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Regulation of Twitching Motility in *Pseudomonas aeruginosa* by the *las*
and *rhl* Quorum-Sensing Systems and The Distribution of the *narG* gene
of *Escherichia coli* Among Denitrifying Bacteria

Dissertation

Submitted to

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The Degree

Doctor of Philosophy in Biology

By

Alex Lee Glessner

UNIVERSITY OF DAYTON

Dayton, Ohio

Spring, 2005

APPROVED BY:



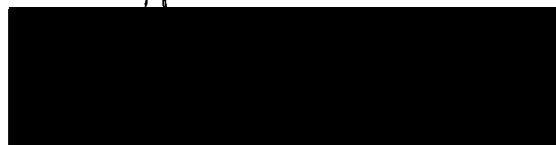
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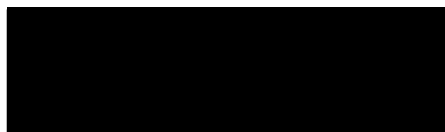
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ABSTRACT

REGULATION OF TWITCHING MOTILITY IN *PSEUDOMONAS AERUGINOSA* BY THE *LAS* AND *RHL* QUORUM-SENSING SYSTEMS AND THE DISTRIBUTION OF THE *NARG* GENE OF *ESCHERICHIA COLI* AMONG DENITRIFYING BACTERIA

Denitrification is a bacterial process in which nitrate is converted to nitrogen gas. The respiratory nitrate reductase (NR) enzyme catalyzes the first step in the pathway of bacterial denitrification. DNA probes constructed from the *narG* gene of *Escherichia coli*, which codes for the α -subunit of the enteric respiratory nitrate reductase (NR), were used in this study to determine the genetic relatedness of this enzyme among known nitrate reducing bacteria and environmental isolates. Of the 15 known nitrate reducing bacteria in our survey, nine representing six genera, were shown to share homology to the *narG* gene of *E. coli* under stringent hybridization conditions. Thirty-nine environmental strains, isolated from denitrifying enrichments were also tested for homology with *narG*. The enrichments were prepared from four different sites, including a pristine fen, a degraded wetland, an agricultural field, and stream sediment. Of the 39 isolates, 35 gave a positive hybridization signal in colony blots under stringent conditions. Southern blots of restricted genomic DNA from each of these 35 isolates were performed and a total of nine different banding patterns, or genotypes, were observed. None of the banding patterns were unique to a specific environmental site. Nitrate uptake studies were conducted to further characterize each of the genotypes. This survey suggests that the *narG* gene of *E. coli* is widely distributed among a variety of enteric nitrate-reducing and denitrifying bacteria.

Pseudomonas aeruginosa is a ubiquitous environmental bacterium and an important human pathogen. The production of several virulence factors by *P. aeruginosa* is controlled through two quorum-sensing systems, *las* and *rhl*. Both the *las* and *rhl* quorum-sensing systems are also required for type-4 pili dependent twitching motility and infection by the pili-specific phage, D3112cts. Mutants which lack the ability to synthesize PAI-1, PAI-2, or both autoinducers were significantly or greatly impaired in twitching motility and in susceptibility to D3112cts. Twitching motility and phage susceptibility were partially restored by exposure of the autoinducer deficient mutants to exogenous PAI-1 and PAI-2. Both twitching motility and infection by pili-specific phage are believed to be dependent on the extension and retraction of polar type-4 pili. Western blot analysis of whole cell lysates and ELISAs of intact cells were used to measure the amounts of pilin on the cell surfaces of *las* and *rhl* mutants relative to the wild type. It appears that *rhl* plays a crucial role in the export and assembly of surface type-4 pili twitching motility and phage infection by affecting the export and assembly of surface type-4 pili. Microscopic analysis of twitching motility indicated that mutants which were unable to synthesize PAI-1, via the *las* gene, were defective in the maintenance of cellular monolayers and migrating packs of cells.

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I would like to thank Dr. Jayne Robinson, my advisor, for providing time, and guidance to able me to complete this dissertation.

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b. Macroscopic stab assay for twitching motility. LB (1% agar) plates were stab inoculated to the bottom of the plate and incubated at 37°C for 24 hours. The twitch zone (diffuse zone), at the petri dish/agar interface, is a measure of twitching motility. The center, dense zone is surface colony growth. Strains courtesy of Dr. John Mattick, University of Queensland. Assays performed by Dr. Jayne Robinson, University of Dayton.....80

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CHAPTER 1.

The Distribution of the *narG* gene of *Escherichia coli*
Among Denitrifying Bacteria

ABSTRACT

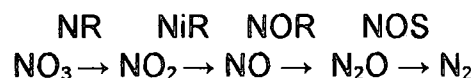
Denitrification is a bacterial process in which nitrate is converted to nitrogen gas. The respiratory nitrate reductase (NR) enzyme catalyzes the first step in the pathway of bacterial denitrification. DNA probes constructed from the *narG* gene of *Escherichia coli*, which codes for the α -subunit of the enteric respiratory nitrate reductase (NR), were used in this study to determine the genetic relatedness of this enzyme among known nitrate reducing bacteria and environmental isolates. Of the 15 known nitrate reducing bacteria in our survey, nine representing six genera, were shown to share homology to the *narG* gene of *E. coli* under stringent hybridization conditions. Thirty-nine environmental strains, isolated from denitrifying enrichments were also tested for homology with *narG*. The enrichments were prepared from four different sites, including a pristine fen, a degraded wetland, an agricultural field, and stream sediment. Of the 39 isolates, 35 gave a positive hybridization signal in colony blots under stringent conditions. Southern blots of restricted genomic DNA from each of these 35 isolates were performed and a total of nine different banding RFLP patterns, or genotypes, were observed. None of the banding patterns were unique to a specific environmental site. Nitrate uptake studies were conducted to further characterize each of the genotypes. This survey suggests that the *narG* gene of *E. coli* is widely distributed among a variety of enteric nitrate-reducing and denitrifying bacteria.

INTRODUCTION

Anaerobic respiration occurs only in bacteria and is much like its aerobic counterpart. Instead of oxygen as the terminal electron acceptor, another inorganic compound is reduced (Zumft, 1997). Nitrate respiration has been studied more completely than any other form of anaerobic respiration. There are two primary modes of dissimilatory nitrate reduction. The first is found in organisms such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. These bacteria, called nitrate respirers, reduce nitrate to nitrite via a membrane-bound dissimilatory nitrate reductase (DNR). The nitrite is then reduced to ammonium by one of the two forms of nitrite reductase (Rowe et al, 1994 and Stewart, 1998). The second mode, termed denitrification, is found in obligate respiratory bacteria such as *Paracoccus denitrificans*, *Pseudomonas stutzeri*, and *P. aeruginosa* and is physiologically very different from the *E. coli* type of nitrate respiration (Hochstein and Tomlinson, 1988).

Biological denitrification is defined as a bacterial respiratory process in which there is a sequential reduction of nitrogenous oxides (e.g., nitrates or nitrites) to gaseous end products, principally N_2O or N_2 (Zumft, 1992). In denitrification, four reactions catalyzed by four different membrane-bound

enzymes are responsible for the reduction of nitrate to dinitrogen:



The first enzyme, nitrate reductase (NR), reduces nitrate to nitrite. Next, nitrite is reduced to nitric oxide by nitrite reductase (NiR). Nitric oxide reductase (NOR) catalyzes the reduction of nitric oxide to nitrous oxide. Finally, nitrous oxide reductase (NOS) converts nitrous oxide to dinitrogen (Hochstein and Tomlinson, 1988). It is this reaction that distinguishes denitrifiers from nitrate respirers (Hochstein and Tomlinson, 1988).

Denitrification is controlled by levels of O_2 , N_2O , and N_2O , as well as the availability of organic compounds because most denitrifying bacteria are heterotrophs, requiring organic carbon sources for growth (Zumft, 1997). The enzymes and membrane proteins involved in denitrification are synthesized at their highest levels in the presence of nitrate, under anaerobic conditions (Stewart et al, 1989). Oxygen inhibits nitrate uptake needed for the denitrification process (Hernandez and Rowe, 1988). Respiratory denitrification is a facultative process, since the bacteria that can use NO_3^- or NO_2^- as a terminal electron acceptor will preferentially use O_2 (Zumft, 1997). Factors controlling denitrification in natural environments are not well understood, and accurate denitrification rates have proven difficult to measure due to high temporal and spatial variation (Murray et al, 1995 and Zumft, 1992).

It has been suggested that the process be called respiratory denitrification based upon the described criteria and methodology for identifying respiratory denitrifiers, which distinguish this group from other groups that reduce nitrate or produce N_2O (Mahne and Tiedje, 1995). The process of denitrification is defined by nitrogen gas (N_2 or N_2O) produced from nitrate or nitrate reduction while increasing growth at a higher rate than if the nitrogenous oxide were an electron sink (Mahne and Tiedje, 1995). Denitrification is carried out by a wide range of bacteria, including members of all subgroups of the proteobacteria, gram-positive bacteria, cyanobacteria, and some of the Archae (Zumft, 1997).

The most well studied bacterial dissimilatory nitrate reductase is that of *E. coli* (Moreno-Vivian, 1999). The nitrate reductase enzyme complex of *E. coli* is coded by the *narGHIJ* operon (Moreno-Vivian, 1999). The nitrate reductase is a three subunit enzyme oriented so the active site faces the cytoplasm (Zumft, 1997). The α subunit, coded by *narG*, contains molybdenum in the form of a Mo-cofactor and is the site of nitrate reduction (Moreno-Vivian, 1999). *NarG* (MoCo) is thought to be highly conserved (Moreno-Vivian, 1999). The Mo-cofactor is noncovalently bound to the protein and functions as a prosthetic group (Moreno-Vivian, 1999).

Membrane bound nitrate reductases are associated with denitrification and anaerobic nitrate respiration. In *E. coli*, nitrate reduction enzymes are encoded by the *narGHJI* operon located in the *chiC* locus at 27 min on the chromosome (Bonnefoy, 1994). Genes homologous to *narGHIJ* of *E. coli* have been reported in other bacteria. Nar enzymes are composed of three subunits: a catalytic α subunit

(*narG*) of 112 to 140kDa with a molybdenum cofactor; a soluble β subunit (*narH*) of 52 to 64 kDa with one [3Fe-4S] and three [4Fe-S] centers; and a 19 to 25 kDa membrane biheme β quinol-oxidizing γ subunit (*narI*) (Zumft, 1997). The soluble α and β subunits are anchored to the cytoplasmic side of the membrane by the γ subunit and can be solubilized by detergents or heat (Moreno-Vivian, 1999). NarI is heat sensitive and can be lost during the purification procedure, leading to the isolation of a soluble $\alpha\beta$ complex that can reduce nitrate (Moreno-Vivian, 1999). A δ polypeptide (NarJ), which is not part of the final enzyme, seems to participate in the assembly or stability of the complex prior to its membrane attachment (Blasco, 1992).

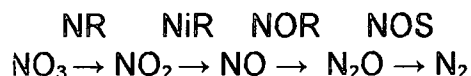
Membrane bound nitrate reductases can reduce chlorate and are inhibited by azide and thiocyanate (Hochstein and Tomlinson, 1988). Nar proteins use the quinol pool as the physiological electron donor and generate a PMF via a redox gradient (Berks et al, 1995). NarI oxidizes quinols at the periplasmic side of the membrane, releasing two protons into the periplasm. Electrons are passed to NarG, via the Fe-S centers of NarH, to reduce nitrate with consumption of two cytoplasmic protons (Moreno-Vivian, 1999).

