Substrate Regulated Microaerophily and Chemotaxis by *Pseudomonas jessenii* strain VT10

by

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Abstract

Low substrate regulated microaerophilic behavior (LSRMB), as measured by changes in microaerophilic band formation in semi-solid medium, was observed in several aerobic bacteria isolated from subsurface soils, Antarctic dry valley soils, an eutrophic pond, a mesophilic pond, an oligotrophic lake and activated sludge. Similar behavior was also exhibited by five Pseudomonas and two Bacillus type strains from culture collection. Isolates identified with LSRMB formed a typical band of growth below the surface of low substrate (10 mg l^{-1} of peptone, tryptone, yeast extract and glucose) semi-solid medium. Surface growth was obtained when the substrate concentration was increased (1000 mg l^{-1} of each of the above mentioned substrates). LSRMB was observed in phylogenetically disparate groups, with all the Pseudomonas and two Bacillus species testing positive for the trait. One of the Gram-negative isolates, strain VT10, was identified by phylogenetic analysis based on its 16S rDNA sequence. High 16S rDNA sequence similarity (99%) was observed with the recently discovered Pseudomonas jessenii (CIP 105274^{T}) type strain. Strain VT10 was used as a model to examine this LSRMB, and show the relationship between oxygen stress and low-substrate growth media. The concentration of 17:0

cyclopropane fatty acid, a common stress indicator, increased 5-fold, and four additional proteins were produced when P. jessenii strain VT10 was grown at low-substrate levels and when the dissolved oxygen concentration was increased from 26 μM to 241 μM. The stress responses by P. jessenii could be due its LSRMB. This study shows that low-substrate regulated microaerophilic behavior helps some microorganisms to track the oxygen minima in their habitat and thus effectively move to an environment, which allows them to thrive. In addition to the above mentioned taxis in response to oxygen concentration, organisms may use chemotaxis to a chemical compound. Quantification of chemotaxis can be extremely difficult. To quantify chemotaxis in an easier fashion, a simplified capillary chemotaxis assay, utilizing a hypodermic needle, syringe and disposable pipette tip was developed. The method was applied to two strains of subsurface microaerophilic bacteria. Strain VT10 was chemotactically attracted toward dextrose, glycerol, and phenol, which could be used as sole carbon sources, and toward maltose, which could not be used. The deep subsurface isolate MR100 (phylogenetically related to P. mendocina) showed no tactic response to these compounds although it could use dextrose, maltose, and glycerol as carbon sources. The chemotaxis results obtained by the new method were verified by using the swarm plate assay technique. The simplified technique may be useful for routine chemotactic testing.

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Introduction

Bacterial motility in subsurface environments is poorly understood though bacterial movement in response to oxygen and substrate concentrations is an important factor in bioremediation. Knowledge about bacterial motility and survival would be useful to evaluate success of *in situ* bioremediation. This study was initiated to increase the body of knowledge on physiological responses by subsurface bacteria in terms of bacterial movement and chemotaxis, to different concentrations of oxygen and substrate. The study was also expected to advance and develop methods, which could be used by other investigators in performing microbiological analysis of the subsurface.

Motility and chemotaxis are mechanisms that bacteria use to respond to stress [Amsler 1993]. Bacterial motility-chemotaxis mechanism is a part of global stress response network, and Amsler et al. suggested that there is a global regulation of motility, chemotaxis, and other stress responses [Amsler 1993]. Evidence in favor of the hypothesis is that the expression of *flh*D operon, the top level flagellar operon whose expression is required for other genes in the flagellar regulon to be activated, is dependent on the presence of heat shock response network proteins [Shi 1992].

In our laboratory we have been studying bacterial survival, motility, and physiological responses to ionizing radiation, hydrogen peroxide, oxygen, and substrates. Based on our studies of surface and subsurface microorganisms, we have found that certain aerobic isolates actively seek microaerophilic environments when they are grown in low

substrate concentration. Isolates exhibiting this low substrate regulated microaerophilic behavior (LSRMB) were found in all the sites that we have surveyed so far, and appear to be a common ecological strategy practiced by many aerobes, to escape from oxygen stress.

I believe that LSRMB is a strategy devised by some bacteria to survive in varying concentration of oxygen and substrates through motility and stress responses. My hypotheis is: organisms that exhibit low substrate regulated microaerophilic behavior are able to move toward a preferred microaerophilic niche in low substrate environment due to their tactic response, alternatively they produce stress molecules to survive in high oxygen-low substrate environment. To test the hypothesis a subsurface strain was chosen and its aerotactic and chemotactic response was evaluated. Furthermore, the stress response of this strain to high oxygen-low substrate was investigated by analyzing its protein and lipid profile.

Literature review

Subsurface microbiology

Microbial life in the deep subsurface was first reported early in the 20th century [Krumholz 1998]. Considering the fact that 20^{0}C is the surface temperature and 100^{0}C being the upper limit of microbial existence, and, there is an increase of 25^{0}C for every kilometer of depth, there could be microorganisms as deep as 3.5 km below the surface of the earth [Krumholz 1998]. Before 1985 extensive microbiological investigations were confined to the upper few meters of the crust. The microbiology of groundwater and subsurface gained importance when it was realized that- a) there was severe groundwater contamination in many places resulting in the loss of potable water; b) clean up may require on site biological remediation; c) therefore, knowledge of ecology, physiological potential, carbon and electron flow, growth, and nutrient prerequisites were required to satisfactorily remediate the contaminated sites.

Ground water accounts for approximately 66% of the fresh water resources of the world and supplies more than 90% of the drinking water of rural populations in the United States [Hicks 1988]. As mentioned above, groundwater is frequently contaminated with compounds from various agricultural and industrial programs. The deteriorating condition of groundwater in the US led the US Department of Energy’s Office of Health and Environmental Research in 1983 to initiate plans for long term, geomicrobiological study of the deep subsurface [Ghiorse 1983]. The research

program was initiated to study the biogeochemical processes governing the subsurface environment. By 1995 there was considerable amount of evidence showing the presence of subsurface microorganisms, and how they influenced the chemistry of the subsurface environments [Benoit 1990, Fredrickson 1988, Fliermans 1989, Phelps 1989, White 1983].

Natural environments like soil and water systems are characterized by their oligotrophic nature. [Williams 1985]. Though it is possible that nutrients and electron acceptors could be limiting factors for microbial existence in the subsurface, there is enough evidence that many microbes can survive in oligotrophic environment with trace amounts of electron acceptors [Balkwill 1985, 1989]. It has been hypothesized that evolutionary process in the deep subsurface favored the survival of organisms, which had an efficient aerobic metabolism in an oligotrophic environment and conservatively managed in a scarce supply of oxygen [Benoit 1990]. Hicks and Fredrickson calculated the rate of mineralization in subsurface sediment samples by the amount $^{14}CO_2$ released from radioabeled acetate, phenol, or 4-methoxybenzoate added to subsurface sediments. They noted that aerobic metabolic potential did not decrease with depth in the Savannah River Plant site studied by them. They also reported a significant lateral continuity with respect to mineralization in the deep subsurface [Hicks 1988]. Fredrickson et al. reported that lithotrophs and heterotrophs were abundant in the subsurface, and interestingly microaerophilic nitrogen fixing bacteria were present in approximately 50% of the subsurface samples from Savannah River Plant site [Fredrickson 1988, 1996]. The tests performed for nitrogen fixing bacteria by Fredrickson et al. were only presumptive tests and it is possible that

these microaerophiles could grow at extremely low concentrations of nitrogen present as impurities in the medium or by utilizing trace amount of ammonia in the atmosphere. Therefore, it is possible that they were not true nitrogen fixing microaerophiles.

Microorganisms are present in a variety of subsurface environments using a variety of mechanisms to support themselves and grow. Microorganisms have been isolated from deep geohydrologically isolated habitats, which implies that these organisms have persisted in situ for millennia [Brockman 1998, Fredrickson 1988, 1996, Halderman 1993, Keift 1995]. Many subsurface microbes have developed strategies to survive in harsh environments encountered in the subsurface [Krumholz 1998]. This survival strategy allows these microorganism to sustain themselves till they encounter conditions favorable for growth and prolifiration. There have been some conflicting reports of the significance of these survival responses for some soil bacteria. For example, van Overbeek et al. showed that adaptation of Pseudomonas fluorescens to oligotrophic conditions in soil by carbon starvation did not affect their survival capabilities [van Overbeek 1995]. Nevertheless, there is ample evidence of metabolic and physiological modification by subsurface bacteria in response to harsh conditions. Fredrickson and Onsott suggested in their article that it is highly possible that the metabolic rate of starved bacteria is much lower than when they have an abundance of nutrients [Fredrickson 1996]. Therefore, they may have extremely low frequency of cell division, such as once in a 100 years or even less [Fredrickson 1996]. One of the fascinating theories which has its basis in starvation and adaptation of organisms to

extremely low levels of nutrients for millions of years is the theory of the origin of eukaryotes. There appears to be enough evidence to claim that prolonged nutrient deprivation 1 billion years ago forced prokaryotes to cooperate and form complex eukaryotic organisms [Holden 1998].

Several researchers have shown, that subsurface microbial communities impact ground water quality [Lovley 1990, Stevens 1995, Tobin 1999]. The microorganisms isolated by Balkwill from the deep subsurface sediments of South East coastal plains were capable of utilizing a variety of carbon sources [Balkwill 1989]. Similar conclusions were reached by Balkwill and Ghiorse regarding bacterial isolates from shallow subsurface aquifer located in Lula, Oklahoma [Balkwill 1985]. Swindoll et al. showed that addition of multiple organic nutrients resulted in enhancement of metabolic activity and degradation than the addition of single substrates [Swindoll 1988]. Furthermore, they also showed that addition of alternate carbon sources such as amino acids or dextrose inhibited the mineralization of xenobiotics [Swindoll 1988]. Chapatwala et al. in 1996 reported that the samples from a shallow subsurface site near Oyster Virginia rapidly increased CO_2 production when yeast extract was added as a micronutrient [Chapatwalla 1996]. Novak et al. 1985 showed that methanol is degraded by subsurface microorganisms much more easily than tertiary butyl alcohol (TBA). The presence of TBA or TBA and benzene, toluene and xylene had no effect on methanol degradation [Novak 1985]. In addition to the above mentioned cases, there are several other examples of microorganims that can be used for cleaning of contaminated subsurface sites as reviewed by Holligner [Hollinger 1997].

Jiminez had earlier pointed out that that deep-subsurface bacteria could be an ideal choice for environmental detoxification owing to their metabolic and genetic capabilities and may offer new strategies for in situ bioremediation of deep aquifers and unsaturated vadose zone sediments [Jiminez 1990].

Role of subsurface microorganisms

Subsurface ecology has been detailed earlier [Ghiorse 1988, Sargent 1989]. An understanding of the ecology of the subsurface is essential to adequately remediate a contaminated subsurface site. It is also essential before introducing any microorganism to destroy compounds that are a threat to the ecosystem. This is because abiotic stresses in the natural environment are different from those in the laboratory. Introduced species may face intense competition, predation, or parasitism. The failure of the inoculation to enhance biodegradation may be due to the following reasons: a) concentration of compound too low to support growth and/or activity; b) inhibitors of growth and/or activity present; c) predation by grazers; d) other organic substrates preferred; and e) failure to move through pores of soil to sites of the organic compound [Goldstein 1985].

The physical and chemical nature of the habitat will usually determine the density and diversity of the microorganisms present. Aerobic, anaerobic and microaerophilic organisms are found in the ground water and subsurface soils [Benoit 1990, Mazumder 1998, Mazumder 1999 a, b, Zhang 1997]. As many subsurface soil environments are often contaminated with potentially toxic wastes (Annual

Progress report 1990, Oak Ridge National Laboratory), biodegradation of these wastes is possible by these aerobic, anaerobic and microaerophilic organisms. The role of microorganisms in subsurface degradation and how they fit into the community structure and their nutritional status in relation to other organisms can help us formulating strategies to combat subsurface contaminants. The microbial ecology of shallow subsurface has revealed that the majority of the microflora is prokaryotic. It consists of microorganisms that are specially adapted for survival in the subsurface, and many may be physiologically inactive for extended periods of time. A significant number of shallow subsurface isolates have a metabolic diversity, which allows them to use a wide range of nutrients and nutrient concentrations [Balkwill 1985, Fliermans 1989]. Similar to the shallow subsurface isolates, most the deep subsurface isolates were prokaryotic [Balkwill 1989]. Some of the characteristics of deep subsurface sediments were unexpected, such as the generally oxidative redox state of some habitats and the microbial diversity and biomass of some aquifers being closer to that of typical surface soils and abiotic sediments [Balkwill 1989]. Arrage et al. showed that the similarity between surface and subsurface bacteria extended to similarities in resistance levels for ionizing radiation. Subsurface bacteria studied were as resistant as surface bacteria [Arrage 1993 a, b]. Blakwill’s study of deep subsurface bacteria from the site in Savannah River Plant in South Carolina revealed that the deep subsurface microorganisms he was studying appeared to be more metabolically active *in situ* than those from the shallow aquifers [Balkwill 1989]. He suggested that if the microorganisms are comparatively active in the deep subsurface then they could play a significant role in

influencing groundwater quality [Balkwill 1989]. Kieft at al. also reported that differences of survival rates between surface and subsurface strains were not significant [Kieft 1997]. They also showed that the phospholipid fatty acid profiles of P. fluorescens returned to preincubation levels after 16 to 32 weeks. This could mean that a long term adjustment or acclimitation to the oligotrophic condition is possible by these microorganisms. Therefore the membrane lipids of bacteria undergoing long-term survival may not differ from those apparently non-stressed cells [Kieft 1997].

Microbial variability in the subsurface

Phelps et al. had reported that the abundance, metabolic activity and transport of subsurface microorganisms are frequently controlled by the physical properties of the subsurface [Phelps 1989]. If there is extensive physical variability within a sediment or formation, this variability is reflected in the microbial abundance and activity. Phelps at al. noted that regardless of depth in the Savannah River Plant site, sediments that contained more than 20% clay exhibited the lowest numbers of microorganisms that could be cultured [Phelps 1989]. Zhang et al. while examining two coastal plain sites in Virginia found that changes in grain size and depth had a significant effect in bacterial abundance and activity at both the sites [Zhang 1998]. Variability in microbial activity and abundance has been observed in scales of centimeters by many researchers [Harvey 1993, Zhang 1998]. Beloin et al. reported that microbial abundance and activity in subsurface sediments of an aquifer in Oklahoma

could vary by a factor of 300 at an interval of 10 cm apart. They also found that the interval containing the maximum amount of clay had the lowest biomass and activity [Beloin 1988]. Tobin et al. studied the interactions of subsurface microorganisms with geological substrates without disrupting the rock texture or the relationship of the microorganisms to the substrate [Tobin 1999]. They used propidium iodide and nucleic acid stains that specifically target double stranded DNA and RNA to visualize cells in surface and subsurface basalts from northeastern Idaho. In addition to the above method they also examined samples incubated with acetic acid-UL-14C via phosphor imagining. They reported that microbial distribution in the rocks exhibited a high degree of variability at the sub-centimeter level [Tobin 1999]. Therefore, to successfully study a subsurface environment and understand its physical, chemical and microbiological characteristics, it is of utmost importance that sampling sites and scales are selected based on careful consideration of variability that may be encountered. In some instances effective sampling would require the capability of sampling microniches to characterize the physical, chemical and microbial variability [Haldeman 1993]. As tens of kilograms of soil samples may be necessary to obtain a representative soils mass for study [Parkin 1987], geophysical techniques such as ground penetration radar could be used to find out the physical parameters and then microbiological characterization could be planned based on that information [Zhang 1998]. In the event such techniques cannot be used, it is necessary to understand the limitations and processing of the sample and reporting of the results should be performed accordingly.

Oxygen toxicity

Reactive oxygen intermidiates

Organisms that use oxygen and therefore are exposed to it and its various reduction products must devise ways to protect themselves from its effects. The free radical theory of oxygen toxicity was first put forward in 1954 by Gerschman et al. [Gerschman 1954]. The toxic species also known as reactive oxygen intermediates (ROI) are formed by one-electron reductions of oxygen. These reduction products include superoxide, hydrogen peroxide and the hydroxyl radical as shown below [Salin 1993].

$O_2 + 1e^- \rightarrow O_2\cdot^-$

$O_2 + 2e^- + 2H \rightarrow H_2O_2$

$O_2 + 3e^- + 3H \rightarrow OH\cdot + H_2O$

Reactive oxygen species can damage DNA, proteins, and lipid membranes [Zheng 1999]. Another toxic form of oxygen is the singlet oxygen, which is formed when the spin restriction of the biradical oxygen is removed and the molecule is excited to the singlet oxygen state [Fridovich 1970, Krinsky 1979].

