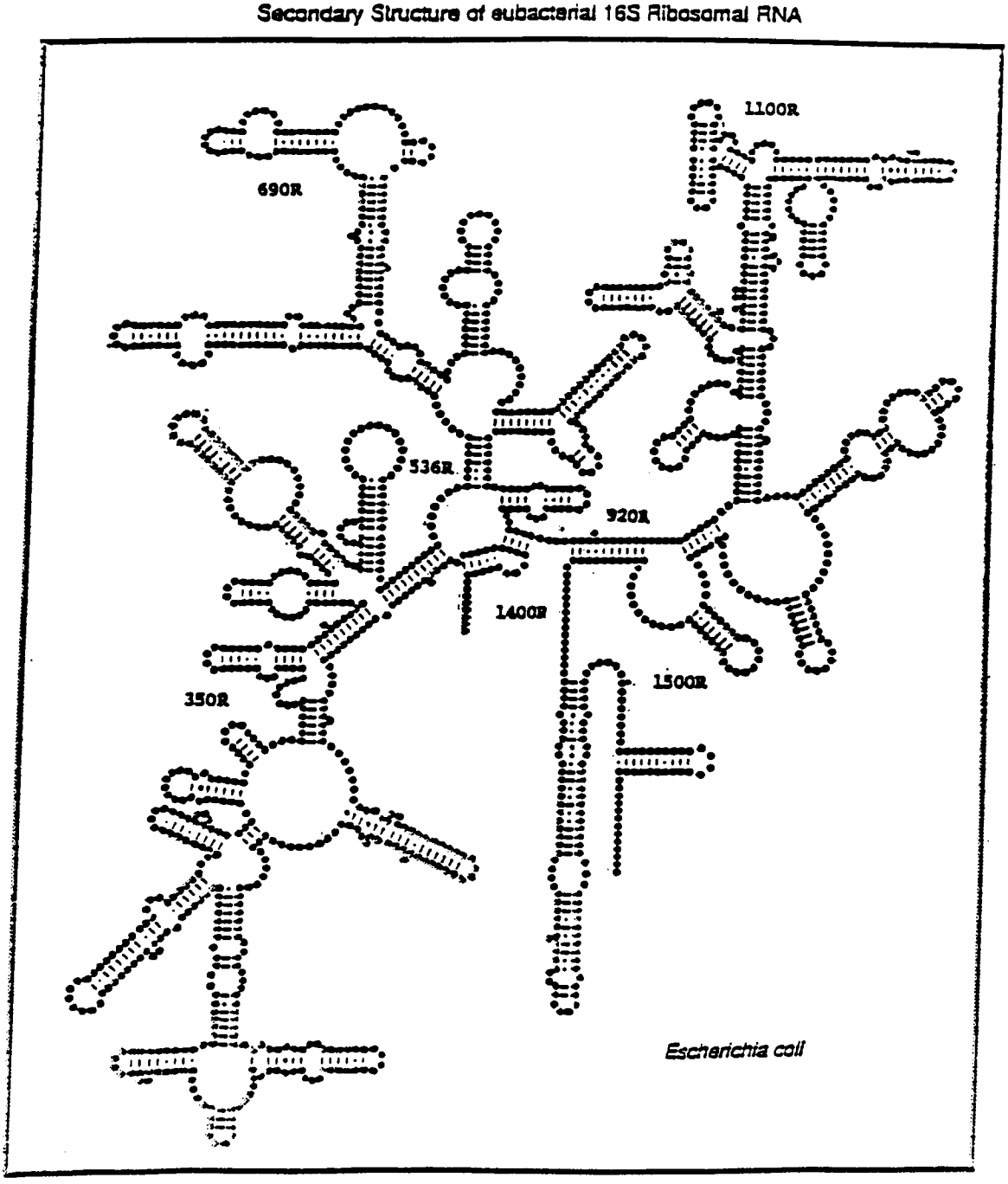


Figure 3. 16S rRNA molecule. Secondary structure of E. coli 16S rRNA molecule showing the priming positions of the "universal" primers. The darker regions of the molecule are the highly conserved regions and the lighter regions are more variable. Taken from Reference 77.

than in the form of complete sequences. Because of this, sequence data must be compared to catalog data in order to accurately type an organism (89).

These methods provide a much more accurate classification of an organism than the traditional approach based on morphological, nutritional, or developmental characteristics (60). In this study ABS10 was characterized and studied as a candidate for strain improvement. A successful candidate would have to be amenable to genetic manipulation. Thus, the goals of this project are the characterization of the isolate ABS10 and the development of a gene delivery system for this organism. Although the organism has thus far proven to be untransformable and no phage have as yet been isolated, the broad host range plasmid RP4 was introduced via conjugation from E. coli and its resistance markers expressed.

CHAPTER III

MATERIALS AND METHODS

Sampling and ABS10 Isolation

The sample material from which ABS10 was isolated came from a shallow aquifer in Lula, Oklahoma, from a depth of 5 meters (Figure 4). The material was obtained through the courtesy of Dr. John Wilson. Core samples were aseptically recovered from the aquifer using a coring device according to the method of McNabb and Mallard (54). The aquifer material was placed in 1% sodium pyrophosphate and mixed on a vortex mixer to attached dislodge attached organisms. The sample was diluted and 0.1 ml aliquots were spread plate inoculated on Soil extract agar (SEA). SEA was made by adding 500 g of soil to 500 mls of distilled H_2O. This SEA mixture was shaken for 30 minutes then autoclaved for 15 minutes. The material was then centrifuged and the supernatant decanted and saved. This was then filtered through a 0.2 µm filter and diluted 10 fold with H_2O. The final solution was then brought to pH 7 and autoclaved. SEA plates were prepared by adding 16 g/l agar (Difco Laboratories, Detroit Mi.) to the SEA mixture. SEA spread plates were incubated at 27 C for 1 week. Following this incubation a predominant colonial type developed, representatives were purified by streak plate isolation and a representative culture, ABS10, was selected for further work.

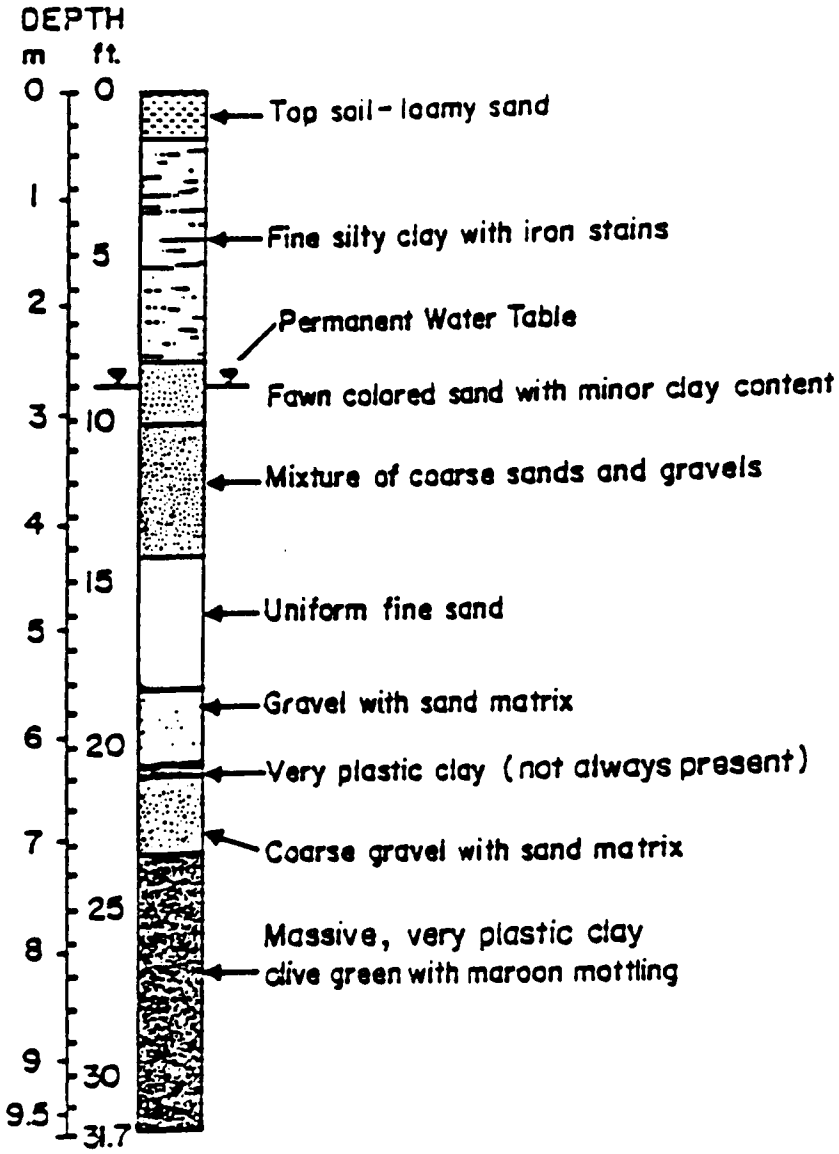


Figure 4. Vertical diagram of the Lula, OK aquifer. Site from which ABS10 was isolated.

Preliminary Characterization of ABS10

Following initial isolation, ABS10 was subjected to preliminary taxonomic characterization, testing for antibiotic sensitivity and determination of plasmid content.

The Gram stain reaction was determined for cultures grown in one tenth strength yeast extract peptone glucose medium (YEPG- 0.1 g/l glucose, 0.2 g/l polypeptone, 0.02 g/l NH_4NO_3, 0.02 g/l yeast extract) broth and on plates at 12, 24, 72 and 120 hours according to the method of Seeley and Van Denmark (72). The oxidase test was carried out by the method of Marquis (25) using colonies scraped from one tenth strength YEPG agar which had been incubated for 24 and 48 hours. The catalase test was carried out by the method described by Seeley and Van Denmark (72) using colonies scraped from one tenth strength YEPG agar after a 20 hr incubation at 27. The presence or absence of flagella was assayed for by negative staining and subsequent viewing under a transmission electron microscope. Negative staining was accomplished by placing a drop of overnight ABS10 culture on a carbon-coated, formvar grid (Ladd Research Industries, Burlington, Vermont). After allowing 1 minute for the culture to settle, the drop was flipped off and a drop of 2% Uranyl Aceteate (Ladd Research Industries) was placed on the grid for 1 minute and flipped off. This was repeated, then excess stain was removed by touching the grid to filter paper. The grid was then viewed on a Zeiss EM9S electron microscope. The Voges-Proskauer test, test for NO_2 reduction, H_2S production, and test for gelatin

hydrolysis were carried out using the API system (Analytab Products, Plainview, New York). Antibiotic sensitivity was checked using the Sensi-Disc Antimicrobial Susceptibility Assay (BBL Microbiology Systems, Cockeysville, MD). Sensitivity was determined by measuring the diameter of the zone of inhibition. Motility was determined using the hanging drop motility test (72). Carbon source utilization was determined by the ability of the organism in question to use a compound as its major carbon source. The compound of interest was supplied at a concentration of 100 mg/l to a minimal salts medium supplemented with 1 ppm yeast extract in order to provide essential salts and vitamins. Chlorobenzoate was purchased from the Aldrich Chemical Company, Milwaukee, WI. Naphthalene and m-Toluate were purchased from the Eastman Kodak Company, Rochester, NY. All other substrates used in carbon source utilization tests were purchased from Fisher Scientific Company, Fairlawn, NJ.