Little is known about the NR enzyme of other bacteria or the diversity among known denitrifying bacteria or environmental isolates. The purpose of this study was to determine how conserved nature of the *narG* gene from *E. coli* and to assess the usefulness of the gene probe based on *narG* in identifying nitrate reducing bacteria from various environments.

Literature Review

Nitrogen is a basic element for life because it is a component of the two preeminent biological macro-molecules: proteins and nucleic acids. Nitrogen exists in the environment in several oxidation states, from N(V) to N(III) (Moreno-Vivian, 1999) and interconversion of these nitrogen states constitutes the global nitrogen cycle. Nitrate reduction plays a key role in the nitrogen cycle and has important agricultural environmental and public health implications. Assimilatory nitrate reduction, performed by bacteria, fungi, algae, and higher plants, is one of those most fundamental biological processes, accounting for 10^4 megatons of inorganic nitrogen transformed each year (Hauck, 1981). The excessive use of fertilizers in agricultural activities has caused concern about contaminated ground and drinking water. Consumption of drinking water with high nitrate levels has been associated with methemoglobinemia and gastric cancer due to endogenous formation of mutagenic N-nitroso compounds by bacteria in the gastrointestinal tract (van Maanen, 1996). A large threat to the environment comes from eutrophication of aquatic ecosystems. Nitrogen oxides generated by denitrification or dissimilatory nitrate reduction are associated with the greenhouse effect and ozone destruction.

Biological denitrification is defined as a bacterial respiratory process in which there is a sequential reduction of nitrogenous oxides (e.g., nitrates or nitrites) to gaseous end products, principally N_2O or N_2 (Zumft, 1992). In denitrification, four reactions catalyzed by four different membrane-bound enzymes are responsible for the reduction of nitrate to dinitrogen:



The first enzyme, nitrate reductase (NR), reduces nitrate to nitrite. Next, nitrite is reduced to nitric oxide by nitrite reductase (NiR). It is this reaction that distinguishes denitrifiers from nitrate respirers. Nitric oxide reductase (NOR) catalyzes the reduction of nitric oxide to nitrous oxide. Finally, nitrous oxide reductase (NOS) converts nitrous oxide to dinitrogen (Hochstein and Tomlinson, 1988).

Denitrification constitutes the last step of the nitrogen cycle returning nitrogen gas to the atmosphere. In denitrification, a nitrogen oxide instead of oxygen serves as the electron donor for the generation of electric chemical gradient across the cytoplasmic membrane (Zumft, 1997). Denitrification is the assimilatory transformation of nitrate or nitrites to a gas species concomitant with energy conservation (Zumft, 1997). Denitrification carries many environmental implications. Nitrate is known to be a pollutant of groundwater and surface water, causing problems for drinking water supplies (Zumft, 1997). Nitrous oxide like carbon dioxide and methane is very important implications in the

greenhouse effect. An increase in nitrous oxide levels has been observed in the environment. Fertilizer application to agricultural fields is known to contribute significantly to this increase (Zumft, 1997).

Denitrification is ecologically important in several ways; First, fixed nitrogen is returned to soil, water, and the atmosphere through denitrification, which accounts for tremendous losses of added nitrogen (*i.e.*, fertilizers) to agricultural soils (Hauck, 1981). Second, denitrification is responsible for the removal of nitrates and carbon from wastewater. Third, many pollutants such as toluene and the herbicide atrazine can be degraded under denitrifying conditions, making bioremediation a possibility. Lastly, denitrification contributes to the accumulation of greenhouse gasses, NO and N₂O in the atmosphere, and is considered the principal source of N₂O released into the atmosphere in terrestrial ecosystems (Scharrong et al, 1984). Denitrification has been extensively studied in agricultural fields, primarily in an effort to understand and control nitrogen losses, which limits crop growth. However, denitrification in wetlands has received little attention.

Wetlands are considered one of the most important ecosystems on earth. The value of wetlands includes the maintenance of biodiversity by providing habitats for diverse populations of flora and fauna, as biofilters and in nutrient cycling are well-recognized (Mitch and Gosselink, 1993). Both natural and constructed wetlands have been used for wastewater treatment (Duncan and Groffman, 1994) and constitute a low cost, low-energy alternative to

conventional tertiary wastewater treatment, removing BOD and nutrients such as nitrates. Despite the recognized value of wetlands in biogeochemical cycling and wastewater treatment, and the obvious role of microorganisms in these processes, the activity and structure of the microbial populations have been largely overlooked, and relatively little data is available on the fluxes of N_2O in wetlands. Fluxes of N_2O from undisturbed northern peatland soils are reportedly very low (Moore, 1994). However, N_2O fluxes have been shown to increase in these systems upon disturbance such as drainage, cropping and the addition of fertilizer (Moore, 1994). If wetlands emit low levels of N_2O in their undisturbed state, and emissions rise with disturbance, then understanding how these disturbances influence the microbial populations is of primary importance in understanding how to control the release of N_2O from terrestrial ecosystems.

Membrane bound nitrate reductase is associated with denitrification and anaerobic nitrate respiration. In *E. coli*, nitrate reduction enzymes are encoded by the *narGHJ* operon located in the *chlC* locus at 27 min on the chromosome (Bonnefoy and Demoss, 1994). Genes homologous to *narGHJ* of *E. coli* have been reported in other bacteria. Nar enzymes are composed of three subunits: a catalytic α subunit(*narG*) of 112 to 140kDa with a molybdenum cofactor, a soluble β subunit(*narH*) of 52 to 64 kDa with one [3Fe-4S] and three [4Fe-S] centers, and a 19 to 25 kDa membrane biheme b quinol-oxidizing γ subunit(*narI*). Soluble α and β subunits are anchored to the cytoplasmic side of the membrane by the γ subunit and can be solubilized by detergents or heat

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Membrane bound nitrate reductases can reduce chlorate and are inhibited by azide, chlorate, azide and thiocyanate (Hochstein and Tomlinson, 1988). Nar proteins use the quinol pool as the physiological electron donor and generate a PMF via a redox gradient (Berks et al, 1995). NarI oxidizes quinols at the periplasmic side of the membrane, releasing two protons into the periplasm. Electrons are passed to NarG, via the Fe-S centers of NarH, to reduce nitrate with consumption of two cytoplasmic protons (Moreno-Vivian, 1999).

Activities within bacterial communities can be examined through the use of gene probes. DNA isolated from bacteria can be screened for the presence and frequency of a gene using gene probes which are detected by radioactive, or nonradioactive labeling. Community composition can be determined by altering the specificity and types of gene probes (Smith and Tiedje, 1992). A drawback to using a particular gene probe is that certain species will be excluded based upon their DNA sequence. Gene probes allow for identification of the presence and diversity of targets within environments all without culture techniques (Smith and Tiedje, 1992).

Studies of nitrate reduction in bacteria have revealed that this complicated process plays a very important role in the global cycling of nitrogen. The advent of gene probes has opened doors to previously uncharacterized environments and those microorganisms which inhabit these environments. These environmental habitats can be part of important nutrient cycles. Through genetic comparison of different nitrate reductases, a better understanding of the regulation and diversity of nitrogen cycling will be learned.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions.

All laboratory strains were grown in Tryptic soy broth with glycerol with the exception of: *Rhizobium meliloti* (YEM-Mannitol MSG), *Azotobacter vinelandii* (Burks-15 mM NH₄Cl), *Thiosphaera pantotropha* (T. pant media-NaOAc as indicated by ATCC), *Rhodobacter capsulatus* (Van Niels-Succinate), *Hyphomicrobium vulgare* and *H. zavarzinii* (337-methanol) (Linne von berg and Bothe, 1992), and *Staphylococcus aureus* (Tryptic soy broth with glycerol). *Escherichia coli*, *Thiosphaera pantotropha*, *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Proteus mirabilis*, *Bacillus licheniformis* and *Staphylococcus aureus* were all grown at 37° C. *Xanthomonas maltophilia*, *Rhodobacter capsulatus*, and *Paracoccus denitrificans* were grown at 30° C. *Rhizobium meliloti*, *Azotobacter vinelandii*, *Hyphomicrobium* spp., *Alcaligenes eutrophus*, *Pseudomonas fluorescens*, and M91-3 were grown at 25° C.

Site description

Environmental strains were isolated from four hydrogologically linked sites. The first of which was the groundwater fed Siebanthaler Fen. The fen was the least disturbed of all three sites. The stream bordered the fen. The Portland Cement site was an ecologically disturbed site. The first three feet of top soil was removed for cement manufacturing. The agricultural field bordered the Portland Cement site and was regularly farmed for corn and soy beans. The field also had regular application of fertilizer.

Environmental strains were isolated by enrichment culture using Glycerol Ammonium Minimal Medium (GAM) or LB supplemented with nitrate (50 mM) or nitrite (100 mM). Environmental strains were initially grown under anaerobic conditions at 30° C to select for denitrifying bacteria.

Cultures for nitrate uptake experiments, with the exception of *Azotobacter vinelandii*, *P. fluorescens* (ATCC 13525), *Xanthomonas maltophilia* and M91 were grown anaerobically in the presence of nitrate (50 mM KNO₃) in sealed Wheaton bottles containing a stir bar to prevent clumping. Culture bottles were flushed with argon for 15 minutes to create anaerobic conditions. The nitrate reducing system of the obligate aerobe *X. maltophilia* was induced under microaerobic conditons in the presence of 50 mM KNO₃. The nitrate reductase enzymes of *Rhizobium meliloti* and *Rhodobacter capsulatus* were induced by growing the organisms semi-aerobically until saturation, then 500 ml of fresh

media added, and the flasks were sealed and flushed with argon to create anaerobic conditions. These cultures were subsequently incubated overnight.

Gene Probe Construction and Southern Hybridization.

Genomic DNA was isolated from organisms that were cultured aerobically with agitation (150-300 rpm) (Tiedje et al, 1989). Laboratory strains were grown in the above media, environmental isolates were grown in LB.

Probes of *narG* were prepared from 3.7 kb gene from *E. coli* cloned in the plasmid pFB71 (with permission from F. Blasco, kindly provided by J.F. Stolz). This plasmid was propagated in *E. coli* strain DH5 α in the presence of chloramphenicol (50 μ g/ml). The 0.84 kb HindIII-HindIII (Probe A) and 2.1 kb HpaI-HpaI (Probe B) probes were prepared by complete endonuclease digestion of pFB71 plasmid DNA (Figures 1 and 2.). Fragments were separated on 0.7% or 1.0% agarose gels and purified using Gene-Clean (Bio 101 Inc.). Probe DNA was labeled by two methods. DNA was random prime labeled (Boehringer Mannheim Biochemicals) with α -³²PdCTP (Amersham). Unincorporated nucleotides were removed with a Sephadex G-50 NICK column (Pharmacia Biotech). Specific activity of radio-labeled probe DNA was determined using a Beckman liquid scintillation counter. Some probes were labeled with non-radioactive, fluorescein dUTP (Gene Images Kit from Amersham). Incorporation of fluorescein-labeled nucleotides was determined by comparison to known standards.