Superoxide: Superoxide is one of the ROIs, which has been implicated in the deleterious effects of oxygen. For example, superoxide generated by xanthine oxidase/purine system can cause accelerated killing of Staphylococcus epidermidis [Babior 1975]. Generation of superoxide *in vitro* has been observed in several systems such as when xanthine oxidase reacts with xanthine or hypoxanthine [Knowles 1969]. Superoxide is also produced by redox-cycling compounds [Hassan 1978]. The reduction of cytochrome c by xanthine and xanthine oxidase occurs in the

presence of oxygen [McCord 1968]. The superoxide and the perhydroxy radical have also been shown to oxidize fatty acids [Bielski 1983]. A major harm that superoxide radicals can cause is DNA strand breaks [Brawn 1981, Lesko 1980]. Some of the harm that appears to be caused by superoxide radicals may be caused by hydrogen peroxide, which is produced by the dismutaion of superoxide. This dismutation generates molecular oxygen and hydrogen peroxide.

Hydrogen peroxide: The enzyme superoxide dismutase, catalyzes the dismutation of superoxide. It has been shown by Steiner et al. that DNA breakage in microaerophile Treponema palladium occurs when exposed to low concentrations of hydrogen peroxide [Steiner 1984]. The lethal effects of hydrogen peroxide on bacteria have been reported by many researchers earlier [Imilay 1988, McCormick 1976]. It has been shown by Keller et al. that low concentrations of H_2O_2 induce resistance of procaryotic cells to degradation of DNA by a future challenge with H_2O_2 [Keller 1977].

Hydroxyl radical: Hydrogen peroxide is not the only reactive species of oxygen formed due to the presence of superoxide radicals. Haber and Weiss detailed in their 1934 paper the production of hydroxyl radical, when hydrogen peroxide reacted with superoxide radical [Haber 1934]. The Haber-Weiss reaction is very slow though chelated iron can increase this reaction rate significantly [Green 1984]. The reduction of paraquat has been shown by gas chromatography to result in the production of hydroxyl radical [Richmond 1982]. Hydroxyl radicals are extremely reactive and they degrade DNA [Keller 1977], and kill bacteria [Rosen 1981].

Singlet oxygen: It has been shown that singlet oxygen can break both plasmid and viral DNA [DiMascio 1989]. Sies

and Menck showed that single and double stranded lesions were produced by singlet oxygen exposure [Sies 1992]. Dahl et al. showed that overproduction of histidine protected Salmonella typhimurium from singlet oxygen [Dahl 1987]. Kellogg and Fridovich showed that lipid peroxidation was inhibited by catalase, superoxide dismutase and scavengers of singlet oxygen, but not of hydroxyl radicals [Kellogg 1977].

In summary, oxygen is poisonous to all organisms. Molecular oxygen is not very reactive however, partially reduced forms of oxygen are very reactive. All these forms of oxygen behave as different levels of oxidants or reductants to a wide variety of compounds *in vivo* and *in vitro*. The partial reduction products of oxygen have been described by Green et al.in detail earlier [Green 1984].

Defenses against reactive oxygen intermidiates

Bacterial cells maintain a variety of defenses against ROIs. Defense mechanisms include a number of enzymes that include superoxide dismutase and catalase, peroxidase, alkylhydroxyperoxidase reductase, glutathione, glucose-6-phosphate dehydrogenase, endonucleases, exonucleases, DNA polymerases, and carotenoids [Alban 1998, Krieg 1986, Krinskey 1979, Zirkle 1996]. Other forms of defense mechanisms include use of host’s protective enzymes, increased respiration rate, thick cell walls, slime production, increased cell numbers, microaerophilic behavior, etc. [Christman 1985, Mazumder 1999 a].

Almost all organisms that can lead an aerobic existence have a complex oxidative stress response system involving several genes acting in concert [Zheng 1999].

Christman et al. discovered a regulatory system the OxyR, which appeared to regulate the group of enzymes that were induced due to oxidative stress [Christman 1985, Storz 1994]. The inducible defense systems that are present against oxidative damage have been best characterized for Escherichia coli and have been reviewed recently by Storz and Imlay [Storz 1999].

Hydrogen peroxide activates the transcription factor OxyR. Activated OxyR induces the transcription of a set of antioxidant genes such as *kat*G (hydrperoxidase I), *ahp*CF (alkylhydroperoxidase), *dps* (nonspecific DNA binding protein), *gor*A (glutathione reductase), *grx*A (glutaredoxin I), and *oxy*S (regulatory RNA). In the case of superoxide-generating compounds, transcription factor SoxR is activated, which then induces SoxS, which in turn activates the transcription of *sod*A (manganese superoxide dismutase), *fpr* (ferrodoxin/flavodoxin $NADP^+$ reductase), *zwf* (glucose 6-phosphate dehydrogenase), *fum*C (fumarase C), *nfo* (endonuclease IV), *acn*A (aconitase A), and micF (regulatory RNA) [Zheng 1999].

There is a significant relationship between iron and oxidative stress. Iron is required as a cofactor for many key metabolic enzymes. Alternatively iron reacts with hydrogen peroxide to form extremely reactive hydroxyl radical via the Fenton reaction. Superoxide accelerates this reaction by elevating the intracellular concentration of iron by releasing iron from iron-sulfur proteins. Cells have evolved regulatory systems to deal with this iron toxicity. In prokaryotes transcription factor Fur (ferric uptake regulation) regulates genes involved in ferric iron uptake to meet the physiological needs of the cell, and avoid iron toxicity [Braun 1997, 1998]. Touati et al.

showed that Δ*fur* mutations are sensitive to hydrogen peroxide and showed increased DNA damage under aerobic conditions [Touati 1995]. Zheng at al. hypothesized that given the wide range of oxidative damage to the cell, it is possible that many cellular activities such as DNA repair, membrane alteration, cell division are all coordinately regulated and oxidative stress response is a concerted effort in the form of a stress response network [Zheng 1999].

Microaerophily and oxygen toxicity: Microaerophilic behavior is a strategy followed by many microorganisms to escape the damaging effects of reactive oxygen species. Many bacteria are permanent or transitional residents of microaerophilic environment [Benoit 1990, Sansone 1978, Kukor 1996]. Isolation of microaerophilic iron oxidizing bacteria by Emerson et al. that grow at the oxic-anoxic interphase [Emerson 1997], suggests that these organisms may have evolved the microaerophilic strategy to combat iron toxicity, a problem faced by all organisms that have developed an aerobic existence. Microaerophily and oxygen toxicity has been reviewed in great detail by Krieg and Hoffman [Krieg 1986]. Krieg and Hoffman noted that the level of oxygen preferred by a microaerophilic bacterium varies with species and strain. Additionally, we have shown that oxygen preference is also dependent upon availability of substrates [Mazumder 1999 a]. Many microaerophiles are pathogens, some are nitrogen fixers while others are iron oxidizers. The microaerophiles can be chemorganotrophs, such as Spirillum volutans and Aquaspirillum magnetotacticum, or chemolithotrophic as Gallionella ferruginea and Beggiatoa. Other forms of microaerophily

have been adopted by organisms, which prefer microaerophilic conditions to enable them to utilize substrates in an efficient manner. This may give them an ecological advantage. Kukor et al. studied the degradation of toluene by Pseudomonas strains isolated from oxygen limited environments. They found that these toluene-degrading strains compensated for a microaerophilic environment by the development of an oxygen-requiring enzyme with kinetic parameters favorable to function in low oxygen environments and also increasing the synthesis of the enzyme in response to oxygen [Kukor 1996]. Sansone and Martens [Sansone 1978] reported a similar form of microaerophily, where methane oxidizers performed better in low oxygen environment. These forms of facultative microaerophilic behavior has not been studied in great detail previously and is of special interest to researchers who are interested in understanding the ecology and physiology of the subsurface environment.

It has been suggested by some researchers that microaerophiles lack some key protective enzymes against ROIs [Krieg 1986, Padgett 1982]. Very little is known about the type of DNA repair employed by microaerophiles. Steiner et al. compared the DNA repair of E. coli and microaerophilic Treponema pallidum and found that T. palladum was more sensitive to DNA damage by hydrogen peroxide than E. coli [Steiner 1984]. Brawn and Fridovich showed that supplements to the media such as mannitol and histidine, which destroys hydroxyl radicals, enhanced the aerotolerance of microaerophiles [Brawn 1981].

Chemotaxis

Chemotaxis enables bacteria to search for nutrients and transport themselves effectively to grow and survive. Chemotaxis is a high energy requiring process. One turn of the flagella requires approximately 1000 protons when it is rotating at an average speed of 15,000 rpm [Wei 1998]. The bacterial cell also invests in a genetic load of more than 50 genes to have the chemotaxis machinery [Wei 1998]. Therefore, it is not surprising that for survival and competitive success in aquatic and soil environments bacteria require sophisticated and probably sub-population level regulation of motility and chemotaxis in response to fluctuating nutrients. Terraciano and Canale-Parola reported that carbon limited Spirochaeta were 10 to 1000 fold more chemotactic to specific sugars used to support growth [Terracciano 1984], while another marine vibrio lost motility after 24 h of starvation [Malmcrona-Friberg 1990]. Macnab and Koshland derived the run and tumble chemotaxis model for enteric bacteria, where the cells have longer runs and they tumble less when they are moving up an attractant gradient [Macnab 1972].

Reasons for chemotaxis

Motility and chemotaxis are mechanisms that bacteria use in response to stress [Amsler 1993]. An ability to move away from stressful environment into microniches more favorable for growth and proliferation is undoubtedly of great adaptive value to microorganisms in most habitats [Amsler 1993]. The motility-chemotaxis mechanism is a part of several global stress response networks as pointed out

by Amsler et al. [Amsler 1993]. The expression of all flagellar genes is dependent on the presence of cyclic AMP-catabolite gene activator protein. This is the regulatory element of the catabolite repression system which is also involved in regulating proteins involved in a variety of functions such as responses to starvation, cell division, and other functions [Botsford 1992]. The expression of the *flh*D operon and therefore all the flagella genes is also dependent on the presence of heat shock proteins DnaK, DnaJ, and GrpE [Shi 1992]. In addition, the chemotaxis proteins are members of the two component regulatory system, which includes systems for nitrogen and phosphorous uptake, oxygen regulation, just to name a few [Amsler 1993]. These systems are very similar and it has been shown that cross talk from one system to the other can occur [Parkinson 1993]. All this proves the global regulation of motility, chemotaxis and other stress responses [Amsler 1993].

Bacterial taxis is believed to enhance survival and growth of bacteria by allowing them to move to favorable environments by responding to favorable and unfavorable chemotactic stimuli [Macnab 1972, Greck 1995]. Taylor et al. described energy taxis which encompass aerotaxis, phototaxis, redox taxis, taxis to alternative electron acceptors, and chemotaxis to carbon source [Taylor 1999]. Bacterial taxis have a significant ecological role in vertical stratification of microorganims in microbial mats and water columns [Taylor 1999]. It has been shown that chemotaxis and bacterial mobility influence transmission of some waterborne disease [Bitton 1992], biorestoration of contaminated aquifers [Wilson 1986], microbially enhanced oil recovery [MacLeod 1988], and in the movement of

genetically engineered microorganisms [Trevors 1990]. In addition to all of the above, another interesting outcome of bacterial motility and chemotaxis is pathogenicity [Panopoulos 1974]. For example, ecological adaptation of phytopathogenic bacteria to the host plant involves flagellar motility. Panopoulas and Schroth showed that motile strains were much more invasive than their non-motile counter parts [Panopoulas 1974]. Studies with Borrelia burgdorferi indicate that motility may be an important factor for pathogenesis for the organism [Sadziene 1996].

Origin of chemotaxis

Since bacterial and archaeal flagella are not homologous, it is difficult to understand and predict the origin and evolution of motility and chemotaxis. Though the flagella of bacteria and archaebacteria are very distinct, the chemotaxis system is very similar [Faguy 1999]. A number of observations based on analysis of bacterial and archaeal genomes by Faguy and Jarrell revealed that the entire chemotaxis system between Gram-positive bacteria and archaea could have been horizontally transferred [Faguy 1999]. Flagellated organisms are proposed to have a controlling chemosensory system. In bacteria, chemotaxis is a two component regulatry system. The chemotaxis signals flows through a sensory transducer or methylable chemotaxis protein (MCP), a histidine kinase transmitter CheA and a response regulator CheY to the switch of the flagellar motor FliM. In addition to the above components there are two other proteins, CheB and CheR, which are responsible for methylation and demethylation of the sensor which

adapts the signal to the chemoattractant or chemorepellant. This allows the cell to respond to chemical gradiants. Complete genome sequencing projects have revealed that homologues of all the above mentioned chemotaxis genes (except *fli*M) have been found in archaebacteria Pyrococcus horikoshii, and Archaeoglobus fulgidus. Furthermore, two other chemotaxis genes *che*C and *che*D have been found in the above two archaebacteria. The gene *che*C has previously been reported only in Bacillus subtilis and *che*D has been found in B. subtilis, Sinorhizobium meliloti and Agrobacterium tumefaciens [Faguy 1999, Greek 1995, Rosario 1995]. Another interesting fact is the absence of homologues of most of the chemotaxis genes in thermophilic chemoautotrophic bacterium Aquifex aeolicus for which the complete genome sequence is available. A. aeolicus has a typical bacterial flagella and motor proteins [Faguy 1999]. It has been hypothesized by Faguy and Jarrell that such organisms may regain the required chemotaxis genes through horizontal transfer when required [Faguy 1999].

Chemosensing in pseudomonads is not well known but it is known that like Escherichia coli they also utilize methyl-accepting chemotaxis proteins as transducers [Craven 1983, Manson 1998].

Quantification of chemotaxis

Chemotactic responses are often quantified by the capillary tube method of Adler [Adler 1973], which involves the preparation of a small U-shaped glass tube over which a cover slip is placed to form a chamber. The chamber is filled with the bacterial suspension and a capillary tube containing the potential attractant is then inserted into

the ‘pond’ of bacterial suspension. After incubation for a suitable amount of time, the capillary is removed, the top broken, and the capillary contents transferred into liquid media for dilution and/or plating. Palleroni modified Adler’s chamber by using a Lucite plate in which the chambers were excavated [Palleroni 1976]. The glass capillaries containing the test substrate were then introduced in these chambers to perform the chemotaxis assay. The Adler and the Palleroni method was initially tried in our laboratory and was found to be difficult to use and required considerable amount of practice. Similar difficulties have been encountered by other researchers, as noted by Han and Cooney [Han 1993].

While studying microbial populations of subsurface environments, our laboratory isolated two bacterial strains, which were microaerophilic, based on the formation of a band of growth below the surface of semi-solid media [Krieg 1986]. The strains grew aerobically on the surface of solid media containing a high nutrient concentration but exhibited spreading motility beneath the surface when a low substrate concentration was provided, which is characteristic of facultative microaerophiles [Benoit 1990, Mazumder 1999 a, b]. These results suggested that substrate concentration might play a role in determining the chemotactic response of these isolates. We measured the chemotaxis responses of the two isolates. We found that the isolate from the deep subsurface was not chemotactic to any of the test substrates, and the shallow subsurface isolate was chemotactic to several of the test substrates at rates comparable to enteric and terrestrial surface bacterial isolates [Mazumder 1999 b]

Chemotactic responses of subsurface bacteria have not been studied quantitatively except by Hazen and our laboratory as mentioned above [Hazen 1990, Mazumder 1999 b]. Hazen found a positive chemotactic response by several deep subsurface bacteria to various sugars and amino acids. Hazen and Lopez-De-Victoria reported a fatal chemotactic attraction of these bacteria to trichloroethylene at concentrations that were toxic to the bacterium [Hazen 1994]. The paucity of information on subsurface bacterial chemotaxis potential could impede the implementation of biorestoration strategies.

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Chapter 1: Low-substrate Regulated Microaerophilic Behavior as a Stress Response of Aquatic and Soil Bacteria

Abstract:

Low-substrate regulated microaerophilic behavior (LSRMB) was observed in 10–54% of the bacteria isolated from several fresh-water lakes or ponds, subsurface soils, activated sludge, and Antarctic dry valley soils. Five Pseudomonas and two Bacillus type species showed LSRMB. A subsurface Pseudomonas jessenii strain was used as a model to show the metabolic interaction between substrate and oxygen concentrations, cell band movement, and the appearance of unique stress lipids and proteins. When the oxygen concentration in the P. jessenii culture medium was increased from 26 μM to 241 μM, the concentration of 17:0 cyclopropane fatty acid, a stress indicator, increased 5-fold, and four unique proteins were also detected. This stress response occurred only in low substrate media. LSRMB is a trait exhibited by many aquatic and soil bacteria.

Introduction

Aerobic-anaerobic interfaces are ubiquitous features in many natural ecosystems such as microbial mats and biofilms, lake chemoclines, microbial flocs, and sediments. Some microaerophiles can gain a survival advantage in that low oxygen niche, or they can survive in zones of higher oxygen concentrations by utilizing oxidative stress mechanisms [Benoit 1989]. The well-documented classical microaerophily model which includes the Campylobacter model, illustrates a selective ability to grow only in habitats containing 0.2 to 12.0% oxygen [Bowdre 1976, Krieg 1986]. It was our hypothesis that aquatic and soil microaerophiles could fit two models: the classic microaerophilic model, or an alternate model that features typical aerobic behavior in high substrate habitats and low-substrate regulated microaerophilic behavior (LSRMB) at low substrate concentrations (10mg l^{-1}). Preliminary evidence using semi-solid media provided evidence for the latter hypothesis. Bacteria that showed an ability to form microaerophilic growth in semi-solid media (Fig 1) also showed the production of cyclopropane fatty acids (CFA), and stress proteins when cultured in low substrate liquid media saturated with oxygen present in atmospheric air.