Plasmid Analysis

Determination of plasmid presence was carried out by the method of Anderson and McKay (1). The method of Kado and Leiu (38) was also effective for the detection of plasmid DNA in ABS10. Exponentially growing cells were used for the Anderson-McKay procedure while colonies (after 20 to 48 hr incubation) were used for the Kado and Liu procedure. Both procedures involve nucleic acid denaturation using NaOH followed by a renaturation step. In theory only plasmid DNA reanneals while denatured chromosomal DNA is removed along with celluar DNA by phenol extraction.

Scanning Electron Microscopy

Scanning electron microscopy was conducted on an ETEC autoscan Scanning Electron microscope at The University of Tennessee, Knoxville. Nucleopore membranes (0.45) were placed on yeast extract peptone glucose agar plates onto which ABS10 was streaked. Samples were fixed at 6 hours and 48 hours of incubation. Colonies growing on the membranes were fixed in 3.0% gluteraldehyde in 0.1M phosphate buffer (pH 7.2) for 1 hr. The samples were then rinsed in phosphate buffer for 5 minutes three times. The samples were then post-fixed in 2.0% OsO_4 in 0.1M phosphate buffer for 1 hr. The samples were then dehydrated through a graded ethanol series as follows: 50% EtOH 15 minutes, 70% EtOH 15 minutes, 95% EtOH 15 minutes, 100% EtOH until critical point drying. The samples were dried to critical point in a Ladd Critical Dryer (Catalog No. 28000, Ladd Research Industries). Samples were then coated with palladium/gold using a denton DV-515 Automatic Evaporator.

Transformation Studies

A number of transformation protocols were used since preliminary data indicated that ABS10 was a Gram-positive bacteria (see Results and Discussion). The procedures used initially were variations of the Cohen procedure for *Bacillus subtilus* which involves protoplasting followed by polyethylene glycol treatment (13). The formation of ABS10 protoplasts was accomplished by lysozyme treatment after which the protoplasts were suspended in SMM buffer (0.5M sucrose/0.02M maleate/0.02M $MgCl_2$, pH 6.5). Five µg of plasmid DNA in a

50 µl volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) was mixed with 50 µl of 2X SMM. Then 0.5 ml of protoplast suspension was added followed by the immediate addition of a 40% PEG solution. After 2 minutes, 5 ml of SMMP (prepared by mixing 2X SMM and 4X Penassay broth (Difco)) was added to dilute the PEG. Protoplasts were harvested by centrifugation, resuspended in 1 ml SMMP, then incubated at 30 C in a slowly shaking water bath for 1.5 hr. Cells were then plated on DM3 regeneration medium (per liter; 200 ml 4% agar, 500 ml 1M sodium succinate (pH 7.3), 100 ml Difco Casamino Acids (Difco Laboratories), 50 ml 10% yeast extract (BBL Microbiology Systems, Cockeysville, MD), 100 ml 3.5% KH_2PO_4 and 1.5% K_2HPO_4, 25 ml 20% glucose, 20 ml 1M $NaCl_2$ and 5 ml filter sterilized Bovine Serum Albumin (BSA-Sigma Chemical Co., St. Louis, MO) added when the mixture is heated to 55 C) without selective pressure. Colonies were then replica plated to a selective medium which contained 25 ppm streptomycin (Sigma Chemical Co.). The transforming DNA was plasmid RSF1010 a broad host range cloning vector with a streptomycin resistance marker (3). The procedure of Katsumata et al. (40) was essentially the same as the Cohen procedure except that it included a penicillin pretreatment.

After the nature of the cell membrane was determined to be of a Gram-negative type, the transformation procedure of Timmis and Bagdasarian was used (3). This procedure is a modification of the classical $CaCl_2$ procedure (17) which has been optimized for Pseudomonas by the addition of MOPS (morpholinopropane sulfonic acid)

buffer and RbCl. The procedure is as follows: Log phase cells were harvested at 0 C and washed with an equal volume of cold buffer I (10 mM MOPS, pH 7.0/10 mM RbCl/100 mM $MgCl_2$), harvested and resuspended in an equal volume of cold buffer II (100 mM MOPS, pH 6.5/ 10 mM RbCl/100 mM $CaCl_2$) and held at 30 C for 30 minutes. Cells were then harvested and resuspended in one-tenth volume of cold buffer II. Then 0.2 ml of the suspension was incubated with plasmid DNA (1 µg) at 0 C for 45 minutes, followed by incubation at 42 C for 1 minute. Three mls of L-Broth (5 g/l NaCl, 5 g/l yeast extract, 10 g/l tryptone) was then added to the mixture which was then incubated at 27 C for 90 minutes. Finally cells were diluted and plated on streptomycin selective media.

Conjugation Experiments

Filter matings between ABS10 and E. coli strain RC709 bearing the RP4 plasmid (Datta et al.) were carried out by filtering 10 mls of log phase cultures (grown in nutrient broth) onto nucleopore membranes and placing the membranes on nonselective medium (nutrient agar) overnight. The membrane was then placed into selective broth containing 50 mg/l streptomycin. Dilutions onto selective plates were then carried out. The same procedure was followed when matings between P. putida bearing the TOL plasmid and ABS10 were carried done except that 50 mM m-toluate minimal medium with 25 mg/l naladixic acid (Sigma Chemical Co.) was used as a selective medium.

DNA Isolation

Plasmid DNA was isolated by the method of Anderson and McKay (1) followed by cesium chloride-ethidium bromide equilibrium centrifugation. Chromosomal DNA preparations were done according to the Marmur procedure (52) with the following modifications; omission of sodium perchlorate and the addition of treatment in a French pressure cell (three passages) following lysozyme treatment. DNA was quantitated and checked for purity on a Beckman DU-7 spectrophotometer.

Guanine and Cytosine Content (% G+C Determination)

The G+C content of ABS10 was determined by midpoint determination of the thermal melting (Tm) profile using a Beckman DU-8 spectrophotometer and monitoring absorbance at 260nM. *E. coli* was used as a reference strain and the following formula was used to calculate the % G+C (GGG):

$$\text{mole \% G+C} = \text{mole \% G+C}_{ref} + 1.99\,(Tm_x - Tm_{ref})$$

DNA Hybridization Studies

Colony hybridization studies were conducted using Plaque Screen hybridization membranes (New England Nuclear, Boston, MA). The manufacturers protocols were followed with modifications of the lysis and washing steps. The lysis procedure involved two NaOH treatments (0.5 M and 1.5 M, respectively) followed by neutralization in 1 M Tris, pH 8. A high stringency wash buffer was used (10 mM

NaCl, 20 mM Tris, 1 mM EDTA and 0.5% SDS) due to the lack of specificity of an entire chromosomal probe and the filters were washed with agitation at 65 C for 2 hours two times. Gene Screen Plus hybridization membranes (New England Nuclear) were used for both dot blot (57) and Southern blot (51) experiments. Vendor protocols were followed although the "high stringency" wash was used as the final wash buffer. Lambda DNA and all restriction endonucleases were purchased from Bethesda Research Laboratories (Gaithersburg, MD).

The ^{32}P-labeled DNA probes were prepared by nick translation (51) using a commercial nick translation kit from Bethesda Research Laboratories. Labeled probe was separated from unincorporated nucleotides by a sephadex G-50 column chromatography. Specific activity was determined by scintillation counting using a Tracor Analytical scintillation counter model 6892. Autoradiography was carried out at -90 C for 16 to 18 hours.

RNA Isolation

For RNA extraction the method of Lane et al. was used (46). Overnight cultures of ABS10 were grown in nutrient broth, harvested and frozen at -20 C. Then 300 mg of frozen cell pellet was resuspended in 50 mM sodium acetate, 10 mM EDTA (pH 5.1). The suspension was mixed on a vortex mixer and 20% SDS was slowly added to a concentration of 1%. An equal volume of buffer saturated phenol was then added and vortexed vigorously. The tube was incubated at 60 C for 10 minutes. The phases were then separated by centrifugation and the upper phase was harvested. Two phenol chloroform extractions

were then done followed by a chloroform extraction. The nucleic acid was precipitated by adding 1/10th volume 0.1 M sodium acetate, and two volumes of 95% ethanol. The precipitate was collected by centrifugation (10,000 RPM at 0 C for 20 minutes) and then suspended in 0.5 ml double distilled water.

16S Ribosomal RNA Sequencing

Determination of 16S ribosomal RNA sequence was done by the method of Lane et al. (46). Ribosomal RNA specific oligonucleotide primers were synthesized manually at the Institute for Molecular and Cellular Biology, Indiana University, Bloomington, IN. Reverse transcriptase from avian myloblastosis virus (10,000 units/ml) was purchased from Seikagku America, Inc. Deoxyadenosine 5'-[-thio] triphosphate, labeled with ^{35}S in the alpha-thio position, was purchased from New England Nuclear. The 2'-deoxy-(d-) and 2',3'-dideoxy-(dd-)nucleotide triphosphates were purchased from P-L Biochemicals. The sequencing method was a base specific dideoxynucleotide chain elongation termination method (51). The procedure is as follows: oligonucleotide primers were hybridized to RNA templates in 7.5 µl reaction mixtures containing 1.5 µl 5X hybridization buffer (500 mM KCl/250 mM Tris.HCl pH 8.7), 2 µl template RNA (2 mg/ml), and 4 µl H_2O. This was then heated to 90 C for 2 minutes and slowly cooled to 37 C. The 5.6 µl of the priming mix was then added to 5.6 µl of reverse transcriptase (3000 µ/ml), and 5.6 µl RT buffer (250 mM Tris.Hcl, pH 8.3/250 mM Kcl/50 mM dithiothrietol/50 mM $MgCl_2$).

Three microliters of this solution were then added to each of five tubes that contained, respectively 2 µl of ddATP, ddCTP, ddTTP, ddGTP or ddCTP. The reactions were then incubated at 37 C for 30 minutes. After this, 1 µl of chase mix (1 mM dATP/1 mM dCTP/1 mM dGTP/ 1 mM dTTP in 10 mM Tris.Hcl) was added to each of the reactions and incubated at 37 C for 15 minutes. The reactions were stopped by the addition of 10 µl of stop mix (86% formamide/10 mM EDTA/0.08% xylene cyanol/0.08% bromphenol blue). Reactions were heated to 90 C for 2 minutes before loading on 6% or 8% polyacrylamide gels. Gels were run at 2,500 volts, 60 amps and 55 watts (8% gels were run for 4 hrs and 6% gels were run for 8 hrs). The gels were then fixed in a methanol, acetic acid, glycerol solution (for 4 liters-400 ml 10% methanol, 400 ml 10% acetic acid and 67.2 g glycerol added to distilled H_2O) for 20 minutes and dried in a Biorad slab gel dryer (Biorad, Rockvillecenter, NY). The dried gels were then put up for autoradiography for 15 hrs using Kodak XAR 5 film (Eastman Kodak, Rochester, NY). The gels were read manually and the sequence was recorded for subsequent analysis. All manipulations of sequence data were performed on a VAX 11/750 computer at the University of Illinois.

Cell Membrane Analysis

Fatty acid profile analysis was carried out by Hewlett-Packard Company using the HP5890A Microbial Identification System (Microbial ID, Inc., Newark, DE). The fatty acid extracts were prepared by

saponification of whole bacterial cells in a strong base, then acidifying to liberate fatty acids. These were then methylated to increase their volatility for subsequent GC analysis. Methyl esters of the fatty acids were then extracted from the acidified aqueous solution. Before injection, the organic extract was washed with a dilute base. The washed extract was then analyzed by GC with a nonpolar capillary column and flame ionization detector (55).