Genomic DNA was restricted with an excess of BamH1 overnight at 37° C. Digestion of genomic DNA was resolved by agarose gel electrophoresis; 0.7% gels at 20-30 volts for 10-15 hours to improve resolution of the larger DNA fragments. DNA was transferred to either supported nitrocellulose membranes (Schleicher and Schuell; BA-S 83, pore size 0.2 µm) or Hybond N⁺. DNA was fixed to the nitrocellulose by baking the membrane in a vacuum oven at 80° C for 1 hour. DNA was fixed to the Hybond N⁺ (Amersham) membrane by UV crosslinking (BioRad GS Gene Linker, 150 mJoules).

Hybridization with the radiolabeled probes was performed at 42° C with at least 10⁸ cpm/µg of labeled probe in 10 ml of 6X SSPE, 5X Denhardt's solution, 0.5% SDS, 100µg/ml salmon sperm DNA and 50% formamide. Membranes were initially washed twice at room temperature in 2X SSPE and 0.1% SDS for 15 minutes. Stringent washes were at 65° C in 0.1-0.5X SSPE and 0.1% SDS for 15 minutes.

Autoradiograph exposure times were as long as 4 days at -70° C for blots that received stringent washing. Hybridization of the fluorescein labeled probe was performed at 62° C with the probe B hybridization mixture. Stringency washes were performed for 15 minutes at 62° C. Membranes were initially washed with 2X SSC and 0.1% SDS followed by 0.2X SSC and 0.1% SDS. Detection was accomplished as per kit instructions (Gene Images Star Detection Module by Amersham).

Measurement of Nitrate Uptake.

Nitrate uptake rates were determined by measuring changes in the concentration of nitrate with an Orion digital ion analyzer (model 601A) connected to an Orion nitrate electrode (model 93-07) and a double junction reference electrode (model 90-02). This device was calibrated directly with known concentrations of KNO_3 in 50 mM KPO_4 , pH 7.0 and 2% ISA (ionic strength adjuster Orion #930711).

Cultures were harvested by centrifugation at 10,000x g for 10 minutes at room temperature. Pellets were washed twice in three volumes of 50 mM KPO_4 pH 7.0 supplemented with 50 mg/ml of chloramphenicol and resuspended to a final concentration of 1 g wet weight/ 5 ml buffer. Each 10 ml reaction vessel contained the following: 9.4 ml 50 mM KPO_4 (pH 7.0), 0.4 ml ISA, 0.2 ml of cell stock suspension (1g/5ml) and 50 μ l of 100 mM KNO_3 . Anaerobic conditions were maintained by continually flushing the vessel with argon. After an initial period of fluctuation, a stable baseline concentration of nitrate was recorded. The uptake of nitrate was initiated by the addition of 500 μ l of 1 M formate, serving as an electron donor. Nitrate concentrations were monitored for one minute (5-15 second intervals) after the addition of the electron donor. For each organism, uptake measurements were done in triplicate. The protein content (mg/ml) of cell suspension was determined by either the coomassie blue or BCA method (Pierce, Rockford, IL). Rates of nitrate uptake are expressed as nmol nitrate/min/mg protein.

RESULTS

Genetic characterization of known nitrate reducing bacteria.

Of the 13 species of laboratory strains surveyed in this study, ten showed homology to *narG* (Table 1). The probe also hybridized to *P. denitrificans* under less stringent conditions (data not shown).

Characterization of environmental nitrate reducing isolates.

Nitrate reducing bacteria were isolated from four hydrologically linked sites. A total of 39 isolates were obtained from the sites. Thirty five (90%) of these isolates showed homology to the 2.1 kb *narG* probe on a colony blot under stringent conditions.

Genomic DNA was isolated from 29 of the 35 showing homology (several became unculturable). The BamHI digested genomic DNA from the environmental isolates was probed with the 2.1 kb *narG* probe (Figures 3 and 4 and Table 2). Environmental isolates were separated based on the molecular weight banding pattern in the Southern blots. Based on the hybridization banding patterns, isolates were assigned to one of ten different hybridization classes. In hybridization classes 1, 7, 8, and 9 a single sampling site represented each class, while for the remaining hybridization classes, multiple sampling sites were represented. There were no obvious or strong correlations

between the environmental site and hybridization class. Ten of the 29 isolates (35%) showed growth when cultured on *Pseudomonas* isolation agar, indicating that at least a third of the isolates are *Pseudomonas* species.

Nitrate uptake rates.

The α -subunit of the dissimilatory nitrate reductase seems to be well conserved over a broad phylogenetic range. Therefore, we sought to determine whether the enzymes were functionally similar by studying nitrate uptake. The nitrate uptake rates showed a high degree of variability among both known nitrate reducers and environmental isolates (Tables 1 and 2). The environmental isolates showed uptake rates higher than the laboratory strains, however, no substantial differences were apparent.

DISCUSSION

Our results show that *narG* gene of *E. coli* has homology to a wide phylogenetic range of bacteria. This implies the nitrate reductase may have been present in the common ancestor from which purple and gram-positive eubacteria diverged (Figure 5). Nitrate reduction was likely present in the ancestor from which the purple and Gram-positive bacteria arose from. Another explanation of the presence of the gene in such a diverse group of bacteria could be the result of horizontal gene transfer. *Rhizobium* is known to have genes involved in denitrification on plasmids (Chan and Wheatcroft, 1993). Denitrifiers are somewhat more frequent within the alpha and beta classes of Proteobacteria, although there is no recognizable pattern of distribution (Zumft, 1997). The process is absent from enteric bacteria which can only convert iron nitrate to nitrite (Zumft, 1997). The lack of hybridization among a number of bacteria that assimilate nitrate (*P. fluorescens* Biotype A, *Azotobacter vinelandii*) indicate that this gene probe is specific for the dissimilatory nitrate reductase.

Nitrate respiring bacteria which did not hybridize to the probes, *S. aureus*, *P. stutzeri* (Presque Isle), *R. meliloti*, *Pa. denitrificans*, *P. mirabilis* and *R. capsulatus*, appear to possess a nitrate reductase genetically distinct from the enzyme found in dominant nitrate-respiring genera such as *Pseudomonas* and *Alcaligenes*. Genetic diversity was further demonstrated between members of

the same genus since a marine strain of *P. stutzeri* failed to hybridize with either probe while the clinical isolate of *P. stutzeri* did. These findings rule out that a single form of nitrate reductase is distributed throughout the entire eubacterial tree. Therefore, *narG* may prove useful in the identification of the nitrate reducing bacteria from various environments. The *narG* gene, coding for the α -subunit of the nitrate reductase enzyme of *E. coli*, showed homology to a wide phylogenetic range of bacteria. *narG* of *E. coli* appears to be specific for dissimilatory nitrate reductase enzymes. High homology to environmental isolates may allow *narG* to be used as an indicator of dissimilatory nitrate reducing bacteria in natural environments. Nitrate uptake rates varied substantially and there was no obvious correlation with genetic homology.

Future Studies

These results indicate that *narG* may be useful in conjunction with other gene probes in the identification and classification of unknown or environmental isolates. Another possibility may be to design degenerate PCR primers from *narG* of *E. coli* and other well conserved nitrate reductases. The degenerate PCR primers would have the capability of detecting many more species at a faster rate than using probes from individual species. Further, if this is the case then environmental rates of denitrification could be estimated by quantitative PCR of the *narG* gene.

Through these molecular methodologies, assessment of the diversity and functionality of different environmental sites could easily be tested and monitored over a period of time. Changes over the time period could easily be tracked and disturbances in the diversity or functionality of these sites be determined and if needed corrected.

CHAPTER II

**Regulation of Twitching Motility in *Pseudomonas aeruginosa* by the
las and *rhl* Quorum-Sensing Systems and The Distribution of the
narG gene of *Escherichia coli* Among Denitrifying Bacteria
Dissertation**

ABSTRACT

Pseudomonas aeruginosa is a ubiquitous environmental bacterium and an important human pathogen. The production of several virulence factors by *P. aeruginosa* is controlled through two quorum-sensing systems, *las* and *rhl*¹. Both the *las* and *rhl* quorum-sensing systems are also required for type-4 pili dependent twitching motility and infection by the pili-specific phage, D3112cts. Mutants which lack the ability to synthesize PAI-1, PAI-2, or both autoinducers were significantly or greatly impaired in twitching motility and in susceptibility to D3112cts. Twitching motility and phage susceptibility were partially restored by exposure of the autoinducer deficient mutants to exogenous PAI-1 and PAI-2. Both twitching motility and infection by pili-specific phage are believed to be dependent on the extension and retraction of polar type-4 pili. Western blot analysis of whole cell lysates and ELISAs of intact cells were used to measure the amounts of pilin on the cell surfaces of *las* and *rhl* mutants relative to the wild type. It appears that *rhl* plays a crucial role in the export and assembly of surface type-4 pili twitching motility and phage infection by affecting the export and assembly of surface type-4 pili. Microscopic analysis of twitching motility indicated that mutants which were unable to synthesize PAI-1, via the *las* gene, were defective in the maintenance of cellular monolayers and migrating packs of cells.

¹ Published in the Journal of Bacteriology (Glessner et al., 1999).

INTRODUCTION

Pseudomonas aeruginosa is known to cause infections in immunocompromised patients and to inhabit very diverse environments (Brock et al, 1994). The infections *P. aeruginosa* causes include respiratory infections, urinary tract infections, burn wound infections, and lung colonization in people suffering from cystic fibrosis (Smith, 1996; Brock et al, 1994). The pathogenicity of *P. aeruginosa* is due to its impressive arsenal of virulence factors which include multiple cell-associated factors such as alginate, pili and lipopolysaccharide (Hahn, 1997) and secreted virulence factors, including toxins (exotoxin A and exoenzyme S), proteases (elastase, LasA protease, and alkaline protease), and hemolysins (phospholipase and rhamnolipid) (Hahn, 1997, Pearson 1995). Two quorum-sensing systems, *las* and *rhl*, have been identified as controlling production of several of these virulence factors and has become a model for studying cell-density control of virulence genes. It has been demonstrated that the quorum-sensing systems of *P. aeruginosa* play a major role in twitching motility (Glessner et al., 1999).

The *las* quorum-sensing system consists of a transcriptional activator, LasR, and LasI, which directs the synthesis of the autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL or PAI-1). When a threshold concentration of PAI-1 is reached, a LasR-PAI-1 complex forms, inducing the production of increased amounts of PAI-1 in a positive feedback loop. The LasR-PAI-1 complex is essential for activation of genes regulated by this system. The *las* system regulates the expression of the virulence genes for elastase (*lasB*), LasA protease (*lasA*), alkaline protease (*apr*) and exotoxin A (*toxA*) (Toder et al., 1991; Gambello et al., 1993; Latifi et al., 1995; Winson et al., 1995). The *las* system has hierarchy over the transcriptional activators (*lasR* and *rhIR*) and *rsaL*, a repressor of *lasI* (Pesci and Iglewski, 1997). Global regulation by quorum sensing is evidenced by LasR-PAI-1 activating the *xcpP,R* genes which encode the proteins of the *P. aeruginosa* general secretory pathway (Chapon-Herve et al, 1997). The Xcp proteins have been shown to belong to a large group of ancillary proteins with prepilin-like leader sequences (Alm and Mattick, 1997).

The *rhl* quorum-sensing system consists of a second transcriptional activator, RhIR, and RhII, which synthesizes *N*-butyryl-L-homoserine lactone (BHL or PAI-2). The *rhl* system functions as a typical quorum sensing system with binding of PAI-2 to RhIR (Pearson et al., 1997). The RhIR-RhII system controls the expression of genes required for rhamnolipid production (*rhlAB*),

elastase (*lasB*), alkaline protease (*apr*), pyocyanin and RpoS, a stationary phase sigma factor involved in the regulation of numerous stress-response genes (Brint and Ohman, 1995; Ochsner and Reiser, 1995; Pearson et al., 1995; Pesci and Iglewski, 1997).