Bacteria are known to form CFA in response to high oxygen tensions, low pH or starvation. [Chang 1999, Grogan 1997, Guckert 1986, Kieft 1997]. Jacques suggested that CFA accumulation is correlated with the reduction state of respiratory components [Jacques 1981]. He showed that Pseudomonas denitrificans produced excess CFAs when grown in an aerated, low substrate medium. CFAs are less reactive to oxidation by singlet oxygen, H_2O_2, or hyperbaric oxygen compared to their unsaturated lipid precursor [Grogan

1997]. In addition to CFAs, stress proteins may be produced under adverse oxygen conditions [Blom 1992]. In this study, we screened for the presence of LSRMB in several local lakes and ponds, subsurface samples, and selected pure cultures in the Virginia Tech Biology Department stock culture collection. A subsurface Pseudomonas jessenii strain was used as a model to demonstrate LSRMB and the production of CFAs as well as stress proteins under various oxygen concentrations.

Material and methods

Culture screen. Aquatic grab samples were collected from: the surface of the eutrophic duck pond on the Virginia Tech campus; surface of pristine oligotrophic Mountain Lake in Giles County, VA; surface of the mesotrophic Pandapas Pond located in Jefferson National Forest, VA. Subsurface soils were taken from formations within the shallow Oyster, VA and deepest Savannah River SC sites [Mazumder 1999]. Surface soils from the Antarctic Victorialand Dry Valleys were collected by Benoit. All samples were serially diluted and plated on 100× PTYG agar medium [Mazumder 1999] and incubated at 24^0C except for Antarctic soils that were incubated at 0^0C [Arrage 1993 a, b, Benoit 1989, Mazumder 1999]. Isolates were inoculated into 15-ml screw cap culture tubes containing 10 ml of 1× PTGY semi-solid medium and incubated at 24^0C. LSRMB was defined as formation of band of growth at least 3 mm below the surface of the 1× PTYG semi-solid medium during a 5-day incubation period.

The pure cultures that demonstrated LSRMB were: Pseudomonas aeruginosa (ATCC 15692), P. fluorescens (ATCC 13525), P. putida (ATCC 17514), P. stutzeri (ATCC 11607),

Bacillus cereus (ATCC 14579), B. sphaericus (ATCC 23857), and P. jessenii (VT10). The pure cultures that did not show LSRMB were Acinetobacter calcoaceticus (VT strain), B. licheniformis (ATCC 10716), B. subtilis (ATCC 6051), Enterobacter aerogenes (ATCC 13048), Escherichia coli (ATCC 9637), Klebsiella pneumoniae (ATCC 13883), Lactobacillus plantarum (ATCC 14917), Micrococcus luteus (ATCC 10204), Proteus mirabilis (ATCC 14153), Salmonella enteritidis (ATCC 13076), Streptococcus lactis (ATCC 11454) and S. pyogenes (ATCC 12344). The Pseudomonas jessenii (VT 10 strain) used in this study was isolated from the Oyster site. This strain closely resembles the P. jessenii (CIP 105274T) isolated from French mineral waters [Verhille 1999], and the band forming microaerophilic strain IpA-2 isolated from a Canadian wastewater treatment plant [Ferrara-Guerrero 1989].

Lipid and protein analysis. P. jessenii was grown with vigorous stirring in 2-liter flasks containing 1 liter of medium. High (241 µM) and low (26 µM) oxygen concentration was maintained by bubbling atmospheric air or 2 % oxygen (balance nitrogen) respectively at a rate of 200 ml min^{-1}. Dissolved oxygen concentrations were measured with a YSI model-57 oxygen probe (Fisher). The high substrate media contained 1000 mg l^{-1} of peptone, tryptone, yeast extract, and glucose each. The low-substrate media contained 10 mg l^{-1} of each of the same ingredients. The growth conditions were:- condition A: low substrate-low oxygen; condition B: low substrate-high oxygen; condition C: high substrate-low oxygen, and; condition D: high substrate-high oxygen. All cultures were grown to the mid-log growth phase. There was no pH change in the media from the time of inoculation

until the cells were harvested. Extraction of cellular fatty acids, and their subsequent saponification, hydrolysis, methanolysis, and esterification for gas chromatography analysis were performed as described by Mayberry [Mayberry 1993] with modification as described by Pinkart et al. [Pinkart 1997]. Further fatty acid verification was made by GC/mass spectrometry [Pinkart 1997]. For protein extraction, the bacterial cell pellet was suspended in 1 ml of 100 mg l^{-1} of MOPS buffer (pH 7.2) with 100 mg of 75-150 µm glass beads (Sigma). The cells were disrupted by sonication. The contents were centrifuged at 30,000 × g at 4^0C for 60 min and the supernatant collected for two-dimensional gel electrophoresis (2-D PAGE). Total protein was quantified and analyzed according to the method of O'Farrell [O'Farrell] with modification as described by Alban et al. [Alban 1998]. The gels were silver-stained as described by Wray et al. [Wray 1981]. At least 3 replicate gels were used for each treatment.

Microaerophilic band movement. Preliminary experiments showed that microaerophilic bands in culture tubes were not static, rather they showed vertical movement during incubation. The variables that control band movement were determined by growing P. jessenii in 15-ml screw cap culture tubes containing 10 ml of 1× PTYG semi-solid medium. After 24 h of incubation at 24^0C, the cell bands formed at the 3-mm depth below the surface of the medium in ambient oxygen (headspace containing atmospheric air). This depth was the baseline used to measure either upward or downward movement of the band based on daily observation of the cultures. In one set of experiments, culture tubes with loosened caps were placed in gas-tight jars and known

quantities of oxygen with nitrogen were added to the jar head space [Smibert 1994]. In another set of experiments, additional substrates (0.5, 1, 2, and 5 g l^{-1} of peptone, tryptone, yeast extract and dextrose each) were added to the culture tubes. Band movements were measured daily for a 5-day period.

Results and Discussion

LSRMB bacteria were a significant portion of the bacteria that grew on PTYG medium that was used to detect viable chemoorganotrophs. The percent of LSRMB bacteria that grew on this PTYG medium from the eutrophic Virginia Tech Duck Pond, the mesotrophic Pandapas Pond and Mountain Lake water samples were 49 (2.5×10^7 CFU ml^{-1}), 34 (1.6×10^2 CFU ml^{-1}), and 54 (6.0×10^2 CFU ml^{-1}) respectively. Pandapas Pond and Mountain Lake are located in pristine forest habitats, whereas, the campus Duck Pond receives surface storm sewer water from the town, campus, golf course, and shopping center. The percent of LSRMB bacteria isolated from the deep subsurface (463 m) depth of the Savannah River site and the Oyster VA site (5 m) was 10 (5.6×10^1 CFU ml^{-1}) and 26 ($\sim 9.1 \times 10^2$ CFU ml^{-1}) respectively. We have also isolated LSRMB bacteria from a Blacksburg wastewater activated sludge tank. Many of the bacteria in Antarctic Victorialand Dry Valley soils originate in cyanobacterial mats dependent on transitory glacial melt water. In these soil samples LSRMB exhibiting bacteria composed over 50% of the viable chemoorganotrophs (Benoit, unpublished data). We previously demonstrated that deep subsurface LSRMB bacteria were quite resistant to the damaging effects of UV and H_2O_2 [Arrage 1993]. The ability of LSRMB bacteria, such as Pseudomonas sp., to form clearly

defined bands in semi-solid media may clue one of several mechanisms used by LSRMB bacteria to survive in oxygenated ecosystems.

The isolation screen for LSRMB bacteria demonstrated that different cultures show a variety of band patterns. Some cultures formed a band of growth near the top of the semi-solid media, while others formed a band near the bottom. In some cases the bands disappeared after several days of incubation. One subsurface culture isolated from Oyster VA, formed bands in liquid 1× PTYG medium without agar. P. jessenii VT10 was the model used to evaluate how the bands of LSRMB bacteria moved in response to environmental conditions. After P. jessenii bands formed at the 3 mm depth in 1× PTYG semi-solid medium in a test tube at ambient oxygen levels, the oxygen concentration in the culture head space was changed, and the culture incubation was continued. If the head space oxygen concentration was changed to 5%, the band movement was minimal. If the oxygen concentration was changed to 0 – 2%, the bands moved toward the surface of the culture tube and the bands disappeared after two days of incubation at the lowest oxygen concentration. If the head space oxygen concentration was changed to 9 – 17%, the bands moved toward the bottom of the tube at speeds proportional to the oxygen concentration. For example, after 5 days of incubation the bands moved down the culture medium 5 mm and 11 mm at the 9% and 17% oxygen concentrations respectively. The maximum downward movement of 20 mm was observed in the control culture tubes with atmospheric air in the headspace. We used an agar plug above the semi-solid medium to show that

bands in inverted culture tubes moved downward in semi-solid media irrespective of gravity.

The effect of substrate concentration on expression of LSRMB was demonstrated by incubating P. jessenii in 1× PTYG semi-solid medium under ambient oxygen until bands formed at the 3-mm depth. At that time, the substrate concentration was increased and the incubation continued for 5 days while band movement was measured. When additional substrate (peptone, tryptone, yeast extract, and glucose each) was added at concentrations of 0.01, 0.1, or 0.5 g l^{-1} to the cultures grown in 1× PTYG medium, the bands moved down the culture tube and mimicked the ambient oxygen controls without added substrate. For example, cultures receiving additional substrate of 0.01 and 0.5 g l^{-1} had bands that moved downward 20 mm and 4 mm respectively over a 5-day period. The downward band movement may reflect a strategy to avoid oxygen toxicity or a mechanism to scavenge substrate. However, when additional substrate was added at concentrations of 1, 2, 3, or 4 g l^{-1}, the cell density in the bands increased as the bands moved upward and formed a typical aerobic surface growth 24 h after the additional substrate was added. Krieg and Hoffman had noted that cell density affected the behavior of some microaerophiles in semi-solid media incubated under atmospheric condition [Krieg 1986]. These experiments proved that P. jessenii shows microaerophilic behavior only at low substrate concentrations and oxygen concentrations greater than 9% concentration. LSRMB bacteria growing in substrate rich media cannot be distinguished from traditional aerobes, which lack a LSRMB capacity by growth patterns. LSRMB appears to be an important survival

strategy for some bacteria living in low substrate, oxygenated environments.

The CFA stress response in P. jessenii was obtained by measuring the lipid pattern after growth at different substrate concentrations and oxygen tensions in liquid media. (Fig 2). CFAs in P. jessenii was more abundant in cells grown in the low substrate conditions (condition A and B) with the maximum being produced when grown under low substrates-high oxygen condition (condition B). High substrate conditions C and D did not appear to induce stress. When the culture was grown in the low substrate medium-high oxygen (growth condition B), there were a five-fold increase in C17:0 CFA, and the percent concentration of C19:0 CFA increased almost three-fold compared to the average of the CFA concentrations detected in the other three growth conditions. During growth condition B the product to monoenoic precursor ratio of C17:0 CFA increased to 0.4, whereas, it was less than 0.1 in cells grown under the conditions A, C and D. Because CFAs are chemically more stable than their unsaturated fatty acid precursors [Grogan 1997], an increase in the CFA concentrations by VT 10 could be a protective mechanisms employed by the bacterium to protect the unsaturated fatty acid double bond against reactive toxic oxygen species [Grogan 1997, Krieg 1986]. The CFA lipid stress response of P. jessenii liquid media paralleled the microaerophilic band stress responses of P. jessenii in semi-solid media.

The stress response of P. jessenii VT10 was further documented by use of silver-stained 2-D PAGE gels (Fig 3) that revealed that the protein profile of isolate VT10 changed when grown in the same culture conditions used in the CFA study. Visual analysis of the silver-stained 2-D

gels revealed that under low substrate states (conditions A and B) there was an increased intensity of four spots (marked by arrows), corresponding to molecular masses of ~26-28 and ~12kDa, and several unique spots were also observed (marked by block arrow). A total of four proteins were induced specifically at low substrate-high oxygen (Fig 3, condition B, circled), corresponding to molecular masses of ~12, 28, 35, 60 kDa. Furthermore, two proteins corresponding to ~43 and 45 kDa were synthesized at higher levels only at condition B (Fig 3, condition B, boxed). A total of ~25 proteins disappeared, and one novel or accumulated protein was observed in response to high substrate conditions (conditions C and D, marked by triangle). The changes in protein profile and the production of CFAs in cells in the mid-log growth phase suggests that these stress factors may represent a physiological response to allow growth under sub-optimum environmental conditions.

This study provides an explanation of the observation of Ferrara-Guerrero and Bianchi [Ferrara-Guerrero 1989] who isolated microaerophiles from Mediterranean marine sediments, which formed bands only under low substrate conditions. The lowest substrate concentrations used in this study approximate the substrate concentrations in many natural ecosystems, therefore, P. jessenii may represent a LSRMB model that is applicable to other aquatic and soil species.

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Chapter 2: Determining Chemotactic Responses by Two Subsurface Microaerophiles Using a Simplified Capillary Assay Method

Abstract

A simplified capillary assay comprising a needle and a syringe and a swarm plate assay were used to measure the motility of two subsurface microaerophiles toward potential carbon sources. One isolate, MR100, was phylogenetically related to Pseudomonas mendocina and was isolated from the deep subsurface; the other, VT10, was a strain of Pseudomonas syringae and was isolated from the shallow subsurface. Both isolates formed a microaerophilic band of growth below the agar surface of tubes and plates of an oligotrophic semi-solid medium but produced surface growth (and colonies on plates) when the substrate concentration was increased. The chemotaxis of these organisms followed a synergistic response to oxygen and substrate. Response of the two isolates although very similar in microaerophilic band formation, was dissimilar in tactic response to various substrates. VT10 was chemotactically attracted toward dextrose, glycerol, and phenol, which could be used as sole carbon sources, and also toward maltose, which could not be used. MR100 showed no tactic response to these compounds although it could use dextrose, maltose, and glycerol as carbon sources. The difference between the two strains might be due to their evolution in different subsurface environments. The simplified chemotaxis assay technique may be useful for studying the tactic responses of other subsurface isolates.

Introduction

While studying microbial populations of subsurface environments, our laboratory isolated two bacterial strains that seemed to be microaerophilic. This was based on the formation of a band of growth below the surface of semi-solid media, which is usually taken as an indicator of microaerophily [Krieg 1986]. The strains grew aerobically on the surface of solid media containing a high concentration of nutrients, but they exhibited spreading motility beneath the surface of aerobically incubated low nutrient agar plates, characteristic of facultative microaerophiles [Benoit 1989]. These results suggested that nutrient concentration may play a role in determining the chemotactic response of these isolates.

Chemotactic responses of subsurface bacteria have not been studied quantitatively except for an investigation by Hazen, and Hazen and Lopez-De-Victoria [Hazen 1989, Hazen 1994], who found a positive chemotactic response by several deep subsurface bacteria to ten sugars and ten amino acids. Hazen and Lopez-De-Victoria also reported a fatal chemotactic attraction of these bacteria to trichloroethylene at concentrations that were toxic to the bacterium.

Chemotactic responses are often quantified by the capillary tube method of Adler [Adler 1973], which involves the preparation of a small U-shaped glass tube over which a cover slip is placed to form a chamber. The chamber is filled with the bacterial suspension of interest and a capillary tube containing the potential attractant or repellent is then inserted into the ‘pond’ of bacterial suspension. After incubation for a suitable amount of time, the capillary is removed, the top broken and its contents

transferred into liquid media. The present report focuses on the development of a taxis response assay for oligotrophic organisms that is simpler and more convenient than the Adler method. The modified method was used to quantify the chemotaxis response of the two subsurface microaerophilic isolates and it could be usefully applied to studies of the tactic responses of other subsurface isolates.

(A preliminary report of these studies was presented at the 97th General Meeting of the American Society for Microbiology [Mazumder R, Lampe RC, Phelps TJ, Hanel JB, Benoit RE (1997) Chemotactic behavior of subsurface “microaerophilic” bacteria. 97th ASM General Meeting, Miami Florida, May, 1997 Session 57, abst Q76, p 468]).