KDO extraction was performed by the method of Karkhanis et al. (51). One liter of ABS10 was harvested from an overnight culture grown in nutrient broth. The culture was then freeze dried in a Labconco Freeze Dryer (Labconco, Inc., Kansas City, MO). The LPS was extracted by chloroform/methanol extraction (25). Freeze dried LPS material was treated with 0.2 N H_2SO_4 at 100 C for 30 minutes. This was followed by treatment with periodic acid, sodium arsenite and thiobarbituic acid. The subsequent reaction forms a red chromophore with an absorbance maxima at 548 nm. The chromophore was kept in solution at room temperature by the addition of dimethylsulfoxide.

CHAPTER IV

RESULTS

Preliminary Characterization of ABS10

ABS10 is a Gram-negative to Gram variable rod that becomes spherical in shape on prolonged incubation. Some characteristics of ABS10 are listed in Table 3. Figure 5 is a transmission electron micrograph showing the absence of flagella. ABS10 showed resistance to a wide array of antibiotics as shown in Table 4. All resistance markers are presumed to be chromosomally encoded as no plasmid DNA was detected in ABS10. Table 5 shows the carbon source utilization of ABS10 as compared with A. globiformis and P. putida bearing the Tol plasmid pWW0. Overall Table 5 shows that the strains can utilize the majority of the substrates tested. Unlike ABS10 and P. putida, A. globiformis could not utilize benzoate as a sole carbon source.

Earlier taxonomic studies of the Lula aquifer showed that Gram-positive Arthrobacter-like organisms predominate (4). Standard taxonomic methods proved inadequate for accurately typing ABS10 into a particular genus although preliminary characterization suggested ABS10 to be an arthrobacter-like organism; however, this characterization was not absolute.

Scanning electron micrographs of ABS10 (Figure 6) show the pleiomorphic variation of ABS10. Young cultures (5-36 hrs on one-tenth strength YEPG agar) are predominantly composed of the rod form.

Table 3. Initial Characterization of ABS10.

Test	Reaction
Gram-reaction	-/+
Oxidase test	+
Flagella	-
Motility	-
NO_2 reduction	-
H_2S production	-
Catalase test	+
Voges-Proskauer	-
Gelatin hydrolysis	-

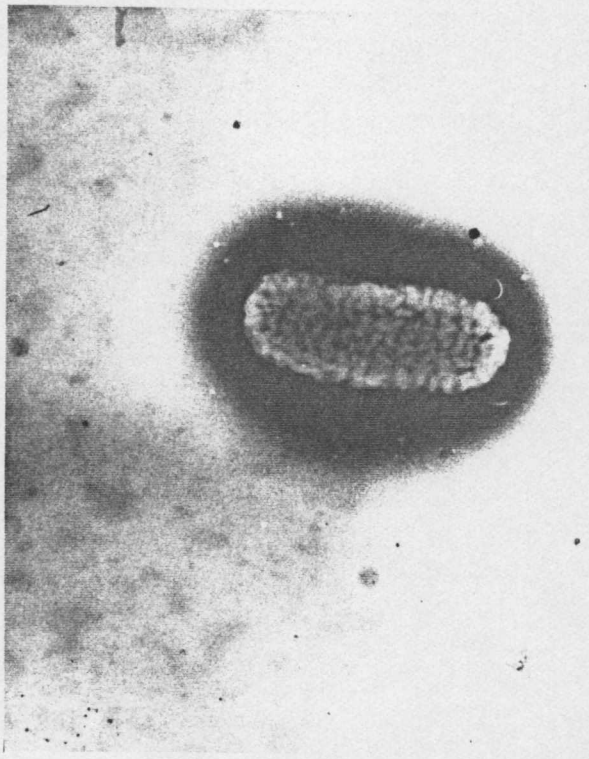


Figure 5. Transmission electron micrograph of ABS10. Magnification 38000. Note that ABS10 lacks flagella.

Table 4. Antibiotic Resistance Profile of ABS10.

Antibiotic	Reaction
Naladixic acid	S
Tetracycline	R
Ampicillin	R
Streptomycin	S
Penicillin	R
Erythromycin	R
Gentamycin	R
Chloramphenicol	R
Clindamycin	R
Novobiocin	R
Neomycin	R
Carbenicillin	R
Kanamycin	S

Table 5. Carbon Source Utilization of ABS10 and Other Strains.

Source	ABS10	A. globinformis	P. putida
Mannitol	+	+	+
Maltose	+	+	+
Lactose	+	+	+
Sucrose	+	+	+
Dextrose	+	+	+
Phenol	-	-	-
Acetate	+	+	+
Citrate	+	+	+
Succinate	+	+	+
Benzoate	+	-	+
Salicylate	-	-	-
Sorbitol	+	+	+
Aniline	-	-	-
Naphthalene	-	-	-
Chlorobenzoate	-	-	-
Styrene	-	-	-
m-Toluate	-	-	+

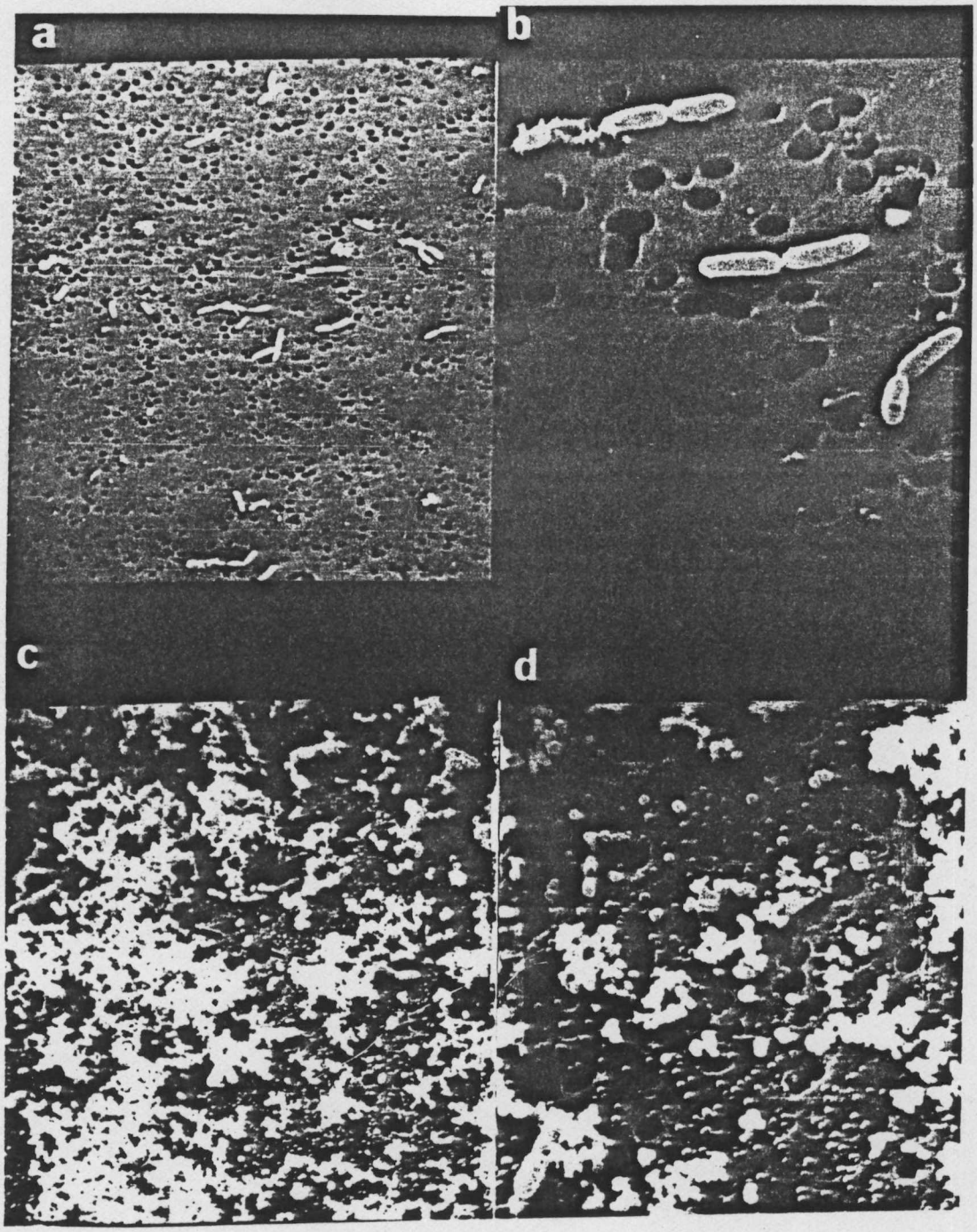


Figure 6. Scanning electron micrographs of ABS10. Morphological change of ABS10. A-rod form magnification 1500. B-rod form magnification 6000. C-cocci form magnification 3000. D-cocci form magnification 6000.

As the culture ages and cell density increases, the cocci form predominates. It was this observation and a Gram variable reaction of 24 hr cultures that led to the initial tentative designation of ABS10 as an Arthrobacter sp. Pleomorphism may be an adaptation to the oligotrophic conditions in the Lula, OK aquifer (4,15).

% G+C Determination

The % G+C content of ABS10, determined in 0.12 M sodium phosphate buffer, pH 6.8, was 65.3. This was determined by Tm determination of both ABS10 and E. coli (which served as a reference). The hyperchromic shift of ABS10 is shown in Figure 7, where absorbance at 260 nm is plotted against temperature. The point of inflection of the curve is the Tm which was 96.4 C for ABS10. The % G+C value of 65.3 was arrived at by comparison with that of E. coli which has a G+C content of 51.0. The value of 65.3% was not useful for distinguishing a Pseudomonas sp. from an Arthrobacter sp. as they have ranges from 58-70% and 60-72%, respectively (10).