Although the *las* and *rhl* systems are separate and complete quorum-sensing systems, there is evidence of interactions between the systems and a hierarchy between them. *RhIR* is controlled by *lasR* (Pesci et al., 1997). The LasR and PAI-1 together activate *rhIR* transcription and PAI-1 blocks PAI-2 from binding to RhIR, indicating that the *las* system exerts control at both the transcriptional and postranslational levels.

Quorum sensing is involved in regulating motility and multicellular behavior of *S. liquefaciens*. AHLs are involved in initiating and controlling swarming motility and multicellular behavior of *Serratia liquifaciens*. In *S. liquefaciens*, swarming motility is abolished in a *swrI* mutant (Eberl et al. 1996). This gene encodes an *N*-acyl-L-homoserine lactone (AHL) synthase, involved in the synthesis of the extracellular signal molecules *N*-butanoyl-L-homoserine lactone (BHL) and *N*-hexanoyl-L-homoserine lactone (HHL).

P. aeruginosa has three forms of motility (1) swimming, a flagella-dependent, pili-independent form of motility, (2) swarming a flagella-dependent, pili-independent form of motility (Rashid and Kornberg, 2000) , and (3) twitching motility a flagella-independent mode of surface translocation which requires

functional type 4 pili (Darzins, 1993, 1994). Twitching motility is characterized by short, intermittent, often jerky cell movements, *i.e.*, 'twitching', of individual cells and the coordinated movement of groups of cells which are organized into the flares or rafts reminiscent of S-motility in *M. xanthus*. Twitching motility has been described in a fairly broad group of Gram-negative bacteria, including *Neisseria*, *Moraxella*, *Dichelobacter*, *Acinetobacter*, *Eikenella*, and *Pseudomonas* (Bradley, 1980; Darzins, 1993; Whitchurch et al., 1991). The most intensively studied of these is *P. aeruginosa*. In contrast to *M. xanthus*, individual cells of *P. aeruginosa* do not appear capable of sustained movement resulting in substantial net translocation, *i.e.*, they do not appear to be capable of adventurous or A-motility. However, the collective coordinated movement of groups of cells results in extensive spreading of a bacterial colony over a solid surface, or interface, with leading edges of the colony taking on a serrated appearance (Henrichsen, 1983).

The cellular mechanics of twitching motility have only recently become known. Net movement of *P. aeruginosa* has been observed to be a result of the extension of pili from the poles of the cell, the attachment of the distal end of the extended pili to a surface and then the retraction of the pili back into the cell, resulting in a pulling type of translocation or movement (Skerker and Berg, 2001). The retraction is believed to be a result of the break down of the pilus into pilin subunits via an ATPase known as PilT (Maier et al., 2002). The pilin

subunits may be recycled from a membrane pool of pilin to cause the extension of a pilus from the cell (Skerker and Berg, 2001).

Although type 4 pili are required and the generation of movement may involve repeated extension and retraction of polar type-4 pili (Mattick et al., 1996), it is not clear why cell-cell interactions in rafts are necessary for net translocation. There are several key similarities between S-motility in *M. xanthus* and twitching motility (Wall and Kaiser, 1999). In S-motile swarms of *M. xanthus* there are spearhead-like clusters of 50 or more cells (Wall and Kaiser, 1999).

The polar, type-4 pili of *P. aeruginosa* are considered virulence factors and are multifunctional structures which play a crucial role in: (1) adherence to, and colonization of, mucosal surfaces (Wong et al., 1995; Hahn, 1997), with the pilus accounting for about 90% of the adherence capability of *P. aeruginosa* to a variety of cell types and the pilin subunit itself carrying the adherence function, (2) the initial stages of infection by bacteriophages (Roncero et al., 1990; Mattick et al., 1996), and (3) twitching motility (Bradley, 1980; Doig et al., 1988). Type 4 pili of *P. aeruginosa* are also necessary for attachment to surfaces in biofilm formation (O'Toole and Kolter, 1998). A large number of these genes (*pilA, B, C, D, E, M, N, O, P, Q, T, U, V, and W*) are believed to be a subset of a supersystem involved in the assembly of surface-associated protein complexes including those involved in TTSS (Type II Secretion system) protein

secretion and DNA uptake as well as pili biogenesis (Mattick et al., 1996).

Since twitching motility involves high densities of cells, we sought to determine if a relationship between twitching motility and quorum sensing existed. We put forth evidence that both the *las* and *rhl* systems are necessary for normal twitching motility. The *las* system seems to have a more prominent role in allowing normal twitching as evidenced by the phage infectivity results.

LITERATURE REVIEW

Pseudomonas aeruginosa is a ubiquitous and important opportunistic pathogen that produces a variety of virulence factors. Two quorum-sensing systems, *las* and *rhl*, have been identified as controlling production of several of these virulence factors and has become a model for studying cell-density control of virulence genes. Recently, *P. aeruginosa* has also become the preferred model system for studies of type-4 pili and twitching motility. The polar, type-4 pili of *P. aeruginosa* are appendages which play a crucial role in the virulence of this organism by promoting adherence to surfaces and are responsible for twitching motility. It has been demonstrated that the quorum-sensing systems of *P. aeruginosa* play a major role in twitching motility (Glessner et al., 1999).

Quorum Sensing is a term used to describe the mechanism used by bacteria to monitor cellular density. Quorum Sensing allows environmental signals to be translated into gene expression. In gram negative bacteria, acyl-homoserine lactone molecules are signaling molecules while lipids, peptides, and amino acids may also play a role as signaling molecules. Bioluminescence is defined as the emission of visible light from living organisms. There are many types of organisms which emit light including bacteria, fish, algae and squid.

Bacteria are the most numerous of the light emitting life forms. The luminescent (*lux*) proteins and their encoding genes have been studied for many years and much is known about them. The expression of luminescence in gram negative bacteria has been determined to coincide with cell-cell interactions and cell density. This cell density-dependent gene regulation, termed quorum sensing, is used by luminescent bacteria to activate their light emitting genes only when the concentration of bacterial cells is high enough (Gray, K.M., et al. 1994).

Quorum sensing was first studied in the bioluminescence of *Photobacterium fischeri* (aka *Vibrio fischeri*), which inhabit the light organs of squid and other marine life. Quorum sensing in Gram-negative bacteria generally involves at least two components. One component is a small intercellular diffusible molecule which is typically an *N*-acyl homoserine lactone compound (AHL), called an autoinducer. After reaching a threshold concentration, the AHL activates a second component, and we now know that these regulators can also down regulate genes thus they are not always "positive" transcriptional activators. The interaction of the AHL and this specific transcriptional activator results as transcription of the genes. AHL-mediated regulation of gene expression was first described in control of bioluminescence in the bacterium *Vibrio fischeri*. The onset of bioluminescence in *V. fischeri* is controlled by *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), which is synthesized by LuxI, an autoinducer synthase. OHHL to a threshold

concentration, transcription of the *lux* operon is upregulated by LuxR. (Fuque et al., 1994, 1996; Salmond et al., 1995). LuxR enables transcription from the promoter of the *lux* operon at *luxI*. The N-terminal domain is the part of the LuxR that binds to the autoinducer. In the absence of any autoinducer, the N-terminus will inhibit the C-domain. The C-domain is responsible for transcribing the *lux* operon. There is a 20 basepair palindrome about 40 basepairs upstream of the transcriptional start of the *lux* operon (promoter). This palindromic sequence is the binding site for LuxR. A Lux palindromic sequence upstream of bacterial genes may provide insight to regulation by autoinduction (Schaefer et al., 1996, Stevens and Greenberg, 1997).

A wide variety of autoinducer analogs can bind to LuxR and either activate the luminescence genes or inhibit the activity of the natural autoinducer (Schaefer et al. 1996). This may suggest something about interspecies communication. Perhaps a given organism at a high cell density can produce an extracellular signal that interferes with the ability of other species to successfully colonize a particular habitat. This kind of quorum sensing would be a distinct advantage for a species that needs to prevent other unwanted species from invading its environment (Schaefer et al. 1996).

P. aeruginosa has at least two complete, semi-independent quorum-sensing systems, designated *las* and *rhl*. These two systems control expression of multiple genes, including several important virulence genes, in response to

cell density (Figure 6). The *las* quorum-sensing system consists of a transcriptional activator, LasR, and LasI, which directs the synthesis of the autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL or PAI-1). PAI-1 unlike many other autoinducer molecules is not freely diffusible, rather active efflux is responsible for the movement of this molecule out of the cell (Pearson et al., 1999). When a threshold concentration of PAI-1 is reached, a LasR-PAI-1 complex forms that induces the production of more PAI-1 in a positive feedback fashion. It is the LasR-PAI-1 complex that is required for activation of genes under the control of this system. The *las* system regulates the expression of the virulence genes for elastase (*lasB*), LasA protease (*lasA*), alkaline protease (*apr*) and exotoxin A (*toxA*) (Toder et al., 1991; Gambello et al., 1993; Latifi et al., 1995; Winson et al., 1995). In addition, the *las* system controls the expression of both transcriptional activators (*lasR* and *rhIR*) and *rsaL* which has recently been shown to be a repressor of *lasI* (Pesci and Iglewski, 1997). Chapon-Herve et al. (1997) have shown that LasR-PAI-1 activates the *xcpP,R* genes which encode the proteins of the *P. aeruginosa* general secretory pathway, demonstrating a global role for the *las* quorum-sensing system. The Xcp proteins have been shown to belong to a large group of ancillary proteins with prepilin-like leader sequences (Alm and Mattick, 1997).

The *rhl* quorum-sensing system consists of a second transcriptional activator, RhIR, and RhII, which directs the synthesis of *N*-butanoyl-L-homoserine lactone (BHL or PAI-2). The *rhl* system appears to be a typical quorum sensing system with binding of PAI-2 to RhIR (Pearson et al., 1997). The RhIR-RhII system controls the expression of genes required for rhamnolipid production (*rhlAB*), elastase (*lasB*), alkaline protease (*apr*), pyocyanin and RpoS, a stationary phase sigma factor involved in the regulation of numerous stress-response genes (Brint and Ohman, 1995; Ochsner and Reiser, 1995; Pearson et al., 1995; Pesci and Iglewski, 1997).

Although the *las* and *rhl* systems are separate and complete quorum-sensing systems, there is evidence of interactions between the systems and a hierarchy between them (Figure 6). *rhIR* is controlled by *lasR* (Pesci et al., 1997). The LasR and PAI-1 together activate *rhIR* transcription and PAI-1 blocks PAI-2 from binding to RhIR, indicating that the *las* system exerts control at both the transcriptional and postranslational levels respectively. Thus, the current view is that *lasR* is at the top of the *P. aeruginosa* quorum-sensing hierarchy (Pesci and Iglewski, 1997). The redundancy of the *las* and *rhl* quorum sensing systems in *P. aeruginosa* in which different target genes can be activated by one, the other, or both autoinducers, e.g., *lasB*, allows for staging in their expression.