Materials and Methods

Bacterial strains and growth conditions. The two subsurface sites from which the strains were isolated were sponsored by the U.S. DOE Subsurface Science program. The drilling and sampling protocols have been detailed elsewhere [Phelps 1989, Zhang 1997 a, b]. Isolate VT10 was isolated from a depth of 5 meters from a site known as the Oyster Virginia DOE site which has been described elsewhere [Zhang 1997 a, b]. The organism was identified as a strain of Pseudomonas syringae by fatty acid analysis. Isolate MR100 was isolated from a depth of 463 meters from a site known as the Savannah River DOE site described by Phelps and Russel [Phelps 1989]. By 16s rRNA sequencing and DNA-DNA reassociation experiments this strain was found to be most closely related to Pseudomonas mendocina, although it did

not belong to this species [Lampe RC, Rippere KE, Mazumder R, Johnson JL, Benoit RE (1996) Characterization of a deep subsurface microaerophile using 16s rRNA sequencing and DNA-DNA reassociation. 96th ASM General Meeting, New Orleans, Louisiana, May, 1996 Session 74, abst Q111, p 404]. The strains were grown aerobically with static incubation to mid to late logarithmic phase in peptone, tryptone, yeast extract, glucose (PTYG) medium. This medium contained (g l^{-1} of deionized water); $NaNO_3$, 0.009; NH_4Cl, 0.055; $Na_2HPO_4 \cdot 12H_2O$, 0.180; $MgSO_4 \cdot 7H_2O$, 0.050; KH_2PO_4, 0.068; $CaCl_2 \cdot 2H_2O$, 0.050; MOPS buffer, 0.100; yeast extract (Difco), 0.010; trypticase peptone (BBL), 0.010; dextrose (Difco), 0.010 and Bacto-Peptone (Difco) 0.010. For solid media, 15 g of Noble agar (Difco) was added. For semi-solid swarm plates 2.5 g l^{-1} of Noble agar was added, and for tubes of semi-solid medium, 1.5 g l^{-1} of Noble agar was added. PTYG 100× medium contained in addition to the above mentioned salts and MOPS buffer, 1 g l^{-1} of yeast extract, trypticase peptone, dextrose and Bacto-peptone. The pH was adjusted to 7.2 before autoclaving. All subsurface isolates were stored at –80^{o}C and working cultures were maintained in semi-solid PTYG medium at 24^{0}C with monthly transfers.

Utilization of specific compounds as the sole source of carbon. For the carbon source medium (CS medium) the following solutions sterilized by filtration were added aseptically to autoclaved PTYG medium lacking peptone, tryptone, yeast extract and dextrose: 3 ml of vitamin stock solution, 10 ml of trace minerals stock solution, and a sufficient volume of the stock solution of the particular carbon source to give a final concentration of 0.01 M. The

vitamin stock solution contained (g l^{-1} of deionized water); biotin, 0.02; folic acid, 0.020; pyridoxine HCl, 0.10; thiamine HCl 0.05; riboflavin, 0.05; nicotinic acid 0.05; panthothenic acid, 0.05; cyanocobalamin 0.001; p-aminobenzoic acid, 0.05; lipoic acid, 0.05. The trace minerals stock solution contained (g l^{-1} of deionized water); nitriloacetic acid, 1.5; $FeCl_2 \cdot 4H_2O$, 0.2; $MgCl_2 \cdot 6H_2O$, 0.1; sodium tungstate, 0.020; $MnCl_2 \cdot 4H_2O$ 0.1; $CoCl_2 \cdot 6H_20$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnCl_2$, 0.05; $CuCl_2 \cdot 2H_2O$, 0.002; H_3BO_3, 0.005; sodium molybdate, 0.01; NaCl, 1; Na_2SeO_3, 0.017 and $NiCl_2 \cdot 6H_2O$ 0.024. The nitriloacetic acid was added to 200 ml of deionized water and the pH was adjusted to 6.5 with KOH solution. Deionized water (600 ml) was added. The components were dissolved in the displayed order and additional deionized water was added to 1000 ml. The solution was autoclaved in serum vials under nitrogen gas.

Modified capillary chemotaxis assay. A disposable 200-µl pipette tip (Fisher Scientific) was used as the chamber for holding 100 µl of the bacterial suspension, which had been grown to late log phase. A disposable 2-cm 25-gauge needle (Becton Dickinson) with a 0.254-mm internal diameter was used as the capillary and was attached to a 1-ml tuberculin syringe (Becton Dickinson). A 100-µl portion of the compound to be tested for a chemotactic response was placed into the syringe-needle chemotaxis chamber (Fig 4). The needle was then inserted into the pipette tip containing the bacterial cell suspension until the neck of the needle formed a tight fit with the base of the pipette tip. This resulted in the needle being inserted to a distance of 3 mm into the

bacterial cell suspension. After an appropriate incubation time at room temperature the needle-syringe was removed from the bacterial suspension chamber and the contents diluted in MOPS buffer (pH 7.0). The dilutions were plated onto PTYG 100× medium. Accumulation in the capillary was calculated from the CFU on the plates. Results were expressed as the mean of at least three separate assays (duplicate plate count for each assay). Control capillaries, which contained the buffer alone, were included with each assay. Error bars were calculated based on a method described by Sackett et al. [Sackett 1997].

Amount of glucose present as a contaminant in maltose was determined enzymatically by using the glucose oxidase and peroxidase assay as described by Bergmeyer and Brent [Bergmeyer 1974]. All the chemicals required for the assay were purchased from Sigma Chemical Co.

Swarm plate assay. Chemotactic responses to dextrose and glycerol were measured in plastic Petri plates (8.5 cm internal diameter) containing 20 ml of CS-semisoft agar medium. Noble agar (0.25% w/v) and appropriate concentrations of the carbon source of interest were added to the CS medium. After the plates were allowed to solidify at room temperature for 3 h, they were inoculated at the center with 10 µl of a culture of strain VT10 or strain MR100 that had been grown to late log phase on PTYG medium. The swarm diameter was measured after 24 h and photographs were taken against a dark background, with illumination from below.

Swimming velocity. To produce chemotaxis, a bacterium must be able to swim fast enough to compensate for rotation from Brownian motion and diffusion of an attractant. Consequently, measurements were done with VT10 and MR100 to compare their velocity with that of two species (Pseudomonas aeruginosa and Escherichia coli) that have been used in chemotaxis studies). All cells were grown to the late log phase of growth in PTYG before observation. A Petroff-Hausser bacteria counting chamber was used instead of the chamber described by Mitchell et al. [Mitchell 1991] which had been created by placing strips of coverslip on a slide and covering this with a whole coverslip. This slight modification reduced the horizontal liquid drift and allowed more accurate cell velocity measurement. The cell suspension (8 μl) was placed in the Petroff-Hausser chamber and the velocity of the cells at room temperature was measured by recording the time required for a cell to swim 50 μm (the length of a Petroff Hausser small square. The cell judged fastest in each field of vision was the one measured. The phase-contrast images were captured by a Sony color video camera (CCD-IRIS/RGB). The velocity of cells in at least 50 fields was measured and the average velocity of the organisms was calculated. The comparison of swimming velocity of VT10, MR100, Pseudomonas aeruginosa ATCC strain 15692, and Escherichia coli ATCC strain 9637), revealed that P. aeruginosa was the fastest of the four with a value of 105.9 μm s^{-1}. VT10 and MR100, however, had average velocities (39.4 and 18.5 μm s^{-1}, respectively) that exceeded that of E. coli (16.5 μm s^{-1}).

Results

Rate of accumulation in the capillary. The capillary tube assay was used to measure the chemotactic response of VT10 and MR100 to various test compounds. The optimal incubation time for chemotactic movement of the two organisms was determined by using 10^{-2} M dextrose. The number of bacteria in capillaries containing 10^{-2} M glucose increased rapidly in the first 15 min, then slowly leveled off until peaking at 45 min of incubation. After 45 min of incubation, 32600 CFUs of VT10 and 13400 CFUs of MR100 entered the individual capillaries. The background accumulation in capillaries containing only buffer reached 7000 for VT10 and 9000 for MR100 after 45 minutes. Based on the dextrose taxis data, a standard incubation time of 45 min was adopted for all subsequent chemotaxis assays.

Carbon sources and growth. The ability of strains VT10 and MR100 to grow when supplied with various compounds as the sole source of carbon is shown in Table 1. VT10 could use dextrose, glycerol, benzoate and phenol as the sole source of carbon but could not use maltose or tannic acid. MR 100 could use dextrose, maltose and glycerol as the sole source of carbon, but could not use phenol, benzoate or tannic acid.

Chemotactic response comparison of VT10 and MR100 to different molarities of dextrose. Using the capillary assay technique (see materials and methods) the bacterial chemotactic responses could be readily quantified. Dextrose elicited a positive tactic response from VT10 but not from MR100 (Fig. 5). Isolate VT10 showed a peak response to dextrose at 10^{-3} M concentration (34700 CFUs per dextrose

filled capillary tube compared with the background accumulation of 7000 CFUs per buffer-filled capillary). Isolate MR100 exhibited only a slight response to dextrose (13100 CFUs/capillary compared to the background accumulation of 9000 CFUs/capillary).

Chemotactic response comparison of VT10 and MR100 to different test compounds. Five other compounds were surveyed for their ability to attract VT10 and MR 100. Isolate VT10 showed a positive tactic response to dextrose, maltose, glycerol and phenol whereas MR100 was not attracted to any of the test compounds (Fig. 6). VT10 was chemotactic to compounds it could utilize as the sole carbon source and showed its strongest chemotactic response towards maltose (10^{-3} M) which it could not use as a carbon source. VT10 showed a slightly lower positive chemotactic response to 10^{-4} M maltose as 39400 CFUs of VT10 were attracted into the capillary (data not shown). To confirm that this chemotactic response was due to maltose and not to the glucose that was present as an impurity, the level of glucose contamination was determined enzymatically and was found to be 0.75%. Since VT10 showed a positive chemotactic response to maltose at 10^{-4} M concentration, the response could not have been due to the presence of glucose because the concentration of glucose in 10^{-4} maltose is 100 times less than the minimum concentration of glucose required to elicit a positive response (Fig. 5).

Swarm plate assays. VT10 produced chemotactic swarm rings when grown on plates containing CS-semisoft dextrose agar. The extent of swarming increased as the concentration of dextrose decreased (Fig 7 a, 7 b). The magnitude of

swarming for VT10 on glycerol plates remained constant at 54 ± 1.5 mm at glycerol concentrations ranging from 0.1 mM to 100 mM at 24 h of incubation. Strain MR100 did not produce any swarm rings on dextrose or glycerol plates.

Discussion

To test the possibility that nutrient concentration may play an important role in determining the chemotactic response of these subsurface isolates, it was necessary to ascertain the background accumulation of VT10 and MR100 when no attractant was present in the capillary. Background accumulation, as estimated with control capillaries containing buffer alone, occurred because of random motility of the bacteria and because of diffusion due to the difference in bacterial concentration between the capillary and the bacterial suspension. The background accumulation of VT10 and MR100 was very similar to that observed by the Adler method by Winterberg and Montie with Pseudomonas aeruginosa (6500 CFUs/capillary in 30 min) [Wintenberg 1994] and by Lynch with Pseudomonas fluorescens (8800 CFUs/capillary at 60 min) [Lynch 1980]. However, it was much higher than that observed by Adler with E. coli (1100 CFUs/capillary in 60 min) [Bergmeyer 1974] and by Cuppels with Pseudomonas syringae (1400CFUs/capillary in 90min) [Cuppels 1988]. These differences in background accumulation could be due to the use of different strains or to differences in the methods used for measuring chemotaxis.

The peak response of VT10 to dextrose at 10^{-3}M concentration (34700 CFUs/capillary of VT10 in 45 min) was similar to the peak response to 10^{-3}M dextrose for Pseudomonas aeruginosa reported by Moulton et al. (34650

CFUs/capillary of Pseudomonas aeruginosa in 30 min) [Moulton 1979]. The peak response of some other organisms to dextrose as reported by other researchers are: 140000 CFUs/capillary of E. coli in 60 min [Adler 1973, Macnab 1987, Melton 1978]; 65000 CFUs/capillary of S. typhimurium in 45 min [Macnab 1987, Melton 1978]; 15850/capillary of Pseudomonas putida in 30 min [Scher 1985] and 8500 CFUs/capillary of P. syringae in 60 min [Cuppels 1988]. Pseudomonas fluorescens has been reported to be unresponsive towards dextrose [Lynch 1980].

It was surprising that strain MR100 failed to exhibit positive chemotaxis toward dextrose, a substrate that it could use as a sole source of carbon (Table 1). However, the failure of other bacteria such as E. coli, S. typhimurium, P. putida and other bacteria to be attracted toward various compounds that they can use as carbon sources has been reported [Shi 1998, Harwood 1984]. For instance, both E. coli and S. typhimurium can transport and utilize maltose but maltose is an attractant only for E. coli [Adler 1973]. The relevant transducer, Tar, of E. coli, but not that of S. typhimurium interacts successfully with the maltose/maltose-binding protein complex, accounting for the differences in the behavior of the two species [Dahl 1985, Mizuno 1986].

Isolate VT10 was also attracted to the aromatic compound phenol. Escherichia coli and some pseudomonads have also been reported to be attracted to phenol and other aromatic compounds [Harwood 1984, Imae 1987, Grimm 1997]. Harwood and Grim observed that 50000 CFUs of benzoate-grown Pseudomonas putida entered a benzoate capillary in 30 min [Harwood 1984, Grimm 1997]. On the other hand, Salmonella

typhimurium has been reported to be repelled by aromatic compounds [Macnab 1987, Tso 1974]

Isolate VT10 could use most of the compounds tested as sole sources of carbon and energy (Table 1) except maltose, and positive chemotaxis towards these compounds could be viewed as advantageous to the organism [Tso 1974]. The apparent anomaly with maltose chemotaxis is not surprising as this form of behavior has been reported before, and the expression of genes for utilization of a particular substrate is not necessarily required for chemotaxis towards that substrate [Harwood 1984]. In the natural environment maltose might serve as an indicator to VT10 of the availability of glucose (which the organism can readily utilize), since maltose is composed of two glucose molecules and can be broken down by many other organisms to glucose. Such specialized taxis towards an otherwise useless compound that serves as a marker of a specific ecological niche has been proposed by Kariman and Ornston [Karimian 1981].

Caraway and Krieg had reported that the movement of the cells of Spirillum volutans was due to self-created oxygen gradients in capillary tubes as the organisms used up an oxidizable substrate [Caraway 1974]. It is likely that in our system and in many *in situ* conditions when metabolizable substrates are present, that migration into a capillary might be due to a combination of both chemotaxis and aerotaxis. When the substrate cannot be metabolized (as with maltose for VT10) then the taxis response must be due to chemotaxis; however, when the substrate can be metabolized, it is necessary to differentiate between chemotaxis and aerotaxis. The Petri plate swarm assay is used for this purpose because of the ready availability of

oxygen and lack of an oxygen gradient. The results obtained in the swarm plate assay confirm the findings from the capillary chemotaxis assay. Isolate VT10 but not MR100 formed swarm rings in response to both dextrose and glycerol and thus was more versatile in its chemotactic response than MR100. The concentric bands formed by VT10 (Fig. 7b) in response to lower concentration of dextrose is similar to the bands of E. coli in tryptone and L-aspartate swarm plates as observed by Wolfe et al. [Wolfe 1989].

Although VT10 formed swarms on the glycerol plate, the swarm response differed from that reported for E. coli by Zulin et al. [Zhulin 1991]. The extent of swarming by VT10 was constant over the range of glycerol concentrations tested, whereas the swarming of E. coli was reported to decrease as the concentration of glycerol increased from 0.5 mM to 5 mM. Zulin et al. proposed that the swarming of E. coli increased more rapidly at the lower concentrations because the bacteria more quickly exhausted the surrounding attractant and migrated in response to the resulting self-created attractant gradient. Although a similar explanation may apply to the swarming of VT 10 on dextrose plates, the constancy of the swarming on glycerol plates is more difficult to explain. The development of similar-size chemotactic rings on glycerol plates at different concentrations suggests that VT10 chemotaxis is not dependent on the concentration of the substrate. It may be that the concentrations of glycerol tested (0.1-100 mM) are saturating for growth of VT10 on glycerol. Alternatively, it is possible the constancy of swarming on glycerol is a response to a gradient of a constant amount of attractant that the cells excrete themselves when growing on glycerol. This type of swarming has been reported in E. coli, which

forms swarm rings in response to aspartate that is excreted when the cells grow on succinate [Budrene 1991, Budrene 1995].

The swimming velocity of E. coli that we obtained is consistent with the results obtained by Frymer, who reported that the average velocity of E. coli ranged from 15 to 25 μm s^{-1} [Berg 1972, Frymier 1995]. VT10 and MR100 do not fall under the category of “high speed bacteria” (> 100 μm s^{-1}) observed by Mitchell et al. but instead their velocity is similar to most “medium speed” bacteria reported so far [Mitchell 1995].

The occurrence of chemotactic responses in VT10 but not in MR100 might be attributable to their different isolation habitats, since the nutrient availability and nutrient composition of the deep subsurface are unlike those of the shallow subsurface [Palumbo 1989]. The mechanisms by which these motile subsurface microaerophiles govern and regulate the magnitude of their taxis response when given oxidizable substrates or non-oxidizable substrates have not been elucidated. It is possible that a fine balance exists between the chemotaxis and aerotaxis depending on the substrate and oxygen availability. Although MR100 failed to exhibit chemotaxis toward the substrates tested, even random motility could promote dispersal from low nutrient regions and prevent dispersal from high nutrient regions, as shown by Lauffenburger et al. in their mathematical model of random motility and growth [Lauffenburger 1981]. Moreover, MR100, which is a deep subsurface bacterium, might benefit from having random motility rather than developing specific chemotaxis

receptors because the substrates it encounters would be few and varied.