Cell Membrane Analysis

The fatty acid methyl ester (FAME) profile of ABS10 is shown in Table 6. Comparison to an extensive library, compiled by Microbial ID, Inc., revealed ABS10 to share 0.74 homology with Pseudomonas putida. FAME profiles have been shown to be distinctive and conserved among groups of related organisms making their analysis useful as a taxonomic tool (55,20). Figure 8 depicts typical bacterial fatty acids. The usual chain length is 10 to 20 carbon atoms. They vary

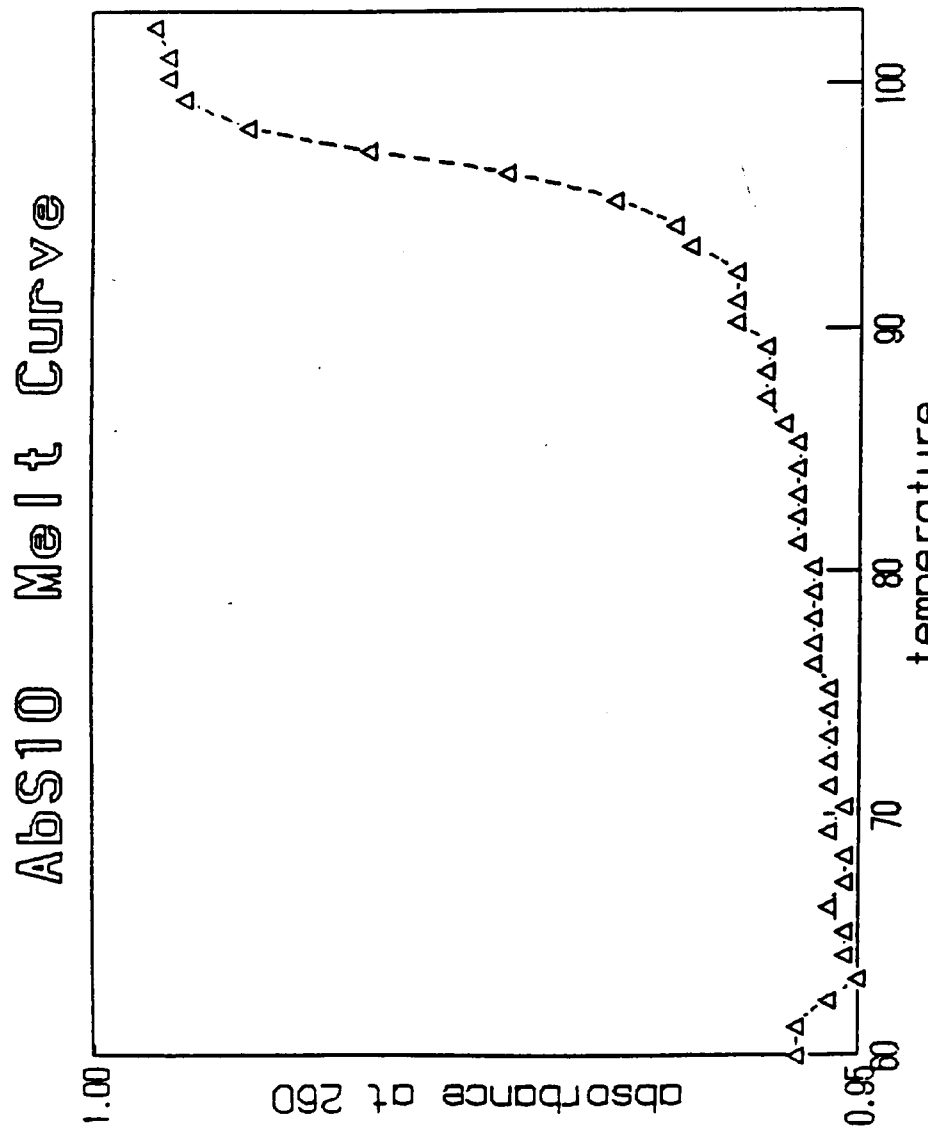


Figure 7. Thermal melt profile of ABS10.

Table 6. Fatty Acid Methyl Ester Profile.

Fatty Acid	Retention Time (Minutes)	Structure
1	3.064	10:00
2	3.392	.....
3	3.923	.....
4	4.129	10:0 3OH
5	4.646	12:0
6	5.041	.....
7	5.195	.....
8	6.012	12:0 2OH
9	6.163	12:1 3OH
10	6.385	12:0 3OH
11	6.862	13:0 iso 2OH
12	7.116	14:0
13	7.531	.....
14	9.093	.....
15	9.443	14:0 3OH/16:1 iso 1
16	9.984	16:1 cis 9
17	10:042	16:1 trans
18	10.283	16:0
19	11.811	17:0 cyclo
20	13.443	18:1 trans
21	13.749	18:0

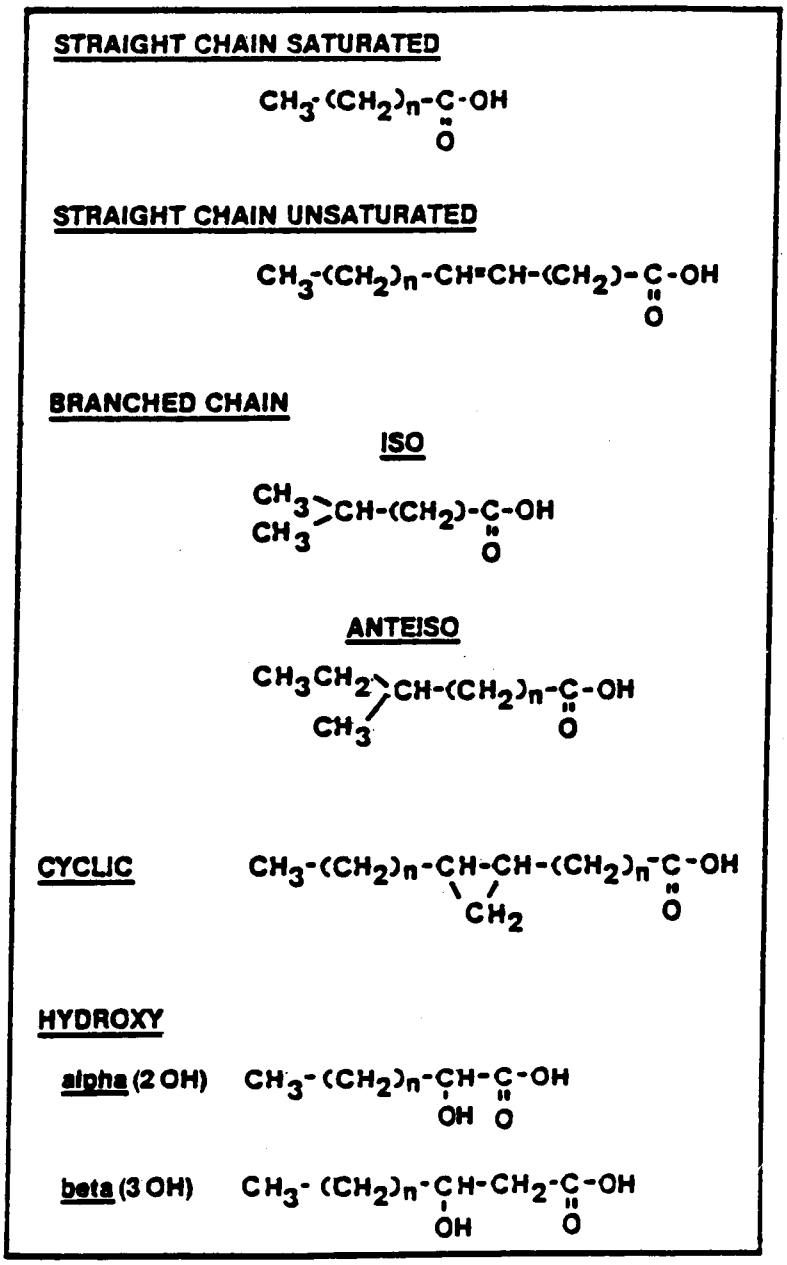


Figure 8. Typical bacterial fatty acids. Taken from Reference 55.

in degree of saturation and may contain hydroxyl groups. The most common fatty acids are straight chain unsaturated molecules. Branched chain fatty acids are abundant in the Gram-positive bacteria while cyclopropanes are not uncommon in the Gram-negative bacteria. Gram-negative bacteria often possess hydroxyl groups bound to the second or third carbon atom (55).

The occurrence of the 17 carbon cyclopropanyl fatty acid, as well as the large proportion of hydroxy fatty acids, indicate that ABS10 has a Gram-negative cell membrane type. There is also an absence of branched chain fatty acids which would be expected to be present in a Gram-positive bacterium (including the Gram-variable Corynebacteria) (6). The similarity value of 0.74 which ABS10 shares with P. putida is not a very high correlation. Environmental bacteria which were isolated on Pseudomonas isolation agar showed similarity values of greater than 0.90 (Dele Ogunseitan, personal communication). This is indicative of substantial differences between ABS10 and P. putida, as well as Pseudomonas in general. Hence fatty acid analysis demonstrates two fundamental properties of the cell membrane of ABS10: i) ABS10, although being isolated from an aquifer where Gram-positive bacteria appear to dominate is in fact a Gram-negative bacterium and ii) the fatty acid profile of ABS10 is indicative of a very unique pseudomonad or possibly a new bacterial species.

The data generated by the KDO assay of Karkanis et al. (39) was consistent with the FAME data in that a Gram-negative membrane type was found to be present in ABS10. A thiobarbituic acid positive

substance formed the characteristic red chromophore when a membrane preparation from ABS10 was treated with periodate and thiobarbituic acid. Figure 9 shows the absorbance profile of ABS10 and S. typhimurium. Polysaccharides (which contain the O-side chain and the core region) are linked to lipid A through KDO. The absorbance maximum of authentic KDO, isolated from S. typhimurium, is 548 nm. Phosphorylated derivatives show a lowering of the maxima (45,8). The absorbance maxima of the chromophore formed by ABS10 had a maxima at 534 nm. KDO with absorbance properties similar to that of S. typhimurium have been found in the Enterobacteriacea, the Neisseriacea, and the Pseudomonadacea; however, this is not true for all Gram-negative bacteria. Vibrio chlolera, a species at first believed to lack KDO, was found to contain phosphorylated KDO (8). Hence, the KDO assay indicated a Gram-negative cell type but not the membrane type of a typical pseudomonad.

DNA Hybridization Studies

A number of DNA hybridization studies were carried out between ABS10 and various other bacteria for taxonomic purposes. A number of hybridization techniques were used, i.e., colony hybridization, Southern blotting and dot blotting. Probes were constructed by labeling chromosomal DNA with 32-P by nick translation. A "high stringency wash" was used due to the inherent lack of specificity of a total genomic probe. The high stringency wash (see Materials and Methods) insured that the fidelity of the hybrids formed would be high and thus impart a greater degree of specificity to the probe.