Over 300 genes have been identified by genechip technology as being part of the quorum controlled regulon in *P. aeruginosa* (Schuster et al., 2003; Wagner et al., 2003). Genes appear to be both promoted and repressed by the quorum sensing systems (Wagner et al., 2003). Genes encoding proteins used for energy metabolism appeared to be under the control of quorum sensing, giving the cell more adaptability to different environments and at the same time complicating therapeutic actions against a specific metabolic pathway (Wagner et al., 2003). These genes appear to be controlled both on a time basis and as a function of the accumulation of the autoinducer receptor *lasR* and *rhIR* transcript levels (Schuster et al., 2003). In conjunction, these two mechanisms allow for a complex regulatory event that results in genes being positively regulated at different points in the cell growth cycle. A quorum sensing interfering compound has tested with genechip technologies and was found to not have an impact on the transcript levels of *lasRI* or *rhIRI*, but appeared to have an impact at the posttranslational level (Hentzer et al., 2003). This impact was apparent with increased sensitivity to antibiotic treatment and higher susceptibility to biofilm maintenance and colonization of surfaces (Hentzer et al., 2003).

The discovery of the quorum sensing hierarchy in which the *las* system is dominant led to further research of the regulation and structure of the *lasR* gene. The LasR-LasI system of *P. aeruginosa* is known to be homologous to the LuxR-

LuxI quorum sensing system of *Vibrio Fischeri* (Albus et al, 1997). In this system *luxR* transcription is controlled by the cyclic AMP (cAMP) receptor protein (CRP), which binds to CRP-binding consensus sequence (CCS) located in the lux operon promoter region. A CCS in the DNA directly upstream from *lasR* and a *P. aeruginosa* CRP homolog Vfr have also been identified. It has been shown that Vfr and CCS are *lasR* regulatory elements such that Vfr is required to bind to the CCS in order for expression of *lasR* to occur (Albus et al, 1997).

The CCS is a 22bp palindrome 100% identical to the sequence in *E.coli* CRP binding sites. Deletions of the first 9bp of this region reduced *lasR* expression by approximately 90%. In addition to this, double point mutations throughout the 22bp region that preserved the palindromic nature of CCS decreased *lasR* expression by approximately 85%. These reports indicate that the CCS is extremely critical for the transcription of *lasR* (Albus et al, 1997).

Vfr was found to be necessary for *lasR* expression. This was seen when a *P. aeruginosa* *vfr* deletion decreased *lasR* transcription by 90% (Albus et al, 1997). Vfr binds to the *lasR* promoter DNA in the region from 324bp to 281bp relative to the *lasR* start codon. This area contains approximately half of the CCS, which demonstrates that the CCS is a *lasR* regulatory element, which serves as a binding site for Vfr (Albus et al, 1997).

The *lasR* gene has additional features that lend themselves to transcriptional regulation. There are two known transcription start sites of *lasR*. T1 is found 201bp upstream from the start of *lasR* translation while T2 is located 231bp upstream from this site (Albus et al, 1997). The T1 transcript is found to be more abundant than that of T2 and does not have a consensus sequence. T2, however, has a -35 and -10 consensus sequence with each region matching 4 out of 6bp with the sigma-70-type promoter. Seventeen nucleotides upstream from the T2 start site is a region very similar to the *V. fischeri lux* box. In fact 13 out of 20bp match that of the *V. fischeri lux* box. This region is where the transcriptional activator LuxR binds to the promoter and serves as a transcriptional regulator. It was found that the *P. aeruginosa lux* box overlaps the T2 transcription start site and is 37bp upstream from the T1 transcription start site. If the *P. aeruginosa lux* box serves the same function as that of *V. fischeri* then LasR most likely binds to the region and therefore overlaps the T2 start site. This implies that LasR, when bound to the lux box could inhibit transcription from the T2 promoter; thus, making the T1 promoter the major start site (Albus et al, 1997).

P. aeruginosa is a ubiquitous and important opportunistic pathogen that produces a variety of virulence factors. Two quorum-sensing systems, *las* and *rhl*, have been identified as controlling production of several of these virulence factors and has become a model for studying cell-density control of virulence

genes. Recently, *P. aeruginosa* has also become the preferred model system for studies of type-4 pili and twitching motility. The polar, type-4 pili of *P. aeruginosa* are appendages which play a crucial role in the virulence of this organism by promoting adherence to surfaces and are responsible for twitching motility. It has been demonstrated that the quorum-sensing systems of *P. aeruginosa* play a major role in twitching motility (Glessner et al., 1999).

AHLs have been shown to be involved in initiating and controlling swarming motility and multicellular behavior of *Serratia liquifaciens*. Swarming motility is abolished in a *swrI* mutant (Eberl et al., 1996). This gene encodes a *N*-acyl-L-homoserine lactone (AHL) synthase which is involved in the synthesis of the extracellular signal molecules *N*-butanoyl-L-homoserine lactone (BHL) and *N*-hexanoyl-L-homoserine lactone (HHL). To date, a single gene, *swrA*, has been shown to be controlled by these AHLs. The *swrA* gene is responsible for the synthesis of serrawettin, an extracellular lipopeptide biosurfactant, which is required for normal swarming motility (Givskov et al., 1998; Lindum et al., 1998). This demonstrates that quorum sensing is involved in regulating motility and multicellular behavior of *S. liquefaciens*. Interestingly, on rich media such as LB or Brain-Heart Infusion (BHI), the *swrI* mutant swarms without exogenous additions of AHL, although swarming is severely delayed, suggesting that AHL-mediated control may be overruled by other stimuli or certain environmental conditions. There have been no reports of AHL involvement in the regulation of

motility and development of *M. xanthus* despite the obvious cell-cell mediated development and the cell-density dependent nature of S-motility.

Although it is clear that AHLs regulate virulence determinants and secondary metabolites in *P. aeruginosa*, a role for AHLs in the regulation of twitching motility in *P. aeruginosa* has not previously been reported. These findings represent the first evidence that quorum-sensing systems are involved in twitching motility. Whether AHLs can directly activate specific cellular processes associated with type 4 pili (such as the rapid extrusion-retraction of pili), or whether they can act only through new gene expression is unknown.

Motility of any kind affects three of the most important things in a bacterium's life: acquisition of nutrients, avoidance of predation/stress and dispersal of populations. Quorum-sensing systems also directly affect the expression of many genes and, therefore, adaptations to specific environments. The molecular specifics of these two behaviors, and their interrelations, are likely to have a major bearing on the population's ability to survive, exploit their habitat and cause disease.

Both surface-associated motility and cell-cell communication are centrally important to the development and function of microbial communities in natural environments, whether those environments be biofilms, soils, sediments or host tissues. The formation and composition of these communities depend on the individual and collective abilities of their members, *i.e.*, their abilities to adhere,

move, metabolize, replicate and transfer genes.

The overall goal of this research was to determine the role of the quorum-sensing systems, *las* and *rhl*, in the flagella-independent mode of surface translocation known as 'twitching motility' in *Pseudomonas aeruginosa*. We want to discover how the chemical signals that convey cell-cell density and proximity interact with their receptors to govern the movement of packs of *P. aeruginosa* cells over surfaces. Such knowledge will improve our understanding of how bacteria disseminate and survive, and is highly relevant to the role that surface motility and taxis play in plant and human disease, horizontal gene transfer, the formation of biofilms and the degradation of xenobiotic compounds.

Two principle, flagella-independent modes of motility are gliding and twitching motility. Gliding motility has been intensively studied in the Gram-negative, nonflagellated bacterium, *Myxococcus xanthus*, which is capable of gliding on solid or semi-solid surfaces and which forms fruiting bodies under conditions of nutritional stress (Bretscher and Kaiser, 1978). The mechanism of gliding motility is not completely understood. However, it is known that it is controlled by three gene systems: the (A)dventurous motility, (S)ocial motility, and Frizzy motility systems. The (A)dventurous system controls the movement of individual cells (Hodgkin and Kaiser, 1979a), while the (S)ocial system controls the movement of cells in a group (Hodgkin and Kaiser, 1979b). The frizzy (*frz*) genes are required for the control of direction of motility and are

homologous to the chemotaxis genes of *E. coli* and *Salmonella typhimurium* (Zusman and McBride, 1991; Kim et al., 1992). S-motility is observed only when high cell densities are achieved and when cells are close together, producing groups or 'flares' of cells while A-motile cells are capable of swarming at very low cell densities as well as at high densities (Kaiser and Crosby, 1983). The two systems of motility are additive *i.e.*, a wild-type (A^+S^+) strain will swarm over a surface at approximately twice the rate as A-motile (A^+S^-) or a S-motile (A^-S^+) strains, while a A^-S^- strain does not swarm and is called non-motile. Type 4 pili are required for (S)ocial, but not (A)dventurous motility in *M. xanthus* (Wu and Kaiser, 1995). Type-4 pili are also required for motility in *P. aeruginosa* and other bacteria including *N. gonorrhoeae*, enteropathogenic *Escherichia coli* and *Synechocystis* PCC6803 (Beiber et al., 1998; Wall and Kaiser, 1999).

Twitching motility is a flagella-independent mode of surface translocation which requires functional type 4 pili (Bradley, 1980; Darzins, 1993, 1994; Henrichson, 1983). Strains lacking polar pili, and those with peritrichous pili are not capable of twitching motility. Twitching motility is characterized by short, intermittent, often jerky cell movements, *i.e.*, 'twitching', of individual cells and the coordinated movement of groups of cells which are organized into the flares or rafts reminiscent of S-motility in *M. xanthus*. Twitching motility has been described in a fairly broad group of Gram-negative bacteria, including *Neisseria*, *Moraxella*, *Dichelobacter*, *Acinetobacter*, *Eikenella*, and *Pseudomonas* (Bradley,

1980; Darzins, 1993; Whitchurch et al., 1991). The most intensively studied of these is *P. aeruginosa*. In contrast to *M. xanthus*, individual cells of *P. aeruginosa* do not appear capable of sustained movement resulting in substantial net translocation, i.e., they do not appear to be capable of A-motility.

However, the collective coordinated movement of groups of cells results in extensive spreading of a bacterial colony over a solid surface, or interface, with leading edges of the colony taking on a serrated appearance (Henrichsen, 1983).

The mechanics of twitching motility have only recently been revealed and involve the extension of pili from the poles of the cell, the attachment of the distal end of the extended pili to a surface and then the retraction of the pili back into the cell, resulting in a pulling type of translocation or movement (Skerker and Berg, 2001). The retraction of a single pilus generates a significant amount of force measuring upwards of 100 pN (Maier et al., 2002). This force is believed to be a result of the break down of the pilus into pilin subunits via an ATPase known as PilT (Maier et al., 2002). These pilin subunits from the retracted pilus may be recycled from a membrane pool of pilin to cause the extension of a pilus from the cell (Skerker and Berg, 2001). Thus, type 4 pili are required and the generation of movement may involve repeated extension and retraction of polar type-4 pili (Mattick et al., 1996).