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Table 1. Ability of the isolates to grow under aerobic conditions using various compounds as the sole source of carbon

Carbon source	Growth response of strains[a]	
	VT10	MR100
Dextrose	+++	+++
Maltose	-	+++
Glycerol	+++	+++
Tannic acid	-	-
Benzoic Acid	+++	-
Phenol	+++	-

[a] The growth response is based on the turbidity of the cultures: ++, A_{620} = 0.03-0.05; +++, A_{620} = >0.06; -, A_{620} = 0.00. Each value represents the results from at least three different experiments using duplicate cultures for each carbon source.

Chapter 3: *Pseudomonas jessenii* VT10: A Model for Low Substrate Regulated Microaerophilic Behavior in Natural Ecosystems

Abstract

Low substrate regulated microaerophilic behavior (LSRMB) exhibited by Pseudomonas jessenii VT10 and other pseudomonads may represent a mechanism to avoid oxygen induced stress. P. jessenii VT10 was identified by phylogenic analysis based on 16S rDNA sequence similarity. High sequence similarity (99%) was observed between strain VT10 and the recently isolated P. jessenii type strain (CIP 105274T), and unidentified strain IpA-2. The fatty acid profiles of VT10 and IpA-2 were similar. P. jessenii VT10 use motility to find substrate and optimum oxygen concentration during the log growth phase and grows as a band underneath the surface of semi-solid media. When the band is disrupted by gentle stirring of the agar medium, the band reforms. The band will not reform if metabolic inhibitors such as mercuric chloride or sodium azide are added to the medium. The respiratory potential of P. jessenii VT10 was demonstrated by increasing the substrate concentration 100× and showing an accompanying 3-fold increase of oxygen uptake. Organisms phylogenetically related and distant to strain VT10 were selected and tested for LSRMB. LSRMB was observed in phylogenetically disparate groups with all the Pseudomonas and two Bacillus species testing positive for the trait. Pseudomonas sp. being ubiquitous in many aquatic habitats, we measured the distribution of LSRMB at several depths of a pristine oligotrophic lake. There were more LSRMB bacteria in the surface water of Mt Lake, VA than the low oxygen hypolimnion water.

Introduction

A diverse group of bacterial species prefer to metabolize at oxygen concentrations below the ambient levels present in air saturated water [Krieg 1986, Mazumder 1999, Mazumder 2000]. According to the classical model of microaerophily, demonstrated by Campylobacter, microaerophiles grow in environments with 0.2 – 12% oxygen. [Bowdre 1976]. We recently proposed another model for microaerophily by demonstrating that some Pseudomonas species exhibit microaerophilic behavior when the substrate concentration is low, whereas, they display aerobic growth when the substrate concentration is high [Mazumder 2000]. The physiological mechanisms available to this microorganism to survive oxygen stress in low substrate environments include production of cyclopropane fatty acids and unique stress proteins, and use of positive chemotaxis to locate scarce substrate in oligotrophic environments [Mazumder 1999, 2000].

Low substrate regulated microaerophilic behavior (LSRMB) exhibiting bacteria grow as a subsurface biofilm on low substrate semi-solid agar media incubated in air, but they form typical surface colonies on these media when the substrate concentration is increased [Mazumder 1999, 2000]. Adaptations to survive in low susbstrate environments may offer a competitive advantage to organisms [Kjelleberg 1983]. We used a semi-solid agar medium to screen LSRMB isolates from natural samples and known species in our culture collection [Mazumder 1999].

The physiological response by LSRMB may represent only one model of microaerophilic behavior of aquatic and terrestrial bacteria. Some microaerophilic bacteria such as Beggiatoa prefer the aerobic/anaerobic interfaces of a

microbial mat or the chemocline of a water column [Krieg 1986]. It was our hypothesis that bacteria such as P. jessenii are not restricted to the aerobic/anaerobic interfaces, rather they use LSRMB to survive in habitats which has fluctuating nutrient levels. When substrate concentrations are low organims capable of LSRMB can survive as a microbial floc in oxygenated areas of the epilimnion; areas near the surface of microbial mats and biofilms; and partially oxygenated areas of an aquifer. The distribution of LSRMB bacteria in a vertical profile of Mountain Lake, VA was used as a test of this hypothesis. The limnology of this pristine, oligotrophic lake has been studied by Beaty and Parker [Beaty 1994] and Cawley et al. [Cawley 1999]. The major sources of substrate to decomposers in this 31 m deep lake is the phyoplankton, surface run-off from the small forest watershed and some human perturbations from a hotel. Summer thermal stratification produces a hypolimenetic oxygen deficit, although bottom anoxia may not occur some years.

We used the 16S rDNA sequence method [Lane 1985, Woose 1987, Boettger 1989] to identify the VT10 strain, and to show its evolutionary relationship to other pseudomonads and bacteria exhibiting LSRMB. The purpose of this study was to determine how widespread LSRMB is, determine if cell numbers and oxygen uptake rates have an effect on the microaerophilic behavior of isolate VT10, and to ascertain the uniqueness of the LSRMB through isolate VT10’s taxonomic classification.

Materials and Methods

Microorganisms and growth conditions. Microbiological investigations at the Subsurface Oyster Virginia site was sponsored by the U.S. DOE as described earlier by Mazumder

et al. [Mazumder 1999]. Isolate VT10 was isolated from a depth of 5 meters from Oyster Virginia site [Mazumder 1999]. Mountain Lake is an alpine, oligotrophic lake located in the Allegheny range of the Southern Appalachians at an elevation of 1180 m. The lake is ovoid in shape with a length of 900 m and a width of 250 m. The mean depth is 9.7 m and the maximum depth is 31 m. The mixed conifer-hardwood watershed surrounding the lake is relatively small and the lake has a replenishment time of 1.6 years [Cawley 1999]. The lake phytoplankton is dominated by chlorophycean algae with microplankton making up the largest fraction of the algal community. The primary source of energy for the lake decomposers is the phytoplankton. The hypolimnion depth during summer stratification begins at the 14 m depth but the bottom is rarely anoxic. The 16 m depth oxygen concentration at the time of sampling was 50 µM, whereas the surface water (21^0C) was 246 µM. The oxygen data and water samples used for this study were taken at the surface, 16m and 25 m by Dr. J Webster. The methods and lake description were reported by Cawley et al. [Cawley 1999].

Bacteria were isolated by serial dilution and grown in peptone, tryptone, yeast extract, glucose (PTYG) medium [Mazumder 1999, 2000]. The PTYG medium composition has been described earlier [Mazumder 1999], contained (g l^{-1} of deionized water), yeast extract (Difco), 0.010; trypticase peptone (BBL), 0.010; dextrose (Difco), 0.010 and Bacto-Peptone (Difco) 0.010 g, and required salts and minerals. For solid media, 15 g of Noble Agar (Difco) was added. For semi-solid medium, 1.5 g l^{-1} of Noble agar was added. The 100× PTYG medium contained in addition to the above mentioned salts and MOPS buffer, 1 g l^{-1} of peptone, tryptone, yeast extract, and dextrose. The pH of the media was adjusted to 7.2 before autoclaving. Strain IpA-2, was

supplied by Dr. WW Mohn. All isolates were stored at –80°C and working cultures were maintained in semi-solid PTYG medium at 24°C with weekly transfers.

Sample processing. The samples were processed following closely the methods detailed earlier to isolate LSRMB exhibiting isolates [Mazumder 1999, 2000]. The samples were serially diluted and plated on 100× PTYG media. After colony growth was observed each colony was inoculated into semi-solid PTYG tubes for observation of microaerophilic band formation capability by the isolate.

Characterization of isolate VT10. General characteristics. Motility was assessed by phase-contrast microscopy. Other strain characteristics were determined using methods as described in Methods for General and Molecular Bacteriology [Johnson 1994]. For the carbon source medium, vitamin and trace mineral solutions were added to autoclaved PTYG medium lacking peptone, tryptone, yeast extract and glucose [Mazumder 1999]. A sufficient volume of the stock solution of the particular carbon source was finally added to give a final concentration of 0.01 M. The use of carbon sources was tested in 10-ml cultures incubated at 24°C. Anaerobic use of carbon source was tested in 10-ml cultures with a head-space of nitrogen. The presence or absence of growth on a particular carbon source was evaluated by microscopy.

16S rRNA gene amplification, sequencing and phylogenetic analysis. Nucleic acids were isolated from late-exponential phase cultures by the procedure of Marmur [Marmur 1961]. Genes for 16S rRNA were amplified by PCR using primers that corresponded to nucleotide positions of Escherichia coli 16S rRNA gene position 8 to 27 in the forward direction (5′-AGA GTT TGA TCC TGG CTC AG-3′), and from position 1541 to 1522 in the reverse direction (5′-AAG GAG GTG ATC CAR CCG CA-3′) [Johnson 1994]. PCR was carried out with a PTC-100 thermocycler (MJ Research, Inc.,

Watertown, MA). Standard PCR reactions were conducted using Ready To Go PCR™ beads in 0.5 ml tubes (Amersham Pharmacia Biotech, Piscataway, NJ)[Amersham 1998]. The reactions reproducibly gave a product of approximately 1.5 kb. Amplification products were electrophoresed on 0.8% agarose gel, excised from the gel and further purified using QIAquick gel extraction column (Qiagen Inc. Santa Clarita, CA) as per manufacturer's instructions [Qiagen 1997]. Purified 16s rDNA were sequenced with an ABI PRISM™ 377 automated sequencer using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS. The PCR products were sequenced from both directions.

The computer program CLUSTALW [Higgins 1996] with default gap-change cost was used for multiple sequence alignment. Some ambiguous alignments were realigned by eye in MacClade [Maddison 1992]. Maximum parsimony (MP) phylogenetic analyses and distance analyses (Jukes – Cantor model of nucleotide substitution) [Jukes 1969] of the aligned sequences were performed using the computer program PAUP* version 4.0b2 [Swofford 1999] run on a Power Macintosh 6500/275. The phylogenetic signal in the data set for MP analysis was evaluated using the g_1 statistic [Hillis 1992] based on 100,000 random trees generated in PAUP*. Heuristic searches were performed using random stepwise addition (1000 replicates) of taxa followed by tree bisection-reconnection (TBR) branch swapping. Branches with a maximum length of zero were collapsed. Branch support for the MP analysis was evaluated using the nonparametric bootstrap procedure [Felsenstein 1985]. The evolution of microaerophily for a subset of the data was reconstructed in MacClade using ACCTRAN optimization accelerated transformation [Swofford 1987].

The 16S rDNA sequence of P. jessenii strain VT10 used in the present study has been deposited with GenBank and

assigned accession number AF191224. Whenever possible 16S rRNA sequences from type strains were used to construct the phylogenetic trees. The accession numbers of the sequences used in this study are as follows: Pseudomonas aeruginosa (LMG 1242T), Z76651; P.agarici (LMG 2112T), Z76652; P.alcaligenes (LMG 1224T), Z76653; P. amygdali (LMG 2123T), Z76654; P.asplenii (LMG 2137T), Z76655; P.caricapapayae (ATCC 33615T), D84010; P.chlororaphis (LMG 5004T), Z76657; P.cichorii (LMG 2162T), Z76658; P. coronafaciens (LMG 13190T) Z76660; P. cichorii (LMG 2162T), Z76658; P. coronafaciens (LMG 13190T) Z76660; P. corrugata (ATCC 29736T) D84012; P. ficuserectae (LMG 5694T), Z76661; P. fluorescens biovar I (DSMZ 50090T), Z76662; P. fragi (ATCC 4973T), D84014; P. fuscovaginae (MAFF 301177T) AB021381; P. jessenii (CIP 105274T), AF068259; P. marginalis (LMG 2210T), Z76663; P. meliae (MAFF 301463T), AB021382; P. mendocina (LMG 1223T), Z76664; P. mucidolens (IAM 12406T), D84017; P. oleovorans (DSMZ 1045T), Z76665; P. pertucinogena (IFO 14163T), AB021380; P. pseudoalcaligenes (LMG 1225T), Z76666; P. putida (DSMZ 291T), Z76667; P. resinovorans (LMG 2274T) Z76668; Pseudomonas sp. (strain IpA-2), X96788; P. stanieri (ATCC 27130T), AB021367; P. stutzeri (CCUG 11256T), X98607; P. synxantha (IAM 12356T), D84025; P. syringae (1247t_1T) Z76669; P. taetrolens (IAM 1653T) D84027; P. tolaasii (LMG 2342T) Z76670; P. viridiflava (LMG 2352T), Z76671; Acinetobacter calcoaceticus (ATCC 23055), Z93434; Bacillus thuringiensis (strain WS 2617), Z84594; B. cereus (ATCC 27877), Z84581; B. licheniformis, D31739; B. sphaericus, D16280; Corynebacterium xerosis, AF024653; Escherichia coli, J01859; Enterococcus faecalis, AF039902; Klebsiella pneumoniae, U33121; Lactobacillus plantarum, X52653; Listeria monocytogenes, X98530; Moraxella osloensis, X95304; Micrococcus luteus, AF057289; Salmonella enteritidis, U90318; Salmonella paratyphi, U88546; Streptococcus lactis, M58837; Shigella boydii, X96965; Streptococcus pyogenes (ATCC 12344), AB002521.

FAME analysis. Isolate VT10 was grown under conditions suggested for FAME analysis (MIDI Inc., Newark, Del)[MIDI 1994]. The cellular fatty acids were saponified, methylated and extracted by using the protocol of the Sherlock Microbial Identification System [MIDI 1994]. FAMEs were analyzed by gas chromatography. Distance based relationships using FAME profiles were evaluated using the MIDI-Sherlock software [MIDI 1994]. Xanthomonas maltophilia was used as the control organism.

Respirometry. An ER-100 batch type electrolytic respirometer was used as per instruction manual (Bioscience Management, Inc., Bethlehem, Pennsylvania) to determine oxygen uptake by VT10 at normal strength and 100× PTYG concentrations at 20^{0}C and pH 7.2. VT10 was grown in 2-liter flasks in 1 liter of PTYG medium for 24 hours at room temperature. The cells were harvested by centrifugation and washed twice with 0.1 g l^{-1} MOPS buffer (pH 7.2) and resuspended in similar strength MOPS buffer for 24 more hours at room temperature with stirring. The cells were then harvested and the cell pellet was resuspended in the 1 liter respirometer bottles containing the following: PTYG medium, 100× PTYG medium and the control was 0.1 g l^{-1} MOPS buffer. Oxygen uptake rates were measured for 120 min. To facilitate comparison, the oxygen uptake rates were calculated per gram of dry weight of VT10. The result shown in Figure 2 is the mean of three separate experiments.

Results

General characteristics of VT10. The strain characteristics of VT10 as seen in Table 2 shows that the isolate shares many characteristics with the genus Pseudomonas which belongs to the gamma subgroup of proteobacteria. Isolate VT10 was found to be motile only during the exponential phase of growth. The cells became progressively non-motile

over time during the stationary phase. Similar phenomena have been reported in Rhizobium meliloti [Wei 1998]. Isolate VT10 was tested for its ability to utilize a variety of compounds as the sole source of carbon under aerobic and anaerobic conditions, and the results are summarized in Table 2. Though VT10 could use a variety of compounds aerobically, it could use only dextrose, ethanol and glycerol anaerobically.

Phylogeny of isolate VT10. The level of sequence similarity between VT10 and the 34 reference species of Pseudomonas ranged from 90.12% to 99.00% (uncorrected p). The phylogenetic signal in this data set is considered to be significant under the assumptions of strict parsimony with all nucleotide positions weighted equally ($g1 = -1.58$; $P < 0.01$). A strict parsimony analysis of these data resulted in 177 equally parsimonious trees (1712 steps, CI = 0.60, RI = 0.71). Figure 8 is the strict consensus of these 177 trees. This phylogeney and the one based on Jukes-Cantor pairwise distances recovers the monophyly of Pseudomonas and clearly places the VT10 isolate within the genus. The monophyly of Pseudomonas and the placement of VT10 (Fig 8) had strong bootstrap support in both parsimony and distance analysis. Isolate VT10 shares a common ancestor with Pseudomonas sp IpA-2 and P. jessenii (Fig 8) and is most similar to these two species (99% similarity).

The cellular fatty acid composition of VT10 have 16:0, 16:1ω7c and isomers of 18:1 as the major constituent like most other pseudomonads [Vancannyet 1996]. These three fatty acids constituted 75.3 % of the total in VT10. Isolate IpA-2 which is very closely related to VT10 based on 16S rDNA analysis has the above mentioned fatty acids at a total concentration of 79.5% [Mohn 1999](Table 3). Isolate VT10's fatty acid profile show similarities to the published fatty acid profile of IpA-2 [Mohn 1999]. Strain VT10 was

identified as closest to Pseudomonas syringae using the MIDI library (isolate IpA-2 and P. jessenii were not present in the library). However, the presence of cyclopropane fatty acids differentiates VT10 from P. syringae in which cyclopropane fatty acids has not been detected [Vancannyet 1996]. Strain IpA-2 was also identified as closest to P. syringae by MIDI analysis [Mohn 99]. Fatty acid profile of type strain P. jessenii was not available for comparison.