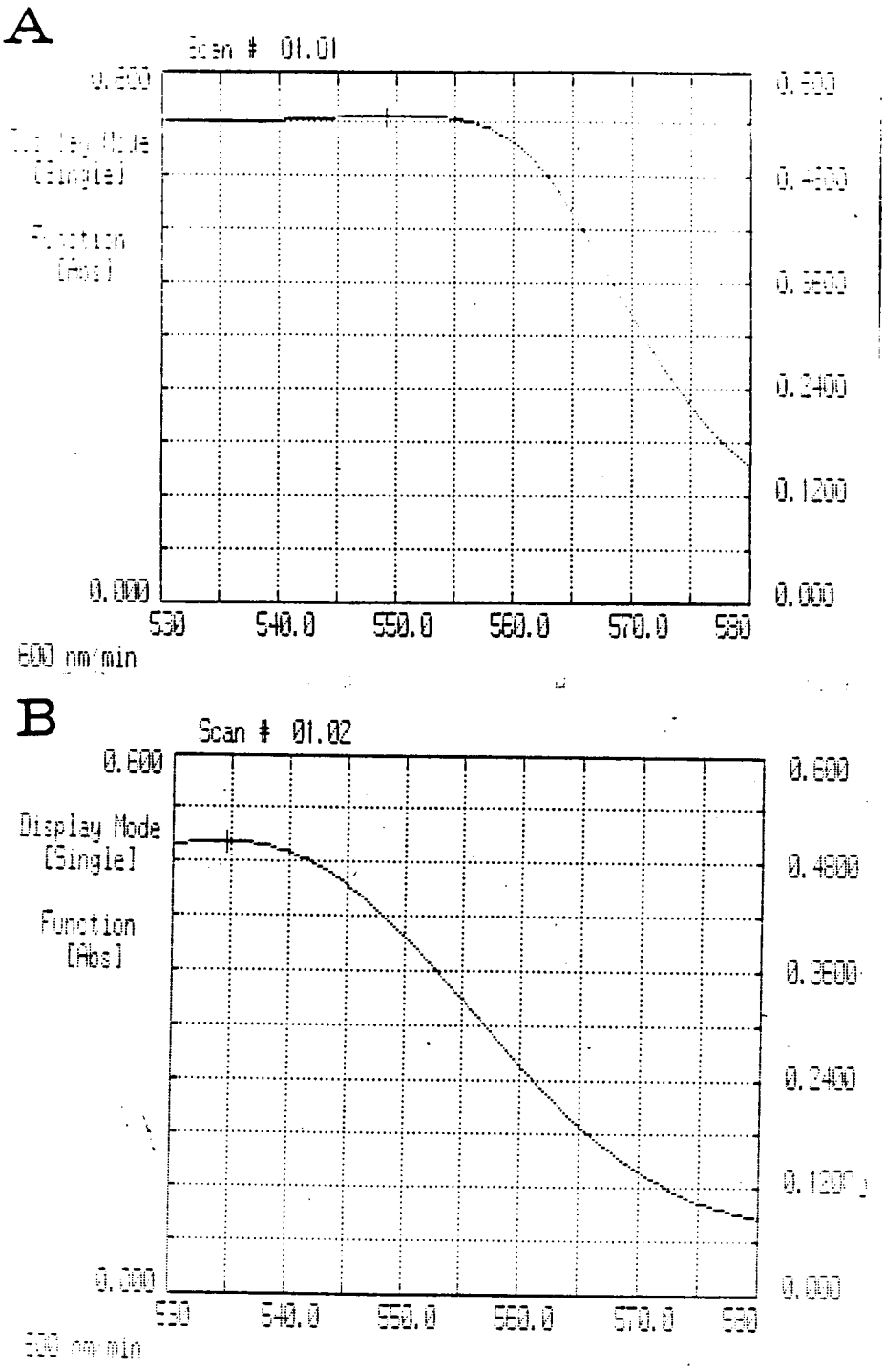


Figure 9. Absorbance profile of KDO. Extraction products of: A. S. typhimurium and B. ABS10.

The results of colony hybridization experiments are described in Figure 10 and Table 7. These data indicate that ABS10 shares a great deal of genetic homology with P. putida and P. aeruginosa. A. globinformis and TM1 showed very weak homology and a newly described Arthrobacter sp., FPC7B2 (23), showed moderate homology. Also presented in Figure 10 and Table 7 are the results of probing different strains with TM1, an Arthrobacter sp. (64). It is interesting to note that TM1 showed only a low level of homology with A. globiformis. Although colony hybridization provides an excellent tool for quick screenings of bacterial strains, the results can be skewed by the different growth rates of the bacteria as well as cellular debris causing non-specific binding of the probe. This may partially explain the weak signal that E. coli showed when probed with TM1.

The results of dot blot hybridization with ABS10 DNA against varying amounts of DNA from known organisms is shown in Figure 11. These data show ABS10's strong homology with P. putida and some weak hybridization with Alcaligenes sp. A5. E. coli and A. globiformis show no homology with ABS10. Once again data on the genetic level supports the cell membrane analysis conclusions that ABS10 has many pseudomonad characteristics.

Further documentation of the relatedness of ABS10 to P. putida on the genetic level was revealed by Southern blot analysis. In this experiment ABS10, bacteriophage lambda, TM1 and P. putida were each subject to restriction endonuclease digestion by EcoR1, BamH1 and EcoR1/BamH1 double digestion. The reactions were run on a 0.75%

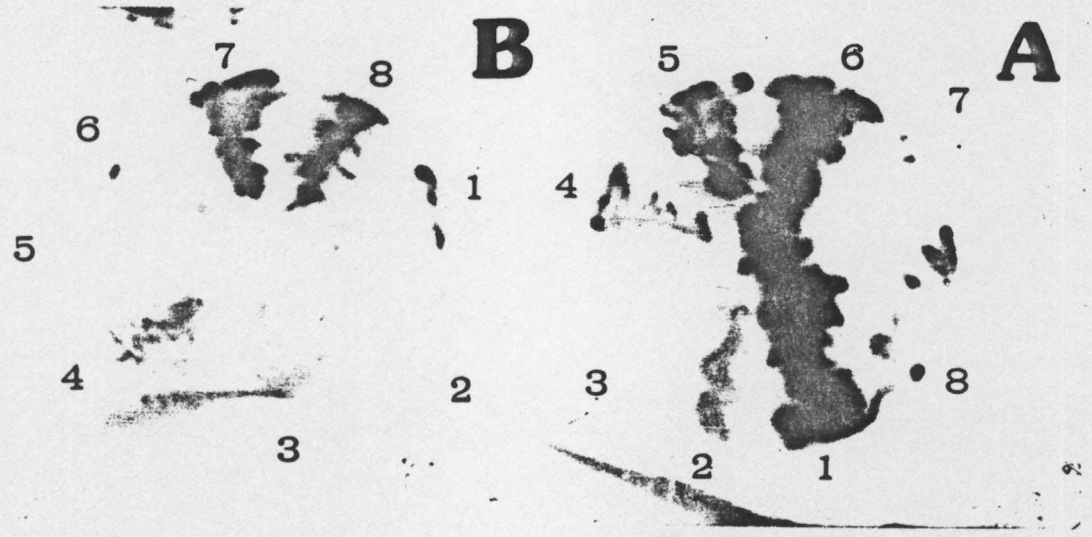


Figure 10. Colony hybridization autoradiogram. Autoradiogram from colony hybridization experiment using ABS10 chromosomal DNA (A) or TM1 chromosomal DNA as a probe. The target colonies are: 1. ABS10, 2. E. coli, 3. A. globiformis, 4. FPC7 B (Arthrobacter sp.), 5. AX2 (Acinetobacter sp.), 6. P. aeruginosa, 7. A. crystallopoietes, 8. TM1.

Table 7. Colony Hybridization; Whole Chromosomal DNA Probe vs. Target Organisms.

Target	Probe	
	ABS10	TM1
ABS10	+++	+
A. globinformis	+	-
E. coli	-	+
FPC7B2 (Arthrobacter)	++	++
P. aeruginosa	+++	++
P. putida	+++	ND
AX2 (Acinetobacter)	+++	+
A. crystallopoietes	-	+++
TM1 (Arthrobacter)	+	+++

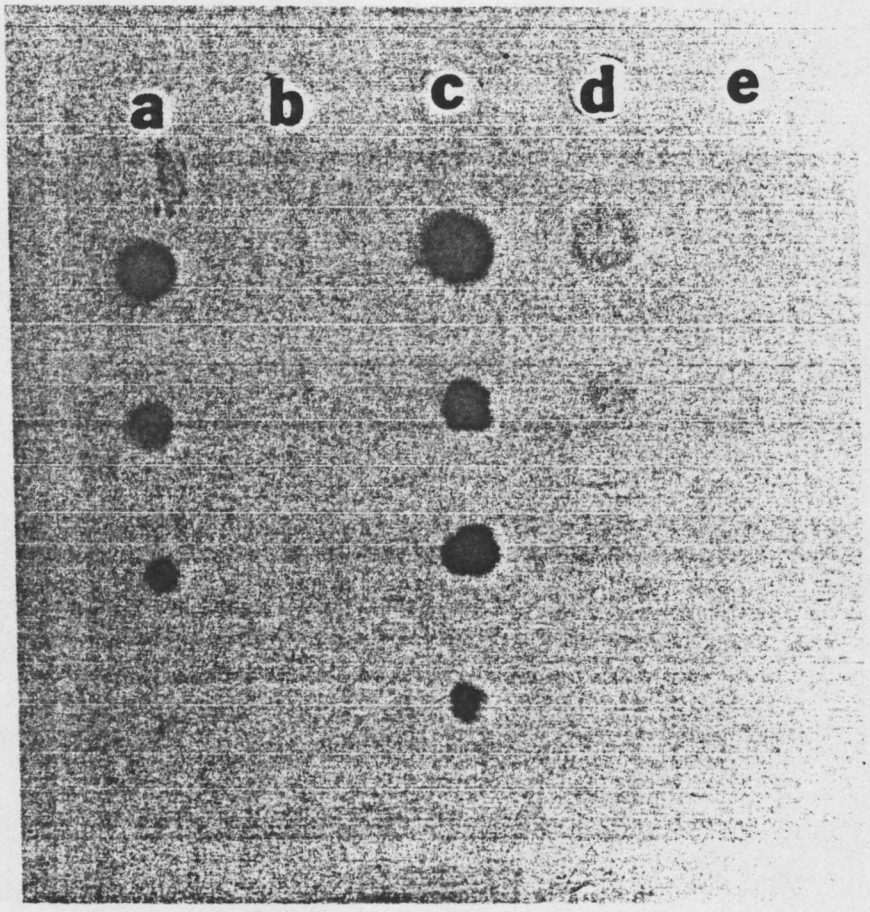


Figure 11. Dot blot using ABS10 as a chromosomal probe. Ln.A ABS10 DNA (1) 1.0 µg (DNA), (2) 0.5 µg, (3) 0.1 µg, (4) 0.01 µg; Ln. B E. coli DNA (1) 10.0 µg, (2) 1.0 µg, (3) 0.5 µg, (4) 0.1 µg; Ln. C P. putida DNA (1) 10.0 µg, (2) 1.0 µg, (3) 0.5 µg, (4) 0.1 µg; Ln. D Alkaligenes sp. DNA (1) 10.0 µg, (2) 1.0 µg, (3) 0.5 µg, (4) 0.1 µg; Ln. E Alcalinges globiformis DNA (1) 10.0 µg, (2) 1.0 µg, (3) 0.5 µg, (4) 0.1 µg.

agarose gel and transferred to Gene Screen by capillary blotting. The filter was then probed with ABS10. Results once again show extensive homology with P. putida and none with lambda or TM1 (see Figure 12).

Thus, ABS10 appears not to be genetically related to three of the Arthrobacter sp. studied--A. globiformis, A. crystallpoites, or TM1. The Pseudomonas sp. studied both show strong homology with ABS10. An Alcaligenes sp., A5 (a genus closely related to Pseudomonas), showed weak homology with ABS10, Figure 11 (73).

16S rRNA Sequence Analysis

16S RNA sequence data on ABS10 was generated by dideoxy sequencing utilizing reverse transcriptase (see Materials and Methods). "Core" or "universal" primers were used to prime the RNA directly, thus eliminating the need for any cloning (usually a prerequisite for most nucleic acid sequencing (46). The primers used and their position on the RNA molecule are shown in Figure 3, page 20. The 1500R and 920R primers failed to prime ABS10 RNA. The most likely explanation for this is secondary structure interfering with the primer. High G+C containing organisms such as ABS10 are generally more difficult to sequence due to inefficient priming and band compression on sequencing gels (46).