P. aeruginosa has become the preferred model system for studies of type-4 pili and twitching motility. The polar, type-4 pili of *P. aeruginosa* are considered virulence factors and are multifunctional structures which play a crucial role in: (1) adherence to, and colonization of, mucosal surfaces (Wong et al., 1995; Hahn, 1997), with the pilus accounting for about 90% of the adherence capability of *P. aeruginosa* to a variety of cell types and the pilin subunit itself carrying the adherence function, (2) the initial stages of infection by bacteriophages (Roncero et al., 1990; Mattick et al., 1996), and (3) twitching motility (Bradley, 1980; Doig et al., 1988). Additionally, O'Toole and Kolter (1998) have recently shown that type 4 pili of *P. aeruginosa* are also necessary for attachment to surfaces in biofilm formation. Recent estimates are that there are over 30 chromosomal genes involved in the biosynthesis and regulation of expression and function of *P. aeruginosa* type-4 pili (Hobbs et al., 1993; Alm and Mattick, 1997; Mattick et al., 1997). These genes can be divided into four groups (Wall and Kaiser, 1999): (i) five transcriptional regulators (*pilS*, *R*, *fimS*, *algR* and *rpoN*); (ii) eight *che*-like genes (*pilG*, *H*, *I*, *J*, *K*, *L*, *chpA* and *B*); (iii) 19 type 4 pili biogenesis genes (*pilA*, *B*, *C*, *D*, *E*, *F*, *M*, *N*, *O*, *P*, *X*, *V*, *W*, *X*, *Y1*, *Y2*, *Z*, *fimT* and *U*); and (iv) two pilus function genes (*pilT* and *pilU*). A large number of these genes (*pilA*, *B*, *C*, *D*, *E*, *M*, *N*, *O*, *P*, *Q*, *T*, *U*, *V*, and *W*) are believed to be a subset of a supersystem involved in the assembly of surface-associated protein complexes including those involved in TTSS protein

secretion and DNA uptake as well as pili biogenesis (Mattick et al., 1996). A recent study shows that type-4 pili actually bind both plasmid and salmon sperm DNA (van Schaik et al., 2005). This binding mechanism is believed to be an interaction between the positively charged pilus and pyrimidine molecules in the net negatively DNA (van Schaik et al., 2005). The DNA binding by pili is important for biofilm formation (van Schaik et al., 2005). The four groups of *pil* genes are distributed among 15 *pil* loci in three separate regions of the chromosome indicating that they are not tightly clustered. For example, one major cluster corresponds to about 71-75 minutes on the genetic map of the *P. aeruginosa* PAO chromosome. This region includes *pilA*, the pilus subunit gene (Sastry et al., 1985), several accessory pilus biosynthetic genes, designated *pilB*, *C* and *D* (Nunn and Lory, 1991; Nunn, et al., 1990), the two-component regulator-sensor pair *pilR* and *S* (Hobbs et al., 1993; Ishimoto and Lory, 1992), and the alternate sigma factor encoded by *rpoN* (Ishimoto and Lory, 1989).

The *pilGHIJKL*, *chpA* and *B* gene cluster encodes proteins that are required for normal pilus production and twitching motility, but their exact roles remain unclear (Darzins, 1993 and 1994; Mattick et al., 1997). This unlinked set of *pil* genes correspond to about 20 minutes on the PAO genetic map. This is one of 5 Che clusters identified on the *P. aeruginosa* genome (Ferrandez, et al., 2002). Two of these clusters (cluster I and cluster V) are essential for chemotaxis in *P. aeruginosa*. Cluster IV is involved in twitching motility; cluster

III has additional homologues of *che* and *mcp* genes, and cluster II is needed for optimal chemotaxis (Ferrandez, et al., 2002).

Defined mutations in each of the *pilG*, *pilI*, and *pilJ* genes results in cells that have no polar pili and are incapable of any twitching motility, whereas *pilK* and *chpAB* are indistinguishable from wild-type and *pilH* mutants produce pili yet exhibit an aberrant type of twitching motility (Darzins, 1993, 1994, 1995). These *pil* genes have significant regions of homology, at the amino acid sequence level, to one of the *che* genes required for flagellar chemotaxis signal transduction in the enteric bacteria (Hazelbauer and Harayama, 1983) and *frz* genes of the gliding bacterium *M. xanthus* (McBride et al., 1989). The following homologies have been proposed: *pilG* (CheY), *pilH* (CheY), *pilI* (CheW), *pilJ* (MCP) and *pilK* (CheR), *pilL* (CheA) and *chpA* (CheA) and *chpB* (CheB). Each of the *pil-che* mutants exhibits normal flagellar motility and chemotaxis, so it appears that the *pilG-L* and *chpAB* genes do not function in flagellar chemotaxis. This pathway may be involved in the control of direction or rate of twitching motility in response to environmental signals. Thus, the homologies to parts of the *che* genes suggest that the *pilG-L* and *chpAB* genes may function in an independent signal transduction pathway that governs expression and regulation of pili biogenesis and twitching motility. The target(s) of the *pil-che* signal transduction pathway (*pilG,H,I, J,K,L* and *chpA,B*) are presently unknown but are presumed to involve the motor which drives twitching motility. Pili biogenesis

and/or function is known to require three nucleotide binding proteins, PilB, PilT and PilU and there is speculation that these genes are the targets of the *pil-che* signal transduction pathway. PilT may well be the signature protein in type 4 twitching motility, perhaps even the motor, as it is one of the best conserved proteins between *M. xanthus*, *P. aeruginosa* and *N. gonorrhoeae* and there is no homolog in the type II secretion system (Wall and Kaiser, 1999).

Mutations in *pilT* and *pilU* confer a hyperpiliated phenotype on the cell and completely block twitching motility (Darzins, 1994; Whitchurch et al., 1991). These mutants are able to synthesize and extrude normal type-4 pili, but appear incapable of retracting them. The *pilT* mutant is also resistant to pili-specific phage while the *pilU* mutant remains susceptible to some pili-specific phage (Whitchurch and Mattick, 1994). Electron microscopic examination and bacteriophage binding of these mutants, in relation to the wild-type, has led to the proposal that pili extension and retraction is the physical basis of twitching motility (Bradley, 1980; Mattick et al., 1996). *pilT* mutants have been observed to attach to surfaces as well as wild-type cells although the mutant cells are not as susceptible to plaque formation nor are the *pilT* mutant cells able to infect cells, suggesting that the retraction of the pilus necessary for infection (Maier et al., 2002). *P. aeruginosa* cells have been observed to move by extending pili, letting the extended pilli attach, and then retracting the attached pili; thus pulling the cell toward the point of attachment (Skerker and Berg, 2001).

Although the *las* and *rhl* systems are separate and complete quorum-sensing systems, there is evidence of interactions between the systems and a hierarchy between them (Figure 6). Recently it has been shown that *rhlR* is controlled by *lasR* (Pesci et al., 1997). The LasR and PAI-1 together activate *rhlR* transcription and PAI-1 blocks PAI-2 from binding to RhlR, indicating that the *las* system exerts control at both the transcriptional and postranslational levels respectively. Thus, the current view is that *lasR* is at the top of the *P. aeruginosa* quorum-sensing hierarchy (Pesci and Iglewski, 1997). The redundancy of the *las* and *rhl* quorum sensing systems in *P. aeruginosa* in which different target genes can be activated by one, the other, or both autoinducers, e.g., *lasB*, allows for staging in their expression. AHLs have recently been shown to be involved in initiating and controlling swarming motility and multicellular behavior of *Serratia liquifaciens*.

Although it is clear that AHLs regulate virulence determinants and secondary metabolites in *P. aeruginosa*, a role for AHLs in the regulation of twitching motility in *P. aeruginosa* has not previously been reported. These findings represent the first evidence that quorum-sensing systems are involved in twitching motility. Whether AHLs can directly activate specific cellular processes associated with type 4 pili (such as the rapid extrusion-retraction of pili), or whether they can act only through new gene expression is unknown.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 3. LB medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl) was routinely used to propagate *P. aeruginosa* strains. Broth was solidified with agar at the appropriate concentrations for the individual assays described below. Wild-type and mutant strains of *Pseudomonas aeruginosa* were maintained as glycerol stocks and stored at -70°C. Freshly isolated colonies on LB agar plates were used for each experiment. Media was supplemented with tetracycline at 50 µg/ml, and mercuric chloride at 15µg/ml, when needed.

Twitching motility assays. Twitching motility was assayed by two methods. LB agar (1%) plates poured to an average depth of 3 mm were prepared, dried briefly and the strains to be tested were stab inoculated with a needle to the bottom of the petri dish. Plates were incubated at 37°C for 48 hours, except where noted otherwise. After the incubation period, the zone between the agar and the bottom of the petri dish, the twitch zone, was measured (Darzins, 1994). A slide culture assay was used to assess twitching

motility at the microscopic level (Darzins 1994). Strains were point inoculated onto the surface of a slab of LB agar (1%) placed on a microscope slide. The inoculated LB agar was covered with a coverslip, and the slide cultures were incubated for 3 to 5 hours at 37°C in a humid petri dish. Twitching motility was visualized under an Olympus BH/2 microscope using Nomarski optics and a 60X objective lens.

Twitching Motility Rescue Assays. In rescue assays wild-type PAO1 cells marked with gfp on a plasmid were mixed in equal amounts with each of the unmarked mutants described above. The cells were grown overnight on LB 1.5% agar plates with the appropriate antibiotics if necessary. The cells were then resuspended in LB broth; the resuspended cells were then counted on a microscope, and mixed in equal proportions. The mixture was then stab inoculated into LB with 1% Agar. The plates were incubated for 8 hours at which time; the edge of the twitch zone was examined and photographed under normal bright light and under fluorescent light to assess the ability of twitch motility in the mutants as compared to wild-type PAO1 cells marked with gfp. Unmarked wild-type cells of PAO1 were included as a positive control in all rescue assays.

Phage sensitivity and adsorption assays. Phage sensitivity was assayed using phage D3112 in the plaque assay. All cultures were incubated for 8 hours at 37°C on LB plates containing 1.5% agar and appropriate antibiotics. Phage were produced and collected as follows. The cells were resuspended in LB to an Abs₆₀₀ of 1.0. The cell suspension was mixed with 0.7 ml of phage 10⁴ pfu/ml in buffer [50 mM Tris.HCl, pH 7.5, 10 mM MgCl₂] and incubated for 40 min at 37 ° C. The mixture was centrifuged at 2,000 x g to remove cells. The resulting supernatant was treated with chloroform by adding to final concentration of 1%. Equal volumes (100 µl) of supernatant and cells from an overnight LB broth culture were mixed together, incubated for 10 minutes and then added to 3 ml of LB containing 0.7% agar. This mixture was poured onto a LB (1.5% agar) petri plate and incubated overnight at 37° C. The total number of plaques was recorded (Roncero et al, 1990). This titre gives an estimation of the sensitivity of the wild-type and mutant cells to the phage.

Western blot analysis. A polyclonal antibody prepared against the type-4 pili of *P. aeruginosa*, kindly provided by Dr. Randall Irvin (University of Alberta, Edmonton) was used to determine the relative amount of pilin protein produced by wild-type and mutant cells. Bacterial strains were grown from frozen stocks on LB agar (2%) plates for 7 to 8 h at 37°C, resuspended in ddH₂O, and adjusted to equivalent protein concentrations. Cells were solubilized by the addition of

Laemmli sample buffer and boiled for 10 min. (Bio-Rad, Cambridge, MA). Whole cell lysates were separated by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% acrylamide [wt/vol]). Prestained molecular weight standards (Bio-Rad, Cambridge, MA) were used to assess efficiency of protein transfer to nitrocellulose membranes. Western immunoblotting was performed using polyclonal PAO1 antipilin antibodies and detection was accomplished using the ProtoBlot II AP system (Bio-Rad, Cambridge, MA).