Oxygen uptake rates. The oxygen uptake rate by strain VT10 was almost 3 times higher at high substrate concentration (100× PTYG) compared to low substrate concentration (1× PTYG) (Fig 2). The control flask with no substrate showed negligible oxygen uptake proving that endogenous respiration was almost absent. These results show that VT10 removes less oxygen from its surroundings when the substrate concentration is low.

Cell numbers as a function of substrate concentration. There were 4.8×10^{8} CFU ml^{-1} at 100× PTYG substrate concentration compared to 2.5×10^{7} CFU ml^{-1} at regular PTYG concentrations which has peptone, tryptone, yeast extract and glucose at 100 times less concentration.

Evidence of LSRMB in isolates from Mountain Lake. In this study the percent of LSRMB in isolates from the Mountain Lake ranged from 5 to 54%. The highest number 54%, was found in the surface waters of the oligotrophic freshwater Mountain Lake [Mazumder 2000], and the lowest number 5% was obtained from ~16 m below the surface of the Mountain Lake. Approximately 18% of the isolates from the bottom of the lake demonstrated LSRMB. We had already reported earlier

that the Oyster site sample obtained 5 m below the surface contained 26% isolates, which had LSRMB [Mazumder 2000].

Proposed LSRMB character evolution. Figure 10 shows the optimization of microaerophily on the proposed phylogeny. This analysis shows that LSRMB may have evolved at least two times within the eubacteria. Although the inclusion of additional taxa may affect the outcome of the character evolution analysis, it appears Pseudomonas and Bacillus has convergently evolved LSRMB.

Discussion

The phylogenetic tree based on 16S rDNA data indicates that VT10 is most likely a strain of Pseudomonas jessenii. Based on our analysis, strain IpA-2 isolated by Mohn et al. [Mohn 1999] is likewise probably a strain of P. jessenii. P. jessenii and isolate VT10 shared the following morphological and physiological characteristics [Verhille 1999]: both were Gram negative motile rods, they formed smooth circular non-pigmented colonies on nutrient agar and they were non hemolytic when grown on blood agar, both produced fluorescence on Kings B medium, they were catalase and oxidase positive, and could reduce nitrate to nitrite, did not liquefy gelatin, did not produce indole from tryptophan and did not produce coagulase. Both IpA-2 and VT10 could use ethanol, benzoate and phenol but could not use methanol. Strain IpA-2 exhibited LSRMB. Therefore, IpA-2 like VT10, is most probably a strain of P. jessenii. P. jessenii was not available for LSRMB testing.

The phylogenies presented in this study are based on MP and distance analyses. I prefer parsimony methods of evolutionary analysis over distance methods because MP trees pose common ancestry based on shared derived characters whereas dendograms based on distance methods are simply graphical representations of pairwise distance matrices and

technically do not convey evolution by descent. However, it is very important to consider multiple methods of phylogenetic reconstruction when horizontal gene transfer is a possibility. Consistency between the distance and parsimony analysis in this study suggests that the phylogenetic placement of strain VT10 close to Pseudomonas jessenii and strain IpA-2 is appropriate.

We have shown earlier that the LSRMB organisms grew as a sharp band underneath the surface of semi-solid medium when the substrate concentrations was low [Mazumder 2000]. The band of growth growing in rings between 5-20 mm in depth in the semi-solid media was most likely to be under microaerophilic conditions (0.2-12% oxygen) as determined earlier by using an oxygen sensor equipped with a platinum mini-electrode by Ferrara-Guerrero and Bianchi [Ferrara 1989]. This trait can easily go unnoticed in normal laboratory conditions where rich substrate media are used. Jannash had reported that some spirilla were microaerophilic when grown under growth limiting substrates [Jannash 1977]. Krieg and Hoffman later noted that cell density affected the behavior of some microaerophiles in semi-solid media incubated under atmospheric conditions [Krieg 1986]. As seen in our previous study the microaerophilic band of growth formed by VT10, moved upward to the surface of the semi-solid medium when the substrate concentration was increased. This behavior by VT10 could be due to two reasons: (a) the higher number of VT10 cells because of higher substrates, resulting in an increase in oxygen requirement and/or; (b) lower rates of oxygen removal by same number of VT10 cells when the nutrient concentrations are low. In defense of the first hypothesis we found that high substrate (100× PTYG) resulted in higher cell numbers. There were 4.8×10^{8} CFU ml^{-1} at 100× PTYG compared to 2.5×10^{7} CFU ml^{-1} at regular PTYG concentrations. The higher number of cells in the high substrate media undoubtedly resulted in higher levels of

oxygen removal from the immediate surroundings of the microorganisms, thus probably keeping the cells under microaerophilic conditions. The second hypothesis was tested by respirometry. We detected that low substrate concentration induced VT10 to reduce the rate of oxygen consumption (Fig 2). This low rate of oxygen uptake could result in higher levels of oxygen around VT10, which in turn could force the organism to seek microaerophilic conditions. We found that the oxygen uptake rate by VT10 under low substrate conditions was almost 3 times lower than when it was placed in high substrate concentration. Most probably the reason why microaerophilic bands disappeared when the substrate concentrations were high [Mazumder 2000] is due to a combination of high cell numbers and higher oxygen uptake rates.

The numbers of LSRMB bacteria in Mountain Lake were 54, 5 and 18 percent at the surface, 16 and 25 m depths respectively. The lake bottom is rarely anoxic and the sediment is the only part of the lake that is consistently anaerobic. The higher number of LSRMB bacteria at the deepest part of the lake compared to the 16 m hypolimnion probably reflects the effect of lateral groundwater seepage flow. The majority of the bacteria isolated from the surface of Mountain Lake show LSRMB and they are largely dependent on the phytoplankton for their energy. If LSRMB bacteria in the epilimnion and metalimnion water gain sufficient substrate from the photosynthetic species, then they can survive the toxic effects of oxygen bypoducts. The several survival mechanisms described for P. jessenii under laboratory conditions may be useful for the survival of Mountain Lake Pseudomonas sp. given that the substrate concentrations of the lake surface water approximates the PTYG liquid and semi-solid media used in this study.

The spacial and metabolic relationships between phytoplankton and LSRMB bacteria in Mountain Lake are unknown but bioflocculation may provide an explanation how the members of the community may seek their niche. For example LSRMB bacteria can escape higher oxygen levels by residing inside a floc. It has been shown that bioflocculation occurs when substrates become depleted [Stanley 1967, Bush 1968, Wu 1978]. Bioflocculation is genetically controlled, and gene expression occurs only in specific environments [Stewart 1977, Johnston 1979]. It remains to be seen if LSRMB is under some form of genetic control whose induction depends on environmental conditions. The positive and negative chemotaxis demonstrated by LSRMB bacteria in semi-solid agar may permit Pseudomonas sp. cells to move within the floc in response to oxygen and substrate concentrations. The P. jessenii used in this study was a subsurface strain, but since the type species strain was isolated from an aquatic habitat, the physiology may be similar. The recognition that the motility-chemotaxis mechanism is a part of several global stress response networks [Amsler 1993] suggests the ecological significance of LSRMB in habitats such as the epilimnion of Mountain Lake.

Bacteria with LSRMB were present in all the environments sampled in this study and a previous study [Mazumder 2000]. It appears to be a character shared by many Pseudomons sp. and some Bacillus sp. and may be much more widespread than previously thought [Mazumder 2000]. Previous research in our laboratory had shown that VT10 produced cyclopropane fatty acid, a membrane component usually associated with stress, and several new proteins, when grown under low substrate and ambient oxygen [Mazumder 2000]. It is possible that physiological stress for these microaerophiles may be manifested by ambient oxygen and low substrate concentrations and their motility response to

microaerophilic conditions is to avoid this oxygen stress. LSRMB may provide organisms a competitive advantage. The ability to move away from stressful environments to microniches more favorable for growth and prolifiration is undoubtedly a great adaptive value to aquatic and soil bacteria.

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Table 2. General characteristics and metabolic range of isolate VT10

Strain characteristics		Substrate use as sole source of carbon		
			Aerobic	Anaerobic
Gram reaction	-	Dextrose	+	+
Motility	+	Maltose	-	-
Length (μm)	1.44 (±0.2)	D-Mannitol	+	-
		Ethanol	+	+
Catalase	+	Methanol	-	-
Oxidase	+	Glycerol	+	+
Doubling time (min)	51.37	Benzoate	+	-
Use of nitrate	+	Acetate	+	-
Growth at 41^{0}C	-	Succinate	+	-
Growth at 4^{0}C	+	Tannic acid	-	-
Fermentation	-	Citrate	+	-
Fluoresces (Kings B medium)	+	Phenol	+	-

Table 3. Fatty acid profile of isolate VT10 and IpA-2. Fatty acid names are total number of carbon atoms, the second number after the colon is the double bonds and position with respect to the alipahatic end (ω end), c denotes cis configuration of the double bond, cyclopropyl (cyc) fatty acids are denoted by the total number of carbons and the position of the hydroxyl from the carboxyl end of the fatty acid is denoted by the number-OH.

Fatty acids	% of total fatty acid	
	VT10[a]	IpA-2[b]
10:0 3OH	3.9 (1.5) [d]	4.7
12:0	4.0 (0.1)	3.9
12:0 2OH	3.0 (0.4)	4.1
12:0 3OH	4.1 (0.7)	5.0
16:0	27.8 (2.8)	27.5
16:1 ω7c	30.7 (5.0)	37.7
17:0 cyc	1.3 (0.1)	-[c]
18:0	1.21 (1.0)	<1
18:1 x[e]	16.8 (2.3)	14.3
19:0 cyc ω8c	1.3 (1.7)	-

[a] Identified as Pseudomonas syringae (0.508 match)

[b] IpA-2 results are from previously published data [Verhille, 1999]

[c] Not detected

[d] Standard deviation

[e] The fatty acids 18:1 ω7c, 18:1 ω9t and 18:1 ω12t could not be separated and was considered together

Conclusions and Future Areas of Investigation

Final conclusions of my research are as follows:

- Low substrate regulated microaerophilic behavior is a common ecological strategy in many aquatic and soil habitats.
- Low substrate regulated microaerophilic behavior is a strategy that bacteria use in response to oxygen stress.
- Strain VT10 produces stress fatty acids and additional proteins when grown in ambient oxygen and low substrate concentrations.
- Chemotaxis is not always related to utilization of the substrate.
- Strain VT10 is closely related to Pseudomonas jessenii and strain IpA-2, which were isolated from French mineral waters and a Canadian wastewater treatment plant respectively.

The physiological response of bacteria in response to low levels of substrate and oxygen will provide better insight into the survival strategies of low substrate regulated microaerophiles. In addition to the physiological observation done during my dissertation work, research into the molecular basis of low substrate regulated microaerophily needs to be done. The study of strains containing mutations in genes which affect the cells

response to oxygen and substrate stress and band formation, may be helpful in assessing the role of such genes in the ecology of these microaerophiles in the environment. Influence of electron transport on the regulation of metabolic process in P. jessenii needs to be studied. Research into the impact of respiratory inhibitors or mutations on gene expression and metabolic process may help us determine the sensory role of the respiratory electron transport system in these microorganisms. Blocking normal electron flow either by inhibitors or by knocking out genes responsible for respiratory enzymes might lead to altered oxygen taxis and hence inability to form microaerophilic bands of growth. For example Grishanin et al. showed that inhibitors of photosynthetic electron transport arrested the elicitation of photoresponse in Rhodobacter sphaeroides [Grishanin RN, DE Gauden, JP Armitage. 1997. Photoresponses in Rhodobacter sphaeroides: role of photosynthetic electron transport. J Bacteriol. 179:24-30]. It is possible that LSRMB is a result of negative chemotaxis to oxygen and reactive oxygen species. Grishanin and Bibikov has written a detailed review on redox taxis and taxis repellent effect of the reactive oxygen species [Grishanin RN, SI Bibikov. 1997. Mechanism of oxygen taxis in bacteria. Biosci Rep. 17:77-83.]. Further experimentation may reveal information to increase our knowledge of how these aerobic bacteria deal with this oxygen paradox.

Appendix A: Lipid extraction

Lipid extraction and analysis.

Sequential alkaline saponification/acid hydrolysis /esterification: a one-tube method with enhanced recovery of both cyclopropane and hydroxylated fatty acid. Modification of the Mayberry and Lane method [Mayberry WR, Lane JR. 1993. Sequential alkaline saponification/acid hydrolysis/esterification: a one-tube method with enhanced recovery of both cyclopropane and hydroxylated fatty acids. J Microbiol Methods 18:21-32].

Method

1. Use fresh reagents and nanopure water.
2. Test tube caps should be rinsed with acetone.
3. Cells grown to exponential phase of growth in 1 l of medium.
4. Tubes and all glassware should be muffled for at least 4 h in a 450^{0}C oven.
5. Procedure for extraction carried out in 30-ml screw caps.
6. Sample harvested by centrifugation and washed three times with nanopure water. Re-suspended in 1 ml of nanopure water.
7. 0.5 ml of 2M KOH added and caps tightened. Heated at 100^{0}C for 1 h. Cooled rapidly by placing in water-bath. 0.94 ml of 6M HCl added. Immediately 1 ml of n-heptane added after that. Caps tightend and tubes placed in 100^{0}C overnight.
8. Extracted with 3 ml of chloroform. Vortexed and centrifuged for phase separation.
9. Aqueous phase removed from the top and to the organic phase the following are added respectively: 0.2 ml

acetic anhydride, 0.2 ml HCl (concentrated), and 4 ml of methanol. Mixture heated at 55^0C for 30 min. Rapidly cooled in water-bath.

10. Partitioned against 4 ml of 1M $NaHCO_3$. 3-ml chloroform added then washed three times by partitioning against 3-ml water.
11. Evaporated to dryness under nitrogen. Residue re-suspend in 0.5 ml of n-heptane.
12. Transfered to GC vials and injected into the GC column.

Appendix B: Figures

Figure Legends

Figure 1. Low substrate regulated microaerophilic band of Pseudomonas jessenii strain VT10 cells located 2 cm below the surface of 1× semi-solid PTYG medium in a 150-ml test-tube (test-tube shown is larger than used in experiments). The band as indicated by the arrow is sharply defined and is ~1 mm thick. Photograph taken after 48 h of incubation at room temperature.

Figure 2. Content of cyclopropane fatty acid (CFA) in the total fatty acid of isolate VT10, when grown under different conditions of substrate concentration and oxygenation as summarised in text. Maximum amount of the stress fatty acid, CFA, was produced when VT10 was grown at low substrate-high oxygen conditions (condition B). Results from at least three replicates. Error bars indicate 1 SD.

Figure 3. 2-D PAGE of VT10 cells when grown under different conditions as summarised in text. Proteins marked by block arrows were low substrate specific; proteins whose synthesis increased in response to low substrate (condition A and B) are marked by arrows; proteins which are circled are low substrate-high oxygen (condition B) specific; proteins whose synthesis increased in response to low substrate-high oxygen (condition B) are boxed; proteins represented by triangles were either high substrate (condition C and D) specific or whose synthesis were higher during growth on high substrate.

Figure 4. Apparatus used in assaying chemotaxis of motile subsurface microaerophilic isolates VT10 and MR100. After

appropriate chemotactic incubation times the contents of the syringe were diluted and then plated on PTYG 100× medium for determining CFUs/ml that had entered the chemotactic apparatus. Components are detailed in text.

Figure 5. A comparison of chemotactic response of isolate VT10 and MR100 using different molarities of dextrose. Duration of chemotactic incubation time was 45 min. Error bars are ± the square root of the number of CFUs counted times the dilution.

Figure 6. A comparison of chemotactic response of isolate VT10 and MR100 using different test compounds. Concentration of chemicals 10^{-3}M. Duration of chemotactic incubation time was 45 min. Error bars are ± the square root of the number of CFUs counted times the dilution.

Figure 7. Swarm response of VT10 and MR100 cells on different concentrations of glucose (a) and glycerol (b) with 0.25% (wt/vol.) semisoft noble agar. Cells (~10^7 cells/ml) grown in PTYG medium were inoculated in the center of the Petri plates. Plates were incubated for 24 h at room temperature, and swarm diameters were measured.

Figure 8. Strict consensus of 42 equally parsimonious trees (1712 steps, CI = 0.60, RI = 0.71) based on 1512 bp of 16S rDNA (phylogenetic signal significant: g_1= -1.60, $P < 0.01$). Numbers above nodes indicate 50% majority rule bootstrap consensus values. The sequence divergence (uncorrected p) in this data set ranged from 13 to 26% with an average divergence of 21%. VT10 shares a common ancestor with Pseudomonas sp. IpA-2 and P. jessenii and is most similar

to these two species (99% similarity). Identity levels ranged from 90.12% to 99.00%.

Figure 9. Oxygen uptake rates by VT10 at low (0.01g l^{-1}) and high nutrient (1 g l^{-1}) concentrations. Equal numbers of starved buffer washed cells were used in all the respirometer bottles. There was no detectable increase in the dry weight of the cells or CFU ml^{-1} in the low and high substrate bottles after the experiment.