The eubacteria are divided into various groups on the basis of 16S RNA sequences (24,92,77) (see Figure 13). The largest of these groups is the purple bacteria which is divided into the Alpha,

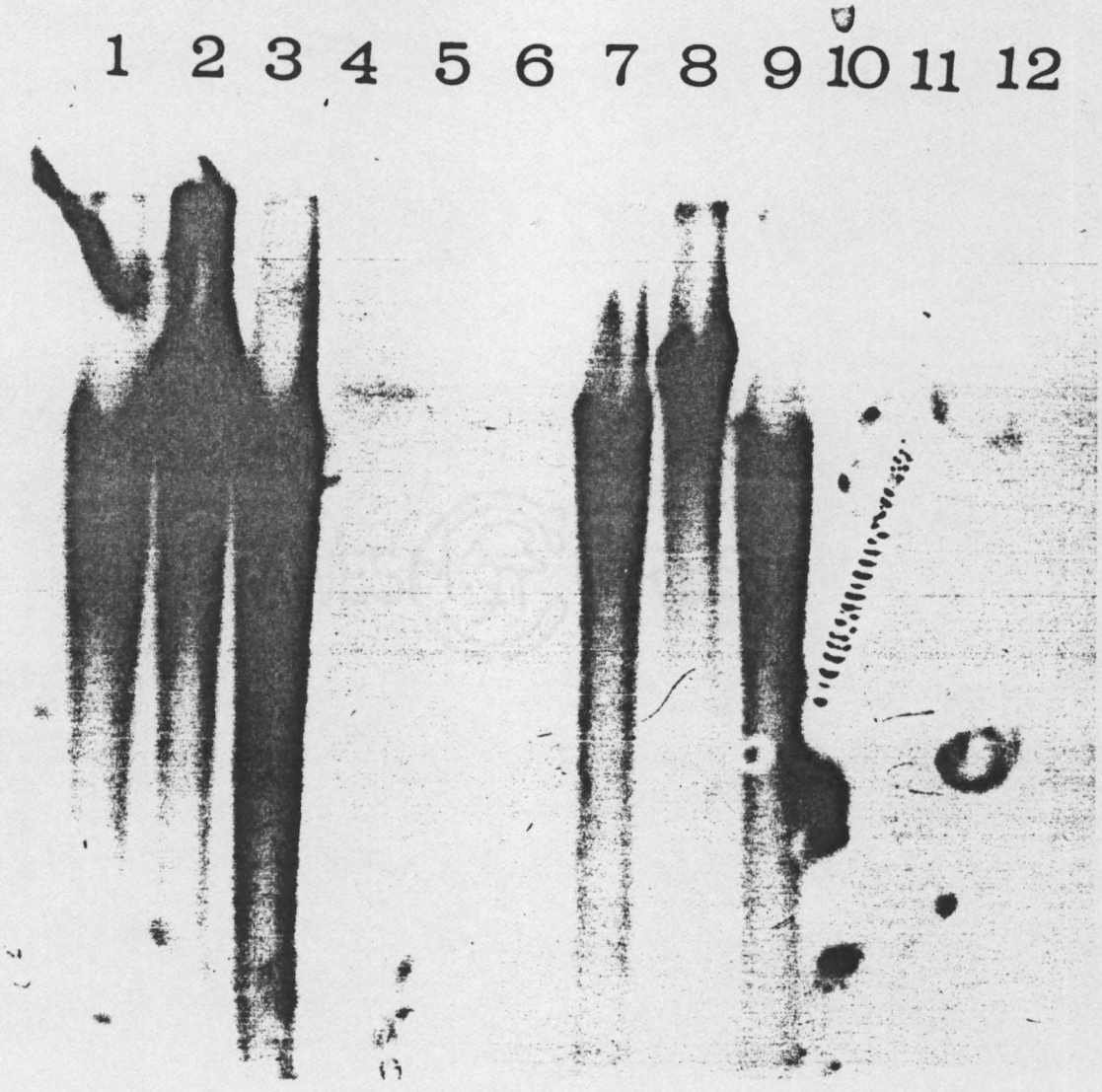


Figure 12. Southern hybridization. Southern blot analysis using the "high stringency" wash and ABS10 chromosomal DNA as a probe. Three DNAs were subject to restriction endonuclease digestion, put on an agarose gel for electrophoresis and blotted. Ln. 1 ABS10 EcoRI digest, Ln. 2 ABS10 Bam HI digest, Ln. 3 ABS10 EcoRI/BamHI digest, Ln. 4 TM1 EcoRI digest, Ln. 5 TM1 BamHI digest, Ln. 6 TM1 EcoRI/BamHI digest, Ln. 7 P. putida EcoRI digest, Ln. 8 P. putida BamHI digest, Ln. 9 P. putida EcoRI/BamHI digest, Ln. 10 Lambda EcoRI digest, Ln. 11 Lambda BamHI digest, Ln. 12 Lambda EcoRI/BamHI digest.

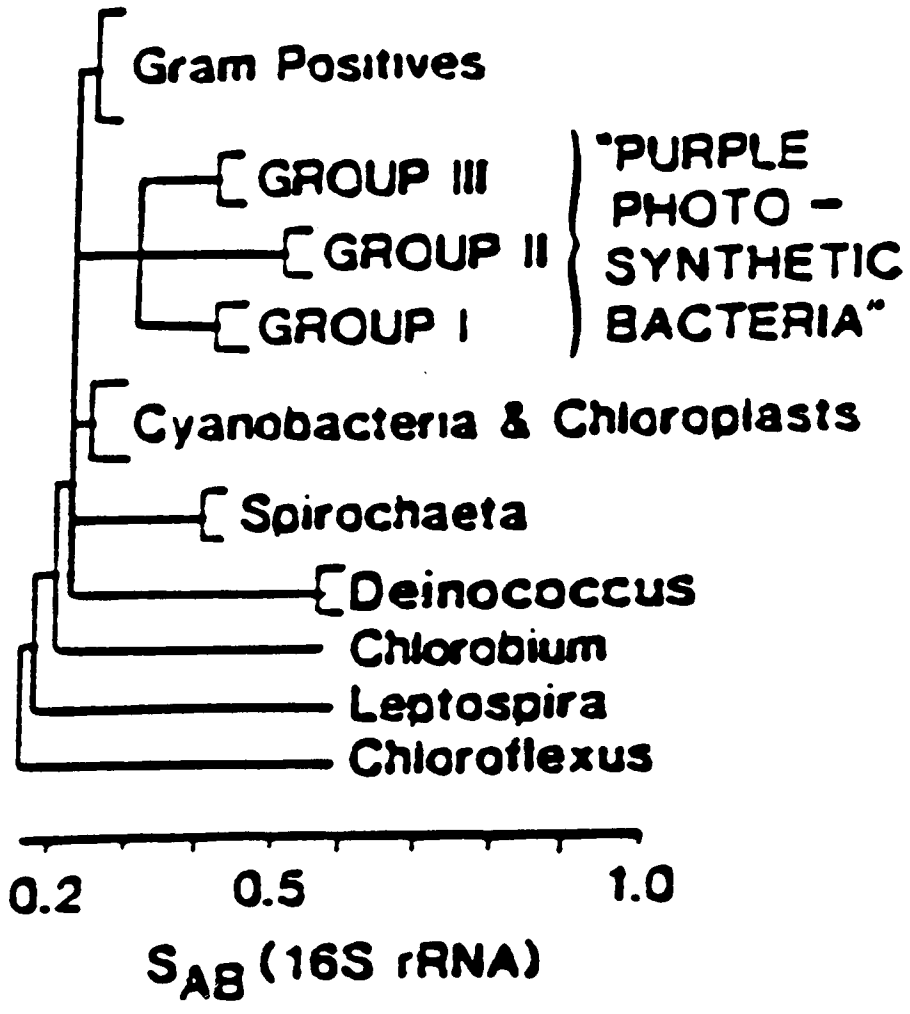


Figure 13. Phylogenetic tree of the Eubacteria derived from ^{16}S rRNA oligonucleotide catalogs. Taken from Reference 78. Organisms can be compared in terms of an association coefficient, S AB. The coefficient is a fraction equal to twice the number of nucleotides in RNA sequences of six bases or longer common to organisms A and B, divided by the total number of bases.

Beta and Gamma subdivisions (91,93,92). Of these subdivisions, the largest is the Gamma division. The 16S RNA data from ABS10 shows it to be most closely related to organisms in the Gamma division. This division is extremely diverse and includes organisms as divergent as E. coli, Legionella jordanis, Pseudomonas putida and Thiomicrospira pelophila.

A comparison of the total sequence data derived from ABS10 versus a bank of strains representative of the various groups of the eubacteria (as well as an archaebacterium) shows ABS10 to share the greatest homology with E coli, a Gamma division representative (see Table 8). Proteus vulgaris, another Gamma division member shares the second greatest amount of homology with ABS10. To demonstrate the significance of these values, Table 8 compares E. coli versus the reference bank. Note the high degree of homology which E. coli shares with Proteus vulgaris as well as the lower value shared with A. tumefaciens (an Alpha group member) and Acholeplasma laidlawii (an archaebacterium).

Although ABS10 seems to be most closely related to the organisms of the Gamma division, total sequence comparison alone is insufficient to place this organism in the Gamma division. Hence, due to the scarcity of complete 16S r RNA sequence data, the ABS10 sequence was scanned for various "signature sequences" specific to various groups of organisms. The signature sequences were derived from oligonucleotide catalog data (24). Table 9 shows the presence or absence of a number of signature sequences in ABS10. Also shown

Table 8. Total Sequence Homology Comparison.

ABS10		E. coli	
Organism	Homology	Organism	Homology
E. coli	0.864	ABS10	0.864
B. succ	0.743	T. nivea	0.829
Pr. vulga	0.850	B. succinogenes	0.746
Myx. xant	0.834	Pr. vulga	0.952
D. radiod	0.766	Myx. xant.	0.828
D. desulf	0.803	D. radiod	0.769
Thiovulu	0.768	D. desulf	0.742
Ps. testo	0.835	Thiovulu	0.705
Rochalim	0.810	Ps. testo	0.793
Agr. tume	0.823	Rochalim	0.826
H. chloru	0.804	Agr. tume	0.833
My. capri	0.792	H. chloru	0.814
Ach. laid	0.779	My. capri	0.783
B. brevis	0.801	Ach. laid	0.772
B. stearo	0.841	B. brevis	0.814
Arth. glo	0.789	B. stearo	0.844
An. nidul	0.785		
Bacteroi	0.754		

Source of sequence:
T. nivea - Thiothrix nivea JP2 David Stahl unpublished.
E. coli - Escherichia coli Brosios et al. (1978) PNAS 75:4801-5.
Bacteroides succinogenes S85 - D. Stahl unpublished.
Pr. Vulga - Proteus vulgaris Carbon, Ebel and Ehresmann (1981) NAR 9:2325.
Myx. xant - Myxococcus xanthus MD207 Oyaizu and Woese (1985) unpub.
D. radiod - Deinococcus radiodurans Woese (1984) unpub.
D. desulf - Desulfovibrio desulfricans Woese (1984) unpub.
Thiovulu - Thiovulum D. Stahl unpublished.
Ps. testo - Pseudomonas testosteroni Woese (1985) unpub.
Rochalim - Rochalimaea quintana (Rickettsiaceae) Woese (1985) unpub.
Agr. tume - Agrobacterium tumefaciens Woese (1985) unpub.
H. chloru - Heliobacterium chlorum Woese, Oyaizu and Debrunner (1985) unpub.
My. capri - Mycoplasma capricolum Iwami (1984) MGG 196:317-322.
Ach. laid - Acholepasma laidlawii Woese (1985) unpub.
B. brevis - Bacillus brevis Kop and Noller (1984) JBC 259:15287-93.
B. stearo - Bacillus stearothermophilus Woese unpub.
Arth. glo - Arthrobacter globiformis Oyaizu and Woese (1985) unpub.
An. nidul - Anacystis nidulans (Cyanobacterium) Tomokoioka and Sugiura (1983) MGG 191:46-50.
Bacterio - Bacteroides fragilis Woese (1985).

Table 9. RNA Signature Sequence Comparison.