Enzyme-linked immunosorbent assays. Cultures were grown as described above for Western blot analysis. Growth was resuspended to an A_{600} of approximately 1.0 in LB medium. Cell suspensions were adjusted to contain the same number of cells as determined by direct microscopic counts. Aliquots of the cell suspensions were transferred to microtitre plate wells (Corning flat bottom, tissue culture treated), and incubated (16 h) at 4°C. All washes and antibody incubations were carried out in the microtitre plate wells following the attachment of cells. The wells were washed 3× with PBS containing 0.05% Tween 20. Blocking was accomplished using 1% BSA in PBS for 1 hour at 37°C. The wells were washed 3× with PBS. A 1/100 dilution of the primary anti-pilin antibody in 0.5% BSA was added to the wells and incubated for 1 hour at 37°C. The developing antibody, an alkaline phosphatase conjugate (Bio-Rad), and the detection substrate (Sigma Fast p-Nitrophenyl Phosphate Tablet

Sets) were used according to the manufacturer's directions. Absorbance was measured using a Dynatech MR500 plate reader. Multiple trials of five replicates of each strain were tested and mean values calculated.

RESULTS

Twitching motility: Macroscopic twitching assays demonstrated that motility in the mutant strains was different from the parent strain (Figures 7 and 9). In contrast, flagellar motility showed no difference between mutant parent strains (data not shown). PAO1 wild-type cells exhibited the expected twitch zones as previously described (Darzins, 1993).

Phenotype of quorum sensing synthase mutants: PDO100 formed no visible twitch zone and did not appear capable of twitching motility (Figure 7). PAO-JP1 showed a decrease in the overall amount of twitch zone formed when compared to wild-type cells of PAO1; however, PAO-JP1 did form a larger surface colony than wild-type cells of PAO1 (Figure 7). PAO-JP2 did not form any significant twitch zone and had a slightly smaller surface colony as compared to wild-type cells of PAO1 (Figure 7). Twitching motility was partially restored with the addition of 20mM of PAI-1 to the plate (Figure 7). The twitch zone after addition of PAI-1 is irregular in shape and does not extend as far from the center when compared to wild-type cells of PAO1 (Figure 7).

Analysis of micrographs taken at the edge of the twitch zones was used to determine if any differences were present at the cell-cell interaction level. Wild-type cells of PAO1 and demonstrated typical single cell layered rafts, which is characteristic of normal twitching motility (Figure 8). PDO100 micrographs revealed rafts containing multi-cell layers (Figure 8). PAO-JP1 does not form single cell layer rafts instead the cells appear stacked several layers deep and unable to organize into the single cell layer rafts similar to that of wild-type cells of PAO1 (Figure 8). PAO-JP2 forms multi-layered rafts of cells which are generally not organized when compared to wild-type cells of PAO1 (Figure 8).

Microscopic analysis of the cells at the edge of twitch zones in the rescue assays revealed equal numbers of wild-type cells of PAO1 marked with gfp mixed with an equal number of unmarked wild-type cells as predicted (Figure 9).

This told us that the presence and expression of gfp in wild-type cells did not alter twitching motility. Thus we proceeded to determine whether wild-type cells could indeed "rescue" the twitching motility phenotype of the various QS mutants. A mixture of equal numbers of PDO-100 cells and the gfp marked wild-type cells of PAO1 revealed that most of the cells at the edge were the wild-type PAO1 cells, some of the PDO-100 cells were rescued (Figure 9). An equal mixture of PAO-JP1 cells and gfp marked wild-type PAO1 cells appeared to have slightly more unmarked PAO-JP1 cells at the edge of the twitch zone as compared to the gfp marked wild-type PAO1 cell mixture indicating that the

PAO-JP1 cells were rescued (Figure 9). Nearly all of the cells at the edge of the twitch zone in the mixture of unmarked PAO-JP2 cells and gfp marked wild-type PAO1 cells were gfp marked PAO1 wild-type cells (Figure 9).

Phenotype of quorum sensing receptor mutants: PDO111 was capable of moving; however, its extent of motility was reduced when compared to wild-type PAO1 (Figure 10). PAO-R1 showed a decrease in the overall twitching motility, but exhibited an increase in surface colony growth (Figure 10).

PAO-JP3 was not able to form noticeable twitch zones in this assay. Upon the introduction of *lasR*, twitching motility was partially restored when compared to the wild-type. The introduction of the other R gene (*rhIR*) had no such effect; twitching motility was still absent (Figure 11). Twitching motility was also restored whenever both R genes were placed into the mutant. The twitching motility resulting from both R genes being present was substantially different from that of the wild-type. The surface colony appeared slightly larger, but the twitch zone was reduced and irregular when compared to the wild-type (Figure 11).

Analysis of micrographs taken at the edge of the twitch zones was used to determine if any differences were present at the cell-cell interaction level. Wild-type cells of PAO1 demonstrated typical single cell layered rafts, which is characteristic of normal twitching motility (Figure 12). PDO111 micrographs

revealed rafts containing multi-cell layers (Figure 12). PAO-R1 forms primarily single cell layer to rafts similar to that of wild-type cells of PAO1 (Figure 12). PAO-JP3 forms multi-layered rafts of cells which are generally not as organized when compared to cells of wild-type PAO1 (Figure 12). Whenever PAO-JP3 is complemented by adding back *lasR*, the cells are stacked many layers deep and are not organized in the same manner as wild-type PAO1 cells (Figure 12). If *rhIR* is added back to the PAO-JP3 mutant, the cells form some single layers in rafts (Figure 12). Whenever both *lasR* and *rhIR* are added back to the mutant, many single layer cell rafts can be seen (Figure 12).

Microscopic analysis of an equal mixture of gfp marked cells and unmarked cells at the edge of twitch zones in the rescue assays showed the expected single cell layer rafts under bright field conditions and equal numbers of wild-type PAO1 cells marked with gfp and unmarked wild-type PAO1 cells under fluorescent conditions (Figure 13). Equal numbers of both unmarked mutant cells and gfp marked wild type PAO1 cells were examined to determine if wild-type PAO1 cells could rescue the twitching motility of the mutant cells. Unmarked PAO-R1 cells mixed with an equal number of wild-type PAO1 cells marked with gfp resulted in equivalent numbers of unmarked PAO-R1 cells and gfp marked wild-type PAO1 cells at the edge of the twitch zone (Figure 13). The equal mixture of unmarked cells of PDO-111 and the gfp marked wild-type PAO1 cells appeared to have slightly more unmarked PDO-111 cells at the edge of the

twitch zone (Figure 13). Nearly all of the cells at the edge of the twitch zone in the unmarked PAO-JP3 and gfp marked wild-type cells of PAO1 mixture twitch zone were gfp marked wild-type cells of PAO1 (Figure 13). The examination of the twitch zone edge in the equal mixture of gfp marked wild-type PAO1 cells with unmarked PAO-JP3 cells with either *lasR* or *rhIR* complemented to the PAO-JP3 unmarked cells appeared to have slightly more PAO-JP3 unmarked cells at the edge of the twitch zone as compared to the wild-type PAO1 cell mixture; although the cells did not appear to be as organized as the wild-type PAO1 cell mixtures (Figure 14). In the mixture of gfp marked wild-type cells with PAO-JP3 unmarked cells with *lasR* and *rhIR* complemented the unmarked PAO-JP3 complemented mutant cells appeared in an equal amount compared to the gfp marked wild-type cells at the edge of the twitch zone, however more non-fluorescent cells were present as the distance from the very edge of the twitch zone increased (Figure 14).

The piliation levels of mutant strains. Western and blot analysis and ELISAs were used in conjunction to determine the level of piliation in all strains. The level of piliation was determined primarily through the antibody detection of the protein pilin.

Synthase mutants: Western blot analysis of whole cell lysates revealed little variation between the mutants and wild-type strains (Figure 7). ELISA assays on whole cells showed a striking difference between wild-type PAO1 cells, PDO-100 cells and PAO-JP2 cells (Figure 7). The PAO-JP1 mutant showed no significant difference when compared to wild-type PAO1 cells (Figure 7).

Receptor mutants: Western blot analysis of whole cell lysates revealed little variation between the mutants and wild-type strains (Figure 10). ELISA assays on whole cells showed a striking difference between wild-type PAO1 cells and PAO-JP3 cells (Figure 10). The single R mutants showed no significant difference when compared to wild-type (Figure 10). Complementation of PAO-JP3 with *rhIR*, *lasR*, or *rhIR* and *lasR* did not increase the ability to attach (Figure 11).

Phage sensitivity. Sensitivity to infection by the pilus-specific phage D3112*cts* was calculated by comparing the titre required to result in clearing of the bacterial lawn. PDO111 showed a slight resistance when compared to wild-type PAO1 cells (Table 4). PAO-R1 exhibited a noticeable resistance to the phage (Table 4). PAO-JP 3 showed a very slight resistance to the phage (Table 4). Complementation of PAO-JP3 with *rhIR*, *lasR*, or *rhIR* and *lasR* did increase the sensitivity to phage infection to levels comparable to wild-type cells of PAO1 (Table 4).

Discussion

The quorum-sensing systems in *Pseudomonas aeruginosa* are known to be complex (Pesci and Iglewski, 1997). The hierarchy, interrelationships between the *las* and *rhl* systems, and diverse number of genes controlled by quorum sensing all make up a complex system of many dependent parts. This illustrates that both the *rhl* and *las* systems provide control over twitching motility as evidenced by the results presented here.

We have previously reported that the *rhl* system seemed to have a very definite role and quorum control of twitching motility (Glessner et al, 1999). The results presented here also indicate that the *las* quorum sensing system seems to be involved in twitching motility and sensitivity to phage infection. The surface piliation and twitch zone formation correlate very well, and absence of both the *rhl* and *las* systems results in a loss of attachment and inability to twitch normally (Figures 7, 10 and 11). Results from the Western blot indicate that the mutant strains to produce normal amounts of pilin (Figures 7, 10 and 11).

Since the cells are producing pilin at roughly equivalent amounts, control by quorum sensing is most likely at the level of secretion of pilin and/or assembly of the pilus. It has been reported that assembly and secretion of pilin is required for normal twitching motility (Alm and Mattick, 1997).

An increased resistance to the phage D3112 by the *rhIR* and *lasR* mutants suggests that the pili are not fully externalized or the pili may have an assembly defect. The attachment and measurement of pilin are contrary to the phage results. However, the inability of the *lasR* mutants to form normal twitch zones supports the phage data (Table 4). The complementation of the PAO-JP3 mutant with *lasR*, *rhIR* or both *lasR* and *rhIR* does not increase the piliation, however does increase the susceptibility to infection by phage D3112 (Table 4). The increase in susceptibility may indicate that the pili are present, however not functioning properly in a manner that allows for successful attachment.

In the *las* and *rhI* quorum sensing systems regulate components of the general secretory pathway (GSP) of *P. aeruginosa* (Chapon-Herve et al, 1997). This secretory pathway is responsible for externalizing exoenzymes responsible for virulence (Pesci et al, 1997). Pili are known to be assembled by a second branch of the GSP, the pilus biogenesis pathway (Lory, 1996). We reported previously, that the GSP involving the pilus formation may be a target for the quorum sensing systems (Glessner et al, 1999). The data presented here further support this claim.

In the complementation studies involving the mutants lacking both *lasR* and *rhIR*, *lasR* seemed to play the dominant role in regulating twitching motility. ng correlates well with the resistance to phage. The finding that complementation with both *lasR* and *rhIR* does not restore the *lasR* and *rhIR* mutant is not surprising. The quorum sensing systems in *Pseudomonas aeruginosa* are very dynamic in requiring precisely controlled ratios of autoinducer to transcriptional activating proteins. Complementation with either or both R genes should allow for speculation as to which system has a dominant effect on twitching motility. Since the complement genes are located on a plasmid and not the usual position in the chromosome, regulation of the entire quorum sensing system may be disturbed enough to not produce wild-type results.