Figure 10. Most parsimonious reconstruction of the evolution of microaerophily using ACCTRAN optimization in MacClade. The phylogeny is based on 1498 bp of 16S rDNA and is one of two MP solutions. This analysis shows that microaerophily has evolved at least two times within the eubacteria and may be synapomorphic for Pseudomonas.

Figures

Figure 1.

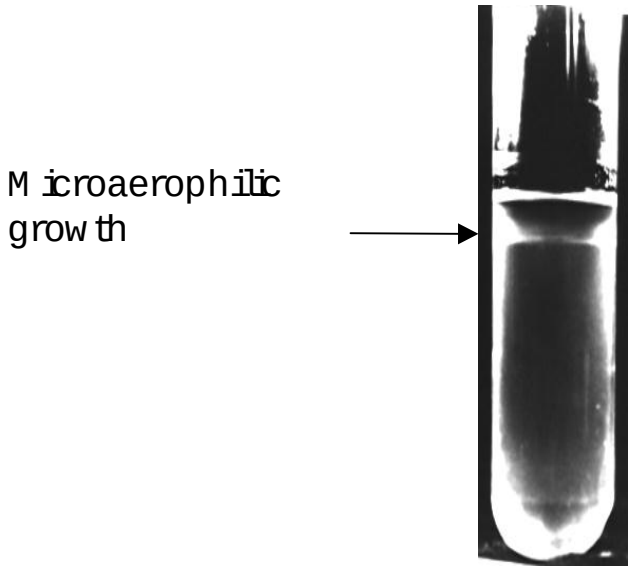


Figure 2.

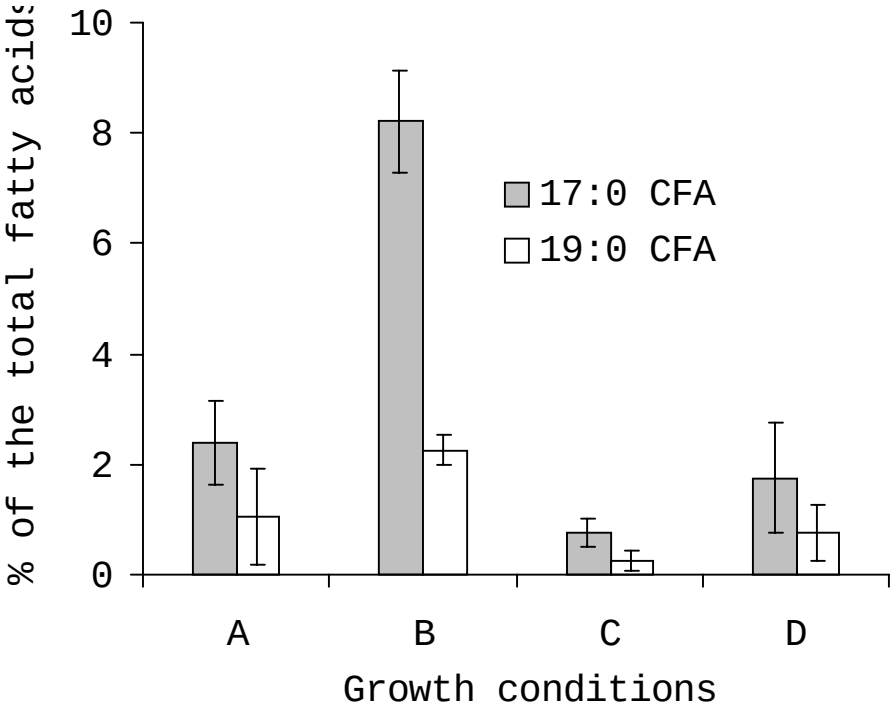

% of the total fatty acids
10
8
6
4
2
0
17:0 CFA
19:0 CFA
A
B
C
D
Growth conditions

Figure 3.

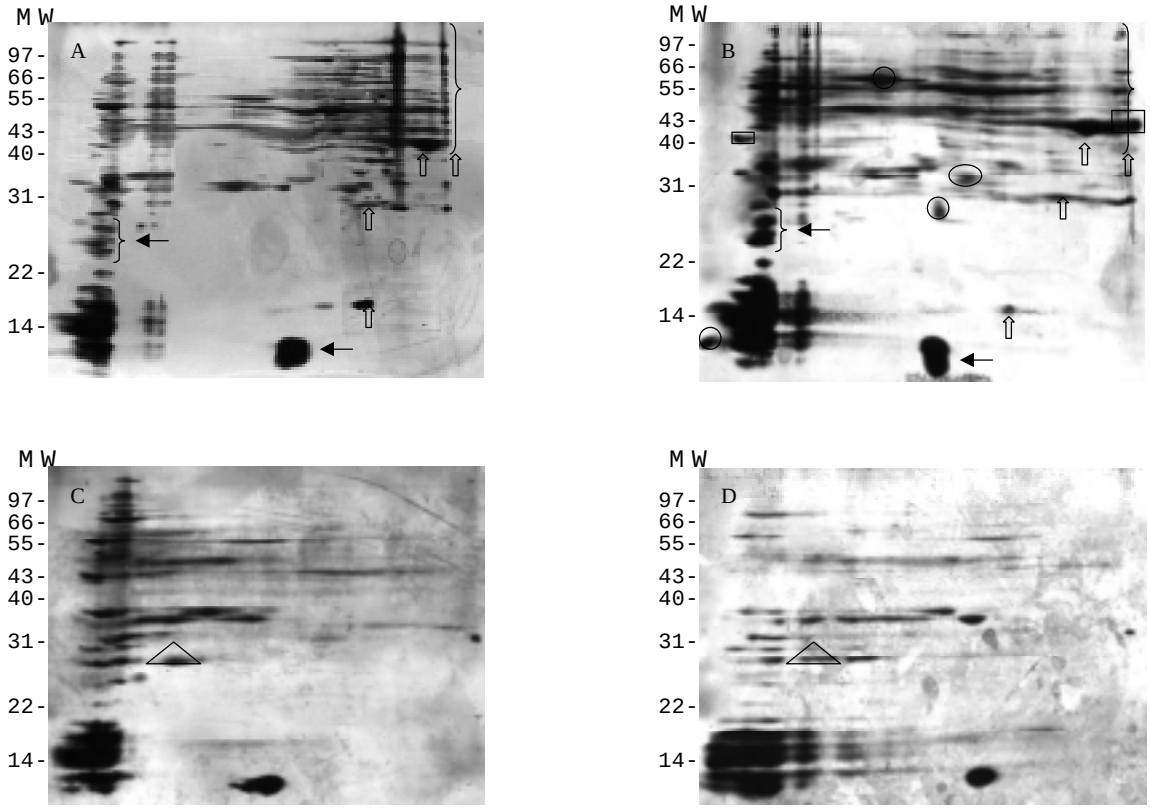


Figure 4.

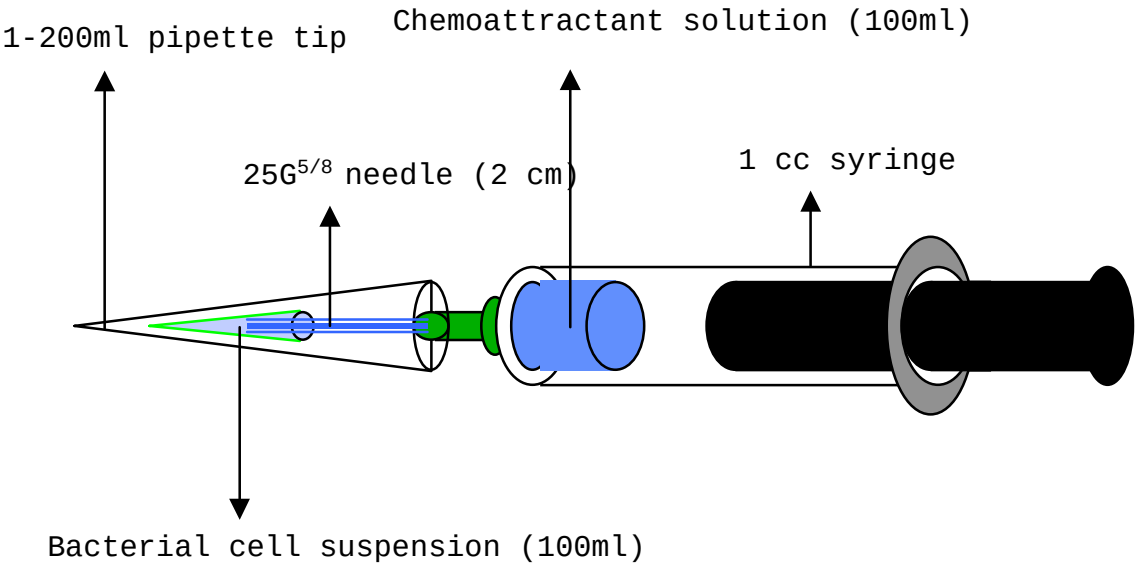

1-200ml pipette tip
Chemoattractant solution (100ml)
25G5/8 needle (2 cm)
1 cc syringe
Bacterial cell suspension (100ml)

Figure 5.

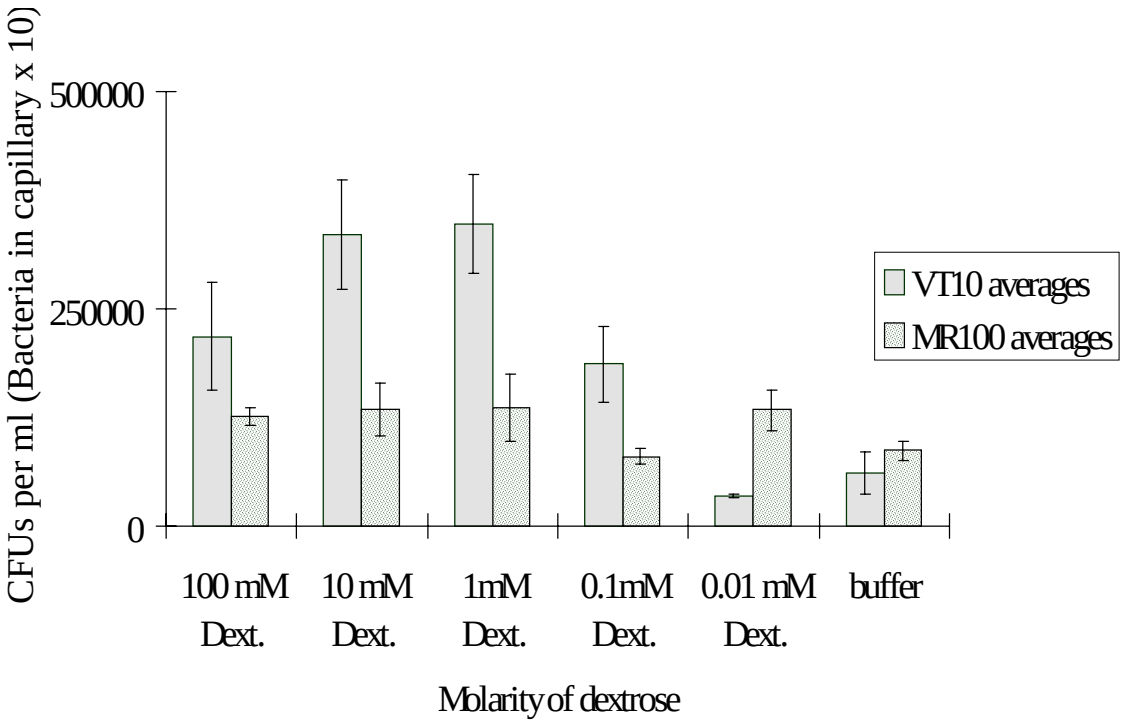

CFUs per ml (Bacteria in capillary x 10)
500000
250000
0
VT10 averages
MR100 averages
100 mM Dext.
10 mM Dext.
1mM Dext.
0.1mM Dext.
0.01 mM Dext.
buffer
Molarity of dextrose

Figure 6.

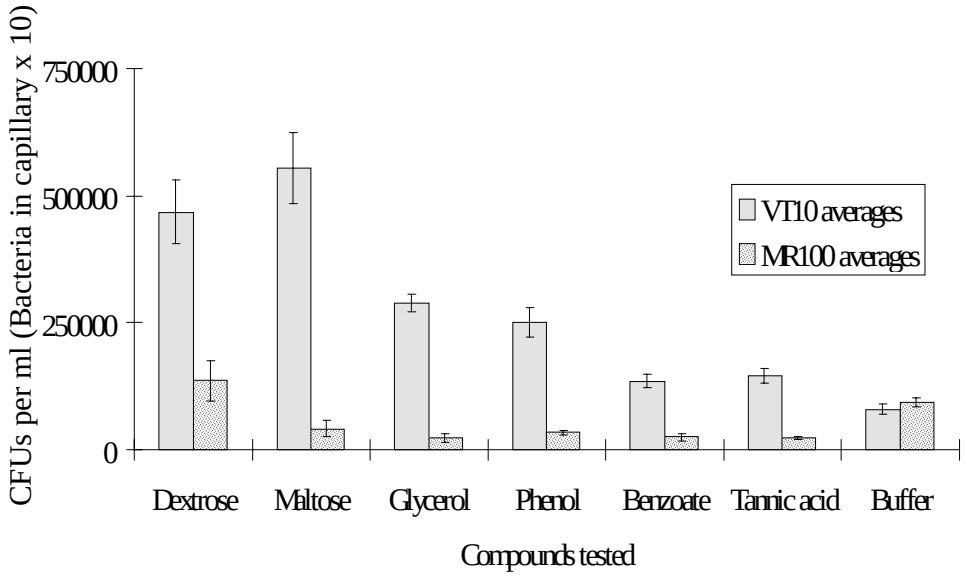

CFUs per ml (Bacteria in capillary x 10)
750000
500000
250000
0
VT10 averages
MR100 averages
Dextrose
Maltose
Glycerol
Phenol
Benzoate
Tannic acid
Buffer
Compounds tested

Figure 7a.

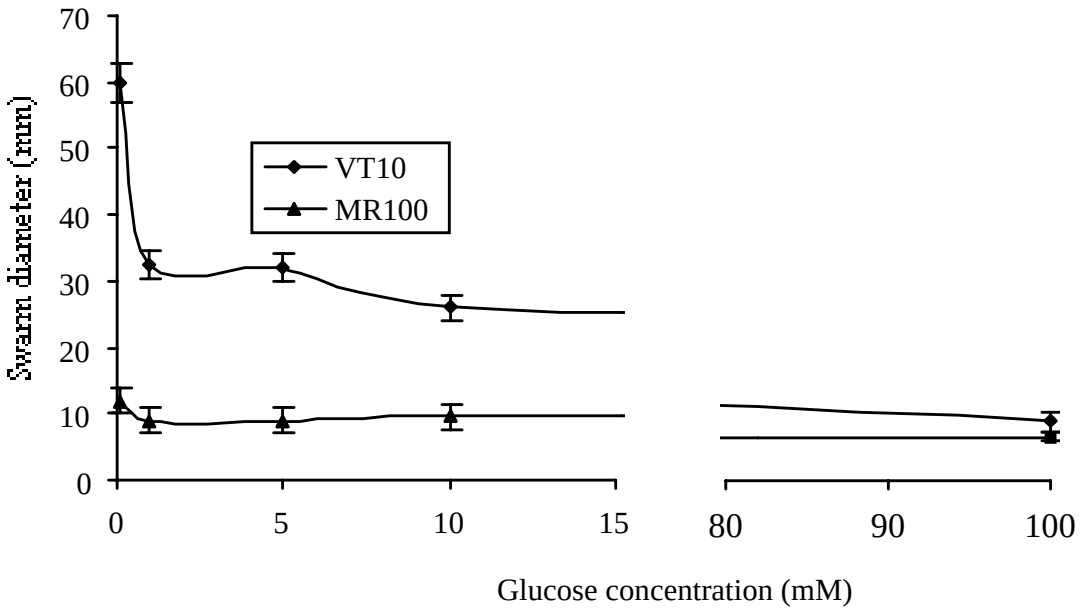

Swarm diameter (mm)
70
60
50
40
30
20
10
0
VT10
MR100
0
5
10
15
80
90
100
Glucose concentration (mM)

Figure 7b.