	Sequence	ABS10	Gram (+) High G+C	Gram (+) Low G+C	Myxobacteria
1.	UCCUCAUG	(-)	(-)	(-)	(+)
2.	CCCYUUAU	(-)	(+)	(+)	(-)
3.	AAACUCAAAG	(-)	(+)	(+)	(+)
4.	CAAAACUCAAAG	(-)	(+)	(+)	(+)
5.	AAACUCAAAUG	(+)	(-)	(-)	(-)
6.	AAAUUCG	(-)	(-)	(-)	(-)

1. Signature sequence for α + β purple photosynthetic bacteria.
2. Signature sequence for Gram (+), high and low G+C bacteria.
3. Signature sequence for Gram (+), low G+C bacteria.
4. Signature sequence for Gram (+), high G+C bacteria.
5. Signature sequence for δ purple photosynthetic bacteria.
6. Signature sequence for Δ purple photosynthetic bacteria.

Source of Sequence:
Gram (+) high G+C - A. globiformis
Gram (+) low G+C - B. brevis
Purple photosynthetic bacteria Δ group - Myxococcus xanthus

in Table 9 are the presence or absence of these sequences in organisms for which complete sequences have been determined. The only sequence present in ABS10 is AAACUCAAAUG. This sequence has been proven to be unique to the Gamma division of the purple bacteria (91). Arthrobacter globiformis shows a very different pattern from ABS10 possessing all the Gram-positive signature sequences.

Table 10 shows the occurrence of particular signatures within the purple bacteria. Table 10 compares the occurrence of particular sequences deduced by cataloging versus organisms representative of the subdivisions of the purple bacteria for which complete sequences have been determined. ABS10 possesses all six of the Gamma division sequences tested. ABS10 also lacks signatures distinctive for the Alpha and Beta divisions. The data in Tables 9 and 10 place ABS10 solidly within the Gamma division of the purple photosynthetic bacteria.

Although ABS10 does fall into the same division as E. coli, there are regions of the molecule where deviation is considerable. One particular region is the segment of the molecule from nucleotide 144 to nucleotide 197. This region is highly variable throughout the kingdoms (77). The structural integrity of the molecule remains intact due to compensatory base changes; however, the sequences vary considerably. Thus, ABS10 does not appear to be in the same branch of the Gamma division as E. coli or Pr. vulgaris (this is consistent with DNA-DNA hybridization data, see DNA-DNA hybridization results, page 48) but rather in some other branch. Figure 14 shows the compensatory base changes in ABS10 vs. E. coli.

Table 10. Signature Sequence Comparison Within the Purple Photosynthetic Bacteria.

Sequence	ABS10	A. tumefaciens (Alpha)	P. testosteroni (Beta)	E. coli (Gamma)
1. AAACUCAAAUG	(+)	(-)	(-)	(+)
2. AACUUUCYAG	(+)	(-)	(-)	(-)
3. CUYYACACAUG	(-)	(-)	(+)	(-)
4. AAAACCUUACC	(-)	(-)	(+)	(-)
5. AUUUAUCG	(-)	(+)	(-)	(-)
6. UUACYY	(+)	(-)	(-)	(-)
7. CCCUUACG	(+)	(+)	(-)	(+)
8. CUCUUUCACCG	(-)	(+)	(-)	(-)
9. CYUAAYACAUG	(+)	(+)	(-)	(+)
10. CAACCYYUR	(+)	(-)	(+)	(+)

1. Sequence for Gamma (1 and 3)
2. Sequence for Gamma (3 only)
3. Sequence for Beta
4. Sequence for Beta
5. Sequence for Alpha
6. Sequence for Gamma
7. Sequence for Alpha and Gamma
8. Sequence for Alpha
9. Sequence for Alpha and Gamma
10. Sequence for Beta and Gamma

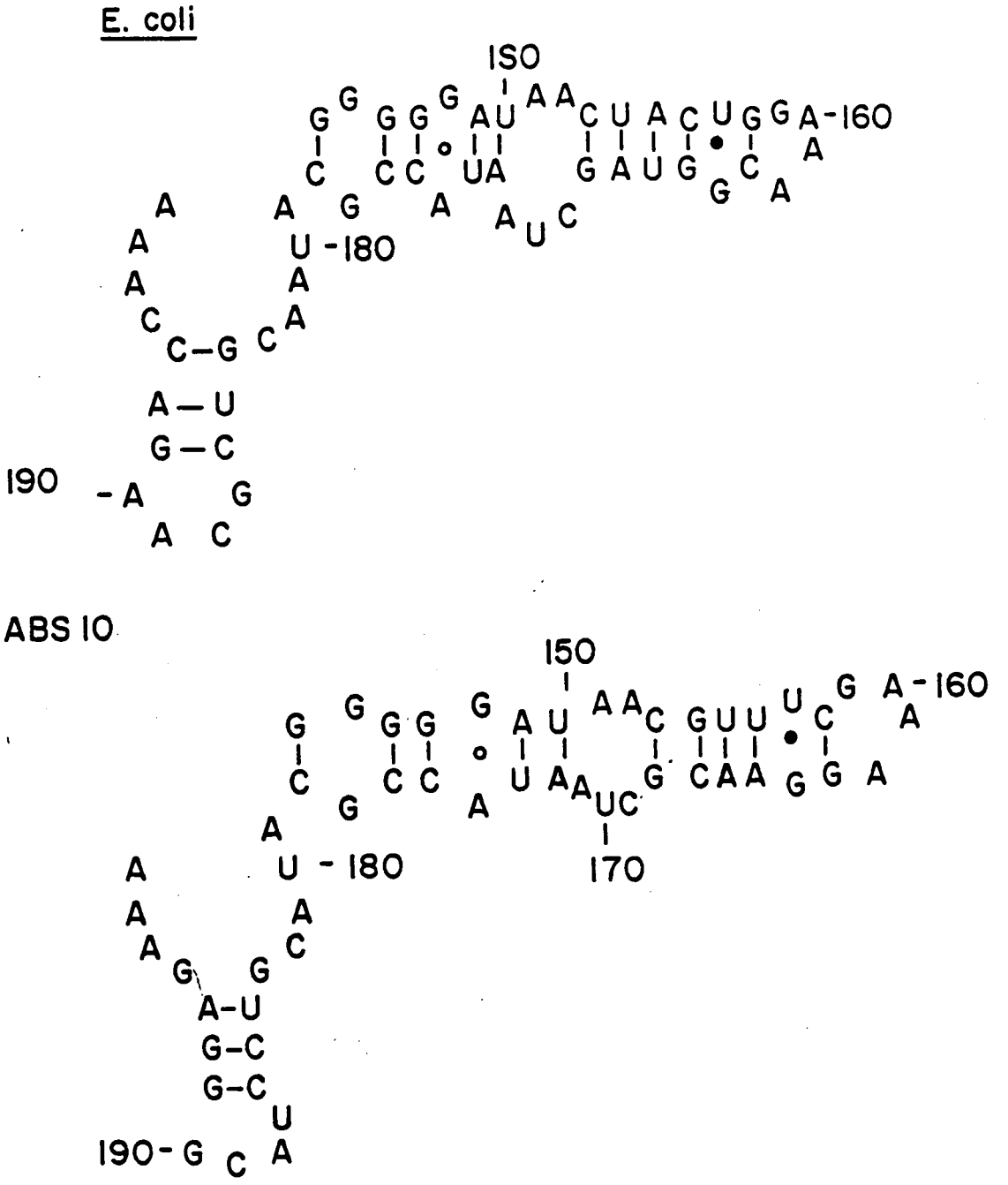


Figure 14. Comparison of the sequence and secondary structure of E. coli and ABS10 16S rRNA molecules from nucleotide 144 to 197.

Gene Transfer Studies

Transformation. Considerable efforts were put into developing a gene transformation system for ABS10. Initially, protocols for Gram-positive bacteria were used as ABS10 was believed to be an Arthrobacter sp. Most Gram-positive transformation protocols are derived from the Chang and Cohen procedure (13). They involve protoplasting followed by polyethylene glycol (PEG) induced DNA uptake. Simple lysozyme, treatment described by Chang and Cohen, would not protoplast ABS10 efficiently. The addition of a penicillin G pretreatment (added at the early log phase of growth at a concentration of 5 μ/ml) resulted in protoplasting greater than 90% of the population (determined by suspension of the protoplasts in distilled water). Replacement of penicillin G by the addition of 2% glycine to the medium was just as effective as penicillin treatment and resulted in a shortened incubation time. Both penicillin pretreatment and the addition of glycine to the growth medium have been used in transformation studies on coryneform bacteria (40).

The transforming DNA in the transformation experiments was the plasmid RSF1010, a broad host range, Gram-negative, cloning vector with a streptomycin resistance marker (3). All manipulations after protoplasting were carried out on ice until the regeneration of the protoplasts. Protoplast regeneration efficiency, using the hypertonic buffers of Katsumata et al. (40), was approximately 1%. Suspect transformants were screened by colony hybridization with 32P-labeled RSF1010. None of the suspect colonies harbored RSF1010. In a

subsequent experiment A. globiformis (a coryneform bacteria) was used as a recipient for RSF1010 and once again no transformants were obtained. Heat shocking at different temperatures (standard heat shock temperature being 37 C), 42 C and 50 C, each for 2 minutes, failed to bring about transformation and the elevated temperatures greatly reduced the regeneration efficiency.

When the pseudomonad characteristics of ABS10 had been documented, transformation procedures described for Gram-negative bacteria were attempted. It is important to note that Gram-negative bacteria such as E. coli can be transformed by protoplast procedures (LLL). In these experiments E. coli DH1 and A. globiformis were subject to the same treatment. The classical $CaCl_2$ transformation procedure of Cohen et al. (17), in which competence is induced by suspension of the recipient in 50 mM $CaCl_2$ for 20 minutes, proved to be unsuccessful in transforming ABS10 and A. globiformis. A modified version of the procedure, developed by Bagdasarian and Timmis (3), was also tried. In this protocol, which was designed for Pseudomonas transformation, the cells were first suspended in 10 mM MOPS, pH 7.0-10 mM RbCl-10 mM $MgCl_2$ and held at 0 C for 30 minutes. Following this the cells are harvested and resuspended in 100 mM MOPS, pH 6.5-10 mM RbCl-10 mM $MgCl_2$, washed, then concentrated 10 fold before the addition of DNA. This also proved unsuccessful; however, a P. aeruginosa strain KT240 (3) had a transformation efficiency of $1.5 \times 10^5/\mu g$ DNA. Although transformation was not accomplished in ABS10 by the methods described here, one cannot conclude that ABS10 is untransformable, rather one can only conclude that it is untransformable

with the methods and transforming DNA used in this set of experiments.

Conjugation. A filter mating experiment between E. coli, bearing the conjugative plasmid RP4, and ABS10 was carried out. Figure 15 shows RP4 plasmid DNA in ABS10. Suspect transconjugants were screened for plasmid DNA and found to harbor RP4. This result not only shows conjugation to be a viable gene transfer system for ABS10, but also supports the data indicating that ABS10 is a Gram-negative bacteria. A filter mating between A. globiformis and E. coli failed to yield any exconjugants. An attempt to use a P. putida strain which harbored the TOL plasmid and RP4 as a mobilizing vector failed to yield any TOL plasmid-bearing exconjugants when filter mated with ABS10. Attempts to conjugate TOL directly into ABS10 were also unsuccessful. RP4 could be stably maintained in ABS10 without selective pressure for a period of months.

Earlier, when the taxonomic status of ABS10 was unclear, filter matings between ABS10 and Strep. mutans DS16 were attempted. DS16 contains a conjugative transposon which has been shown to undergo inter-species transfer (i.e., S. faecalis to Staph. aureus) (16). Hindsight shows that this experiment was probably doomed from the start given the pseudomonad characteristics of ABS10.

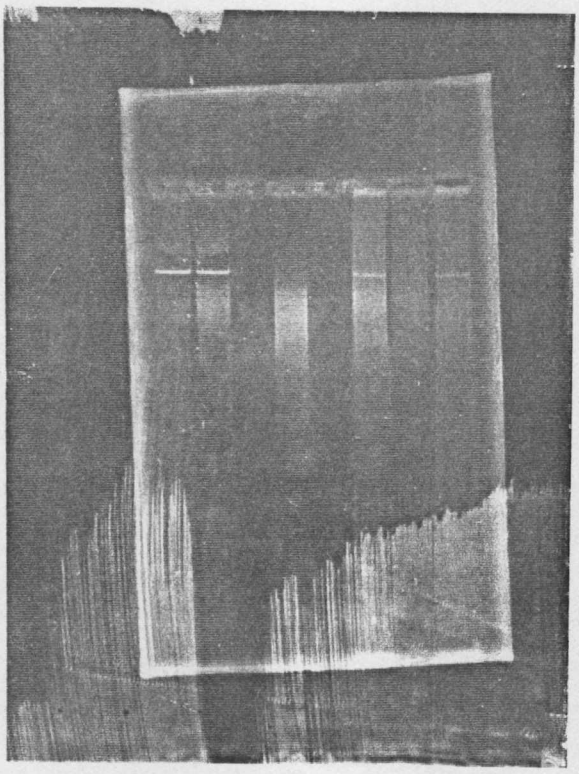


Figure 15. RP4/ABS10 conjugation experiment. Agarose gel. Ln. 1 - suspect exconjugant. Ln. 2 - suspect exconjugant. Ln. 4 - ABS10. Ln. 6 - E. coli donor bearing RP4. Ln. 7 - ABS10. Ln. 8 - same as Ln. 6.

CHAPTER V

DISCUSSION

The isolate ABS10 has been shown to have a number of pseudomonad characteristics. FAME analysis shows ABS10 to share considerable similarity to P. putida, while DNA-DNA hybridization shows great homology with both P. putida and P. aeruginosa. Most conclusively 16S rRNA sequence data shows ABS10 to be in the Gamma division of the purple bacteria, a division that includes P. putida, P. aeruginosa and P. fluorescens. Thus a number of different molecular techniques support the conclusion that ABS10 shares a number of pseudomonad characteristics. ABS10 does, however, have a number of characteristics not usually found in pseudomonads; it appears to have modified KDO, it is not flagellated and it is pleiomorphic. It is important to note that although the FAME analysis of ABS10 shows greatest homology with P. putida the homology value of 0.74 is low. A value of 0.90 or higher would be expected for a typical pseudomonad. The fact that the KDO is modified also suggests that ABS10 is considerably different from characterized members of the genus Pseudomonas.

These factors may well account for the fact that ABS10 proved to be untransformable by both the classical $CaCl_2$ procedure (17) as well as the Pseudomonas transformation procedure of Bagdasarian and Timmis (3). Induction of artificial competence is to a large extent dependent on the effectiveness of the treatment in permeabilizing

the cell membrane to DNA. This is only a hypothesis since one cannot rule out such factors as an efficient restriction system or inability to express the RSF1010 phenotype. The fact that RP4, a plasmid with a broad host range similar to that of RSF1010, is maintained is indicative of the fact that gene expression is probably not the barrier.

The difficulty encountered in attempts to transform ABS10 exemplifies a problem which is chronically encountered in environmental microbiology; many isolates are not amenable to standard laboratory methods of characterization and manipulation. These methods may be as simple as the Gram-stain or as complicated as genetic engineering protocols. Naturally this presents an obstacle when trying to type a strain taxonomically. It is not unusual, when applying traditional taxonomic procedures to environmental isolates, to generate data that is ambiguous or contradictory. This is due to the current chaotic state of bacterial taxonomy which stems from an adherence to a very arbitrary and inappropriate system of classification (89).

This state of confusion reaches its apex in the genus Pseudomonas. When an organism is designated as a "Pseudomonas sp." or a "pseudomonad," it is more often than not an extremely vague designation. Members of the genus Pseudomonas fall into five subgroups which are genetically unrelated (90). Obviously the classification of an organism affects experimental design as well as interpretation of results. The situation in regard to Pseudomonas has caused some

to suggest that nothing should ever be called Pseudomonas. Strangely enough Pseudomonas is one of the more thoroughly accepted bacterial categories. When designating an organism as a pseudomonad, it implies that the organism has a number of exclusively pseudomonad characteristics. The characteristics are often vague and contradictory. As Woese states, "It would seem that the initial choice of the name pseudomonad (i.e., false unit) was an especially wise one" (90).

The molecular data gathered on ABS10 shows that this organism belongs in the Gamma subdivision of the purple bacteria. The rRNA sequence AAACUCAAAUG is diagnostic for this group (89). The purple bacteria comprise the largest division of the eubacteria. This group is divided into three subdivisions called Alpha, Beta and Gamma. The origin of this group is believed to be photosynthetic (89,91). Strains called Pseudomonas are scattered throughout the Alpha, Beta and Gamma divisions of the purple bacteria. By comparing ABS10 sequence data with oligonucleotide signature sequences, ABS10 was found to be a Gamma division member. The data generated by FAME analysis and DNA-DNA hybridization support the rRNA sequence data.

DNA-DNA hybridization data shows ABS10 to share extensive homology with P. putida and P. aeruginosa which are both Gamma division members. FAME analysis also shows ABS10 to share similarity with P. putida. Consequently, three different molecular methodologies all point to ABS10 being a Gamma division member.

ABS10 also features a number of unique characteristics which made its classification difficult. Among these are lack of flagella,

modified KDO and pleiomorphism. All of these characteristics are atypical of the "generic pseudomonad." It is interesting to speculate that these characteristics may be the result of natural selection in the environment.

One could make a strong case for pleiomorphism being an adaptive response to the oligotrophic conditions of the groundwater environment. It is hypothesized that the change in cell morphology from a long rod to a spherical form offers an optimal surface to volume ratio for efficient utilization of nutrients under starvation conditions (15, 22). This fact is believed to account for the dominance of the genus Arthrobacter in soils (15). Incidentally, Arthrobacter is believed to be the dominant organism in the Lula aquifer (4). One may speculate that pleiomorphism is an adaptive strategy enabling ABS10 to survive and compete with organisms such as Arthrobacter in the oligotrophic groundwater environment.

The introduction and maintenance of the plasmid RP4 in ABS10 demonstrates that this strain will be amenable to some degree of genetic engineering. ABS10's carbon source utilization spectrum could conceivably be broadened to include groundwater contaminants thus making ABS10 a tool in biological groundwater reclamation. A number of authors have pointed out the fact that nonindigenous or even strains only briefly maintained in the laboratory have difficulty surviving outside the lab (32,50,74). ABS10 is a strain which is indigenous to the groundwater and able to cope with conditions in that environment. It is a logical strain to experiment with even if it has become somewhat "laboratory acclimated."

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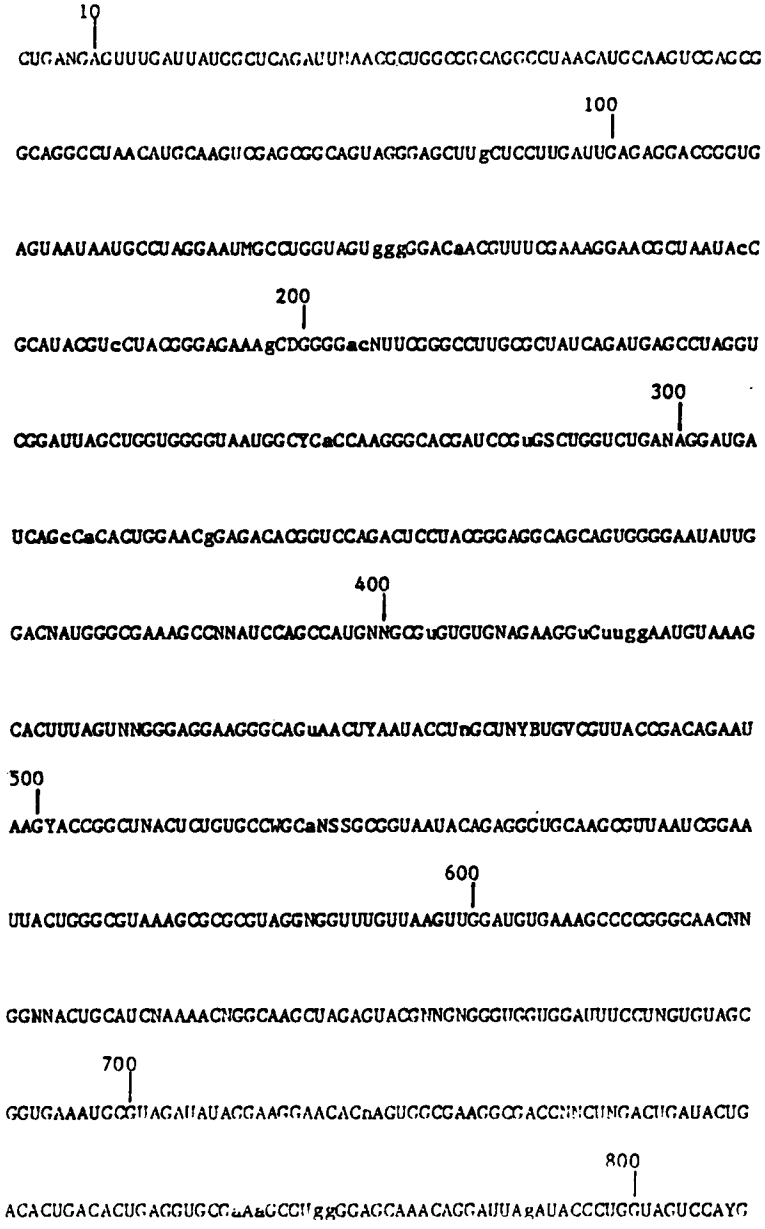
APPENDIX

APPENDIX

16S rRNA SEQUENCE OF ABS10



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1200

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1300

CAANcNNCGAGGUGGRGCUAAUCUCACAAAACCGAUCGUAGUCCGGAUCGCAGUCUGCAACUC

GACUGCGUAAGUCGGAAUCGCUAGUAAUCGCGAAUCAGAAUGUCGCGGUNAA

A = adenine
a = most probably adenine
U = uridine
u = most probably uridine
C = cytosine
c = most probably cytosine
G = guanosine
g = most probably guanosine
N = nucleotide position
n = most probably a nucleotide position
M = A or C
R = A or G
W = A or U
S = C or G
Y = C or U
D = A,G, or U

VITA

Alexander W. Breen was born on August 29, 1959, in Chicago, Illinois. In 1963 he moved to Teaneck, New Jersey. He attended St. Anastasia Grammar School and St. Joseph Regional High School. He enrolled at The University of Tennessee, Knoxville, in 1978 and majored in Microbiology. He received his degree in June 1982. From 1982 until 1984 he worked as a research technician on the "Sludge Project" under the direction of Dr. Gary Sayler. In 1984 he enrolled in the Master's program in the Department of Microbiology and finished in 1987.