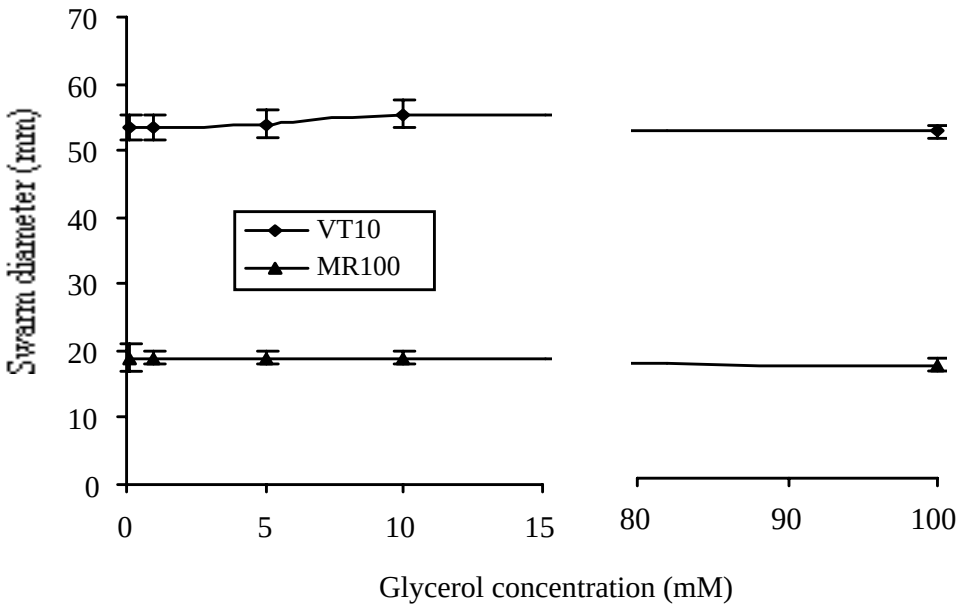

70
60
50
40
30
20
10
0
Swarm diameter (mm)
VT10
MR100
0
5
10
15
80
90
100
Glycerol concentration (mM)

Figure 8.

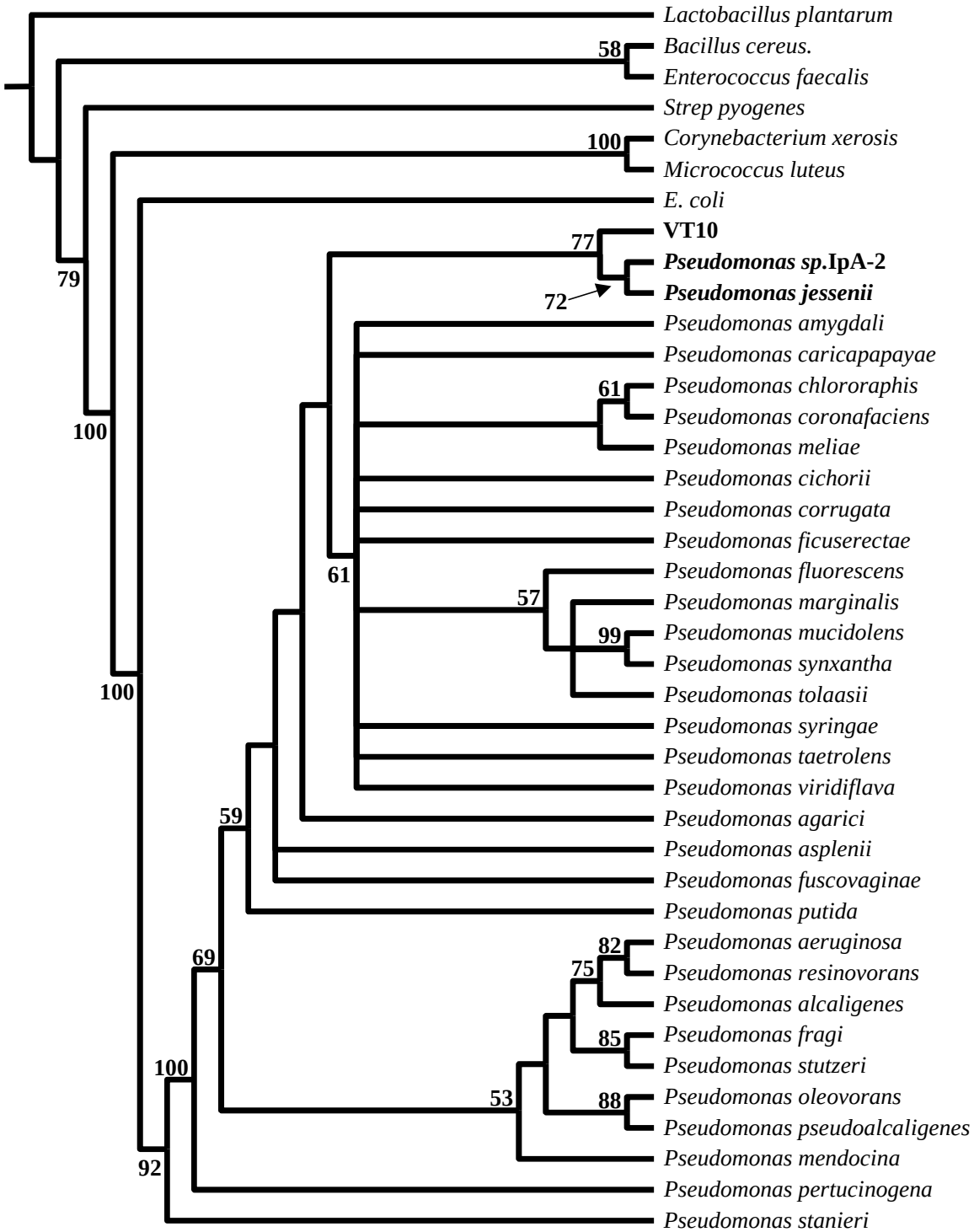

Lactobacillus plantarum
58
Bacillus cereus.
Enterococcus faecalis
Strep pyogenes
100
Corynebacterium xerosis
Micrococcus luteus
E. coli
77
VT10
Pseudomonas sp.IpA-2
79
72
Pseudomonas jessenii
Pseudomonas amygdali
Pseudomonas caricapapayae
61
Pseudomonas chlororaphis
100
Pseudomonas coronafaciens
Pseudomonas meliae
Pseudomonas cichorii
Pseudomonas corrugata
Pseudomonas ficuserectae
61
Pseudomonas fluorescens
57
Pseudomonas marginalis
99
Pseudomonas mucidolens
100
Pseudomonas synxantha
Pseudomonas tolaasii
Pseudomonas syringae
Pseudomonas taetrolens
Pseudomonas viridiflava
59
Pseudomonas agarici
Pseudomonas asplenii
Pseudomonas fuscovaginae
Pseudomonas putida
82
Pseudomonas aeruginosa
69
75
Pseudomonas resinovorans
Pseudomonas alcaligenes
85
Pseudomonas fragi
100
Pseudomonas stutzeri
53
88
Pseudomonas oleovorans
Pseudomonas pseudoalcaligenes
Pseudomonas mendocina
92
Pseudomonas pertucinogena
Pseudomonas stanieri

Figure 9.

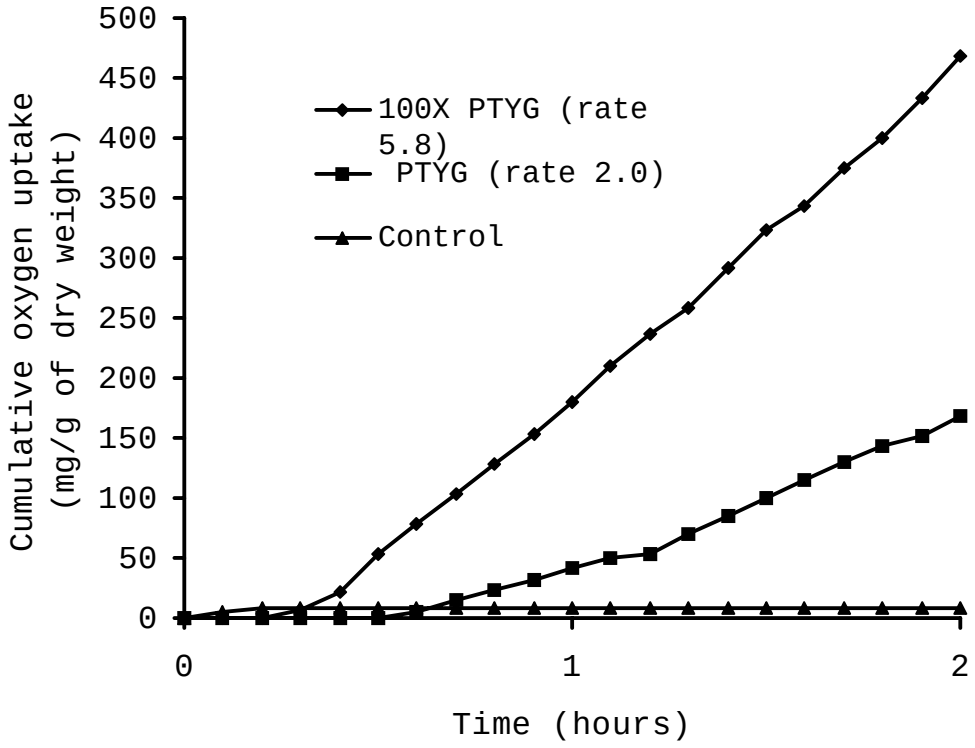
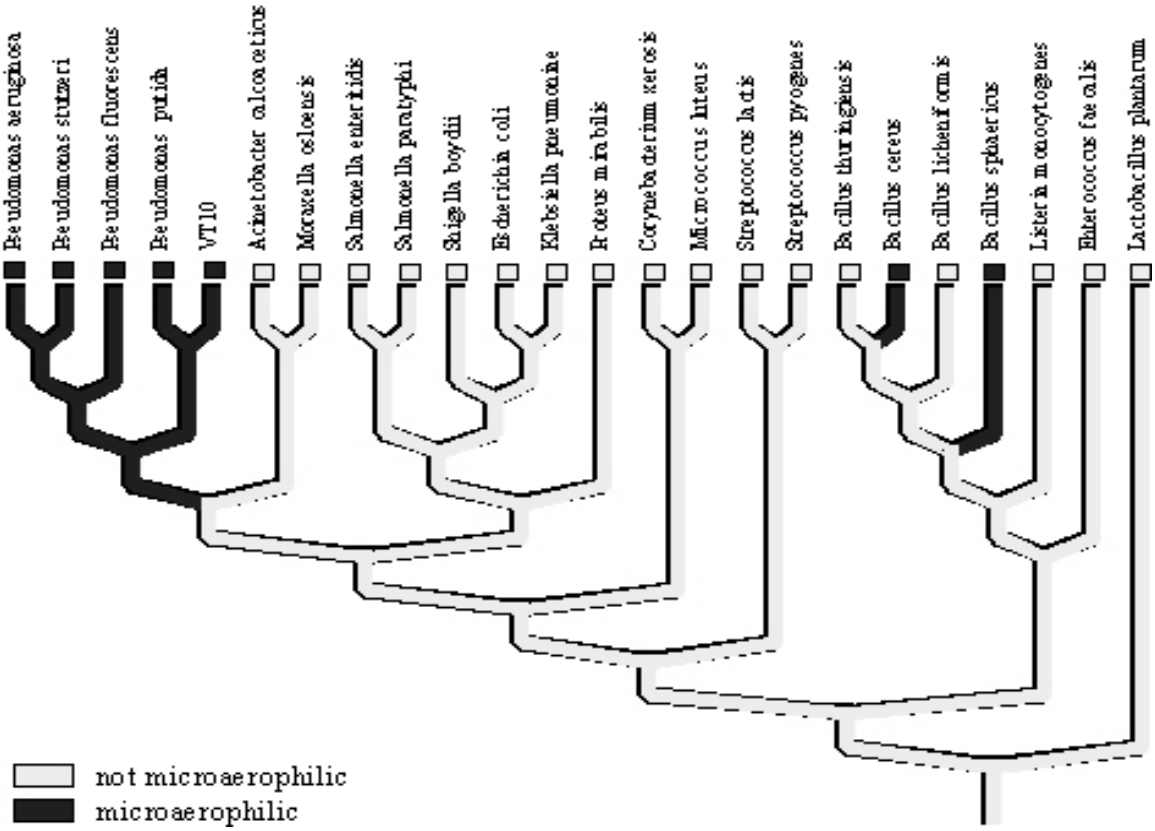

500
450
400
350
300
250
200
150
100
50
0
Cumulative oxygen uptake
(mg/g of dry weight)
100X PTYG (rate 5.8)
PTYG (rate 2.0)
Control
0
1
2
Time (hours)

Figure 10.


Pseudomonas aeruginosa
Pseudomonas stutzeri
Pseudomonas fluorescens
Pseudomonas putida
VT10
Acinetobacter calcoaceticus
Moraxella osloensis
Salmonella enteritidis
Salmonella paratyphi
Shigella boydii
Escherichia coli
Klebsiella pneumoniae
Proteus mirabilis
Corynebacterium xerosis
Micrococcus luteus
Streptococcus lactis
Streptococcus pyogenes
Bacillus thuringiensis
Bacillus cereus
Bacillus licheniformis
Bacillus sphaericus
Listeria monocytogenes
Enterococcus faecalis
Lactobacillus plantarum
not microaerophilic
microaerophilic

Curriculum Vitae

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Background Microbiology, Biotechnology, Molecular Biology.

Education

Ph.D. Microbiology, Virginia Tech, Virginia, 2000.
Dissertation: Substrate regulated microaerophily and chemotaxis by Pseudomonas jessenii strain VT10

M.S. Microbiology, University of Mississippi, Mississippi, 1994.
Thesis: Biodegradation of azo dyes by bacterial isolate C7

B.S. Immunology, University of Calcutta, India, 1990.

Skills

Microbiology:

Bacteria isolation, identification, enrichment and maintenance; batch and continuous culture operation; media design; microbiological profiling.

Molecular/Analytical:

DNA manipulation; genomic library prep.; PCR; cloning; enzyme assays; enzyme kinetics; radioisotope assays; fatty acid analysis; immunological assays; SDS-PAGE, 2-D PAGE; GC/MS & FID; HPLC; TLC; Familiar with Oligonucleotide synthesis.

Computer:

Operating systems: Windows, MacOS, UNIX, DOS. Programming language: C++. Computer modeling, and various research and analytical software use.

Professional Experience

1996-present: Ph.D. candidate, **Virginia Tech,** Blacksburg, VA.

Developed a novel way to measure chemotaxis and traced the evolution of microaerophily.

Developed techniques to biologically monitor oxygen and nutrient stress.

Taught laboratory sections of microbiology (BIOL2614 and CE/BIOL4154).

Supervised 6 undergraduate research projects (1996-1999).

1998 May-Sept.: Primary investigator, Tech. Ops., **Merck and Co. Inc.** Elkton, VA.

Performed microbial profiling of wastewater unit operations and wrote Standard Operating Procedures.

1995: Research technician, Oak Ridge National Laboratory (**ORNL**), Oak Ridge, TN.

Evaluated the metabolic potential of bacteria associated with a pristine site in Virginia.

1993 - 1994: M.S. student. **University of Mississippi**, University, MS.

Characterized a novel oxygen insensitive azoreductase enzyme.

Training

Training Workshop in Teaching. Virginia Tech. 1996

24-hr Hazwoper. International Chemical Workers Union & ORNL, 1995

Training Program in Public Relations & Time Management. MARG, 1991

Honors

Sigma Xi Grants-in-Aid of Research Award, 1998; Department of Biology, Virginia Tech. Grants-in Aid of Research Award, 1998; Post Masters Fellowship, Oak Ridge Associated Universities, ORNL, 1995; Doctoral Research Grant ORNL, 1996-1997. Honors Fellowship, Graduate School, University of Mississippi, 1993-94.

Affiliations

Member of American Society for Microbiology; GSA representative to the Intellectual Property Rights Committee, Virginia Tech. 1998-1999; International programs director for Rotaract Club. 1989-1991

PUBLICATIONS

1. **Mazumder, R**., H. Pinkart, P. S. Alban, T. J. Phelps. R. E. Benoit. 1999. Low nutrient and ambient oxygen levels induce excess cyclopropane fatty acid production and protein profile changes in facultative microaerophile Pseudomonas jessenii strain VT10. Current Microbiology. Manuscript in press.
2. **Mazumder, R**., J. E. Bond, T. J. Phelps, R. E. Benoit. Facultative microaerophily a common ecological strategy. Will be submitted in April 2000.
3. **Mazumder, R**., A. Mikell, J. R. Logan, S. W. Hooper. 1999. Characteristics and purification of an oxygen-insensitive azoreductase from Caulobacter subvibrioides strain C7-D. Journal of Industrial Microbiology and Biotechnology. 23:476-483.
4. **Mazumder, R**., T. J. Phelps, N. R. Krieg, R. E. Benoit. 1999. Determining chemotactic responses by two subsurface microaerophiles using a simplified capillary assay method. Journal of Microbiological Methods. 37:255-263.
5. Zhang, C., S. Liu, J. Logan, **R. Mazumder**, and T. J. Phelps. 1996. Enhancement of Fe (III), and Cr (VI) reduction at elevated temperatures and by a thermophilic bacterium. Applied Biochemistry and Biotechnology. 57/58:923-932.
6. **Mazumder, R**. 1994. Biodegradation of azo dyes by bacterial isolate C7. M.S. Thesis. The University of Mississippi, University, MS. December 1994.

CONFERENCE PRESENTATIONS

1. Baki, K, **R. Mazumder**, RE Benoit. 1999. A survey of low substrate regulated microaerophiles. Meeting of the VA

Branch of the American Society for Microbiology, Blacksburg, Virginia, November, 1999.

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