Recent published results suggest that the *las* and *rhI* systems do not control twitching motility (Mattick et al., 2002). The results published by Mattick et al. (2002) state that defective variants of the $\Delta lasI$ and $\Delta rhII$ mutants used for the preceding studies had arisen through culturing in the absence of autoinducers leading to second site mutations (Mattick et al., 2002). However, they show only the results for QS mutants in the PAK strain. These researchers go on to state that the PDO100 and PAO-JP2 mutants contain a deletion in the *pheC* gene adjacent to the *rhII* gene and that complementation is needed to

verify the certainty of a mutant strain. Complementation, albeit partial and solely with AHLs added back, has been shown with the PAO-JP2 mutant indicating that this is a valid mutant for use in this study (Figure 7). Upon knock out of the *pheC* gene, Mattick et al. (2002) found no effect on twitching motility. If the *pheC* gene does not play a role in twitching motility then the mutants used in this study were valid and the *las* and *rhl* systems do play a major role in twitching motility. To be sure that the *las* and *rhl* systems do play a role in twitching motility the

Δ *las* *rhl* mutant was reconstructed in the PAO1 strain and tested for twitching motility (Mutants provided by Dr. Barbara Iglewski). As shown in Figure 15a, JM-2 (Δ *las* *rhl*) is indeed deficient in twitching motility. Complementation by the wild type genes added back on a plasmid is clear with this newly constructed mutant. These mutants illustrate that the QS systems do play a role in twitching motility in the PAO1 strain. We were able to obtain the QS mutants Mattick constructed in the PAO1 background. A mutation in the *rhl* gene shows no twitch zone when compared to wild-type PAO1 (Figure 15b). The Δ *rhl* Δ *las* mutant created by the Mattick lab shows a decreased twitch zone as compared to wild-type PAO1, but a twitch zone is still apparent (Figure 15b). The presence of twitching in this double mutant is surprising since a mutation in the *rhl* gene by itself rendered the Δ *rhl* mutant unable to form a twitch zone as compared to wild-type PAO1 (Figure 15b). The presence of the twitch zone in the double

mutant raises an important question about the integrity of this double mutant and more importantly the conclusions these researchers reached in their publication regarding twitching motility not being controlled by quorum sensing (Mattick et al., 2002). Further, the lack of a twitch zone as seen in the $\Delta rhII$ mutant (Figure 15b.) seems to contradict the findings listed in their recent publication and supports the findings previously documented by this lab (Glessner et al., 1999).

FUTURE STUDIES

The continuing study of quorum control of twitching motility in *Pseudomonas aeruginosa* is of importance. Quorum control of pili dependent biofilm formation is of practical and economic value (Davies et al., 1998). The quorum and sensing systems control of biofilms can easily be realized in industry, but biofilms serve many important ecological functions as well. How quorum sensing affects twitching motility and processes involving twitching motility awaits further study.

How quorum sensing can be used in medicine is also an important question that needs exploring. If the quorum sensing systems control a variety of genes, analogs or autoinducer blocking compounds could potentially aid in the treatment of a variety of ailments caused by *P. aeruginosa* as well as other bacteria where the quorum sensing systems have been elucidated.

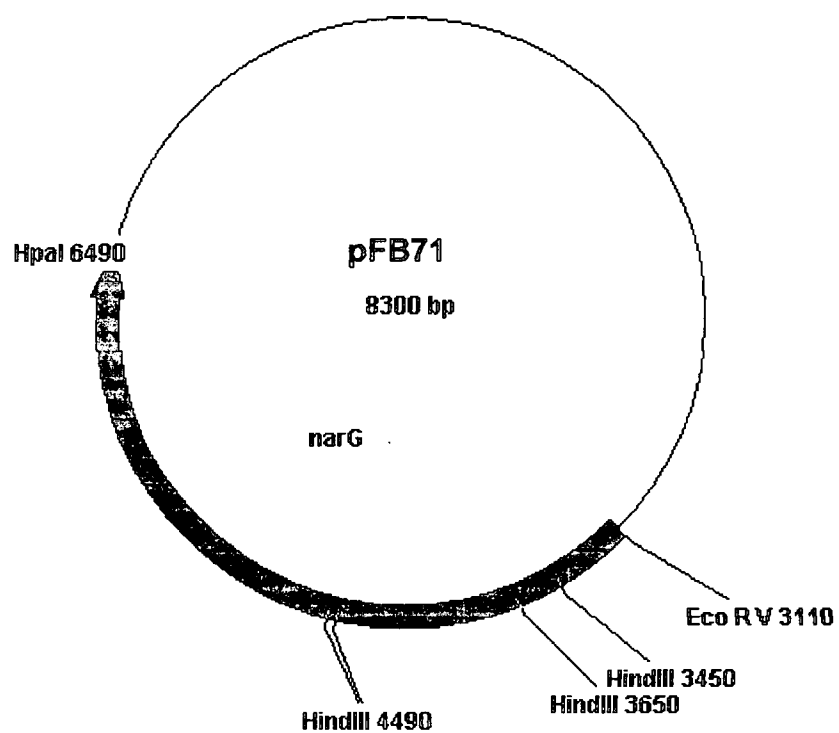


Figure1. Restriction map of pFB71 containing *narG* from *E. coli*.

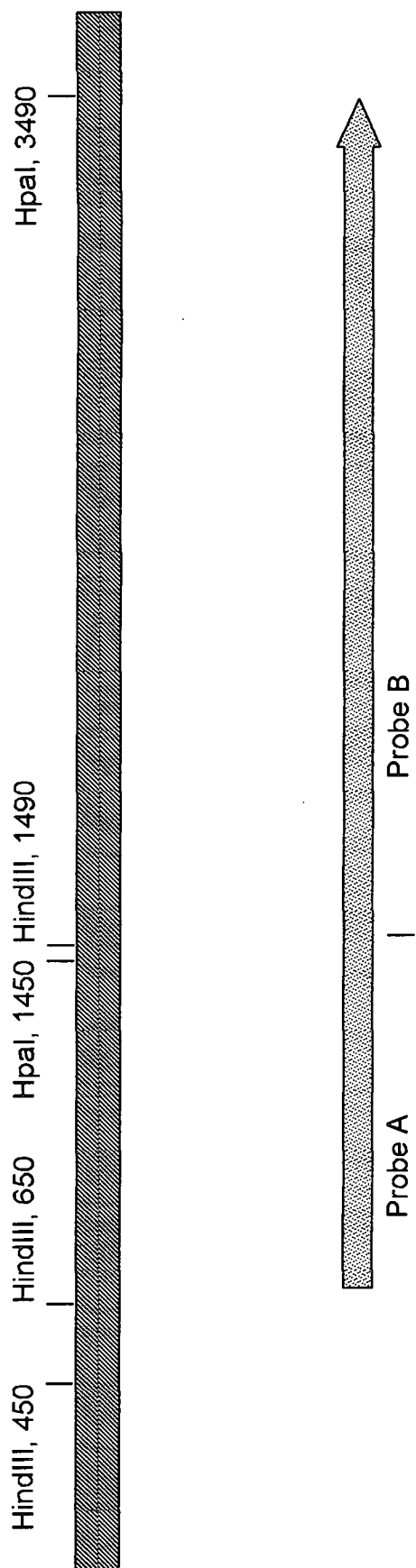


Figure2. Restriction map of *narG* and internal position of probe A (0.84 kb HindIII) and probe B (2.1 kb HpaI) relative to *narG*.

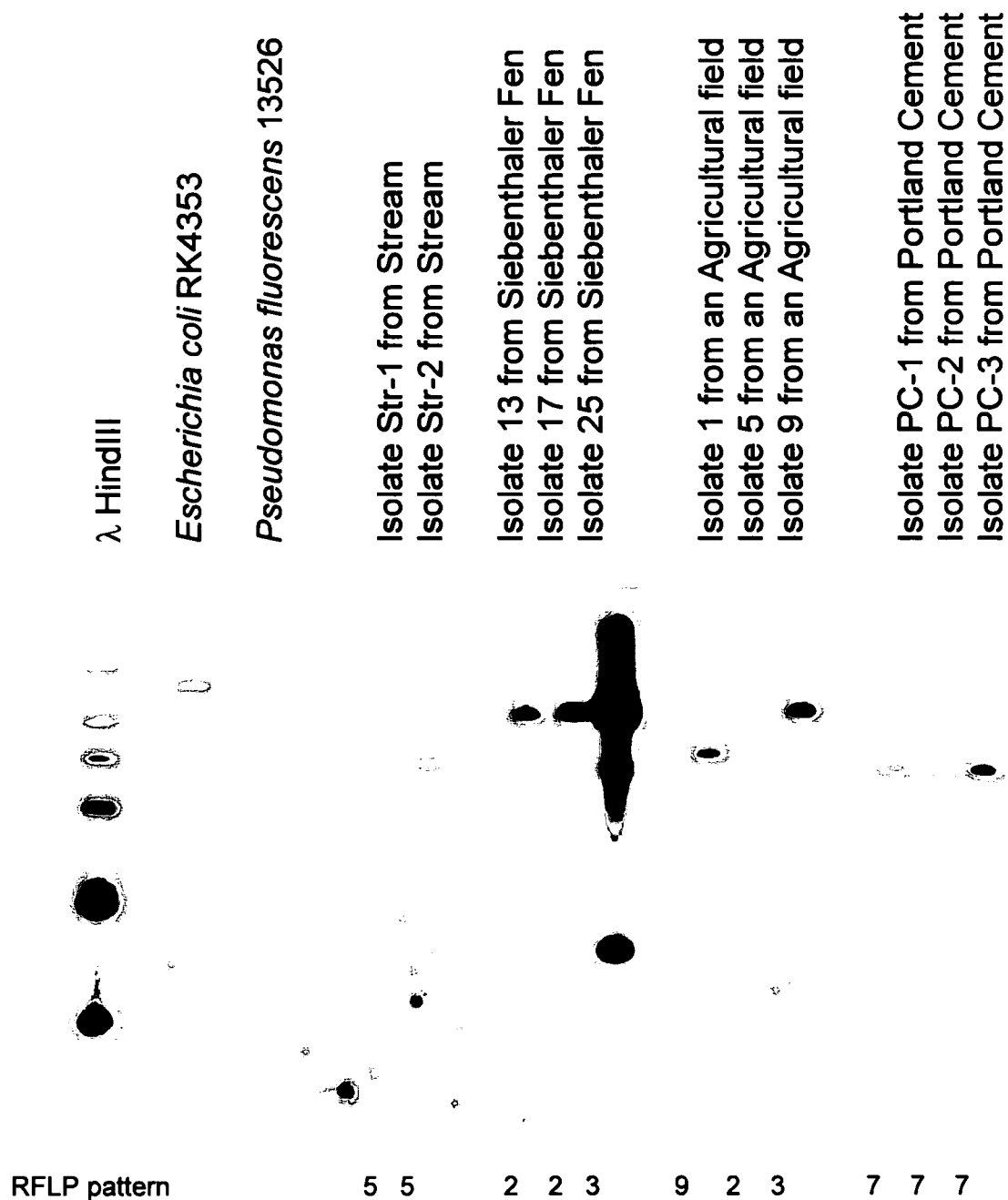


Figure 3. Southern Blot analysis of genomic DNA isolated from environmental isolates probed with *narG* (Probe B 2.1 kb) from *E. coli*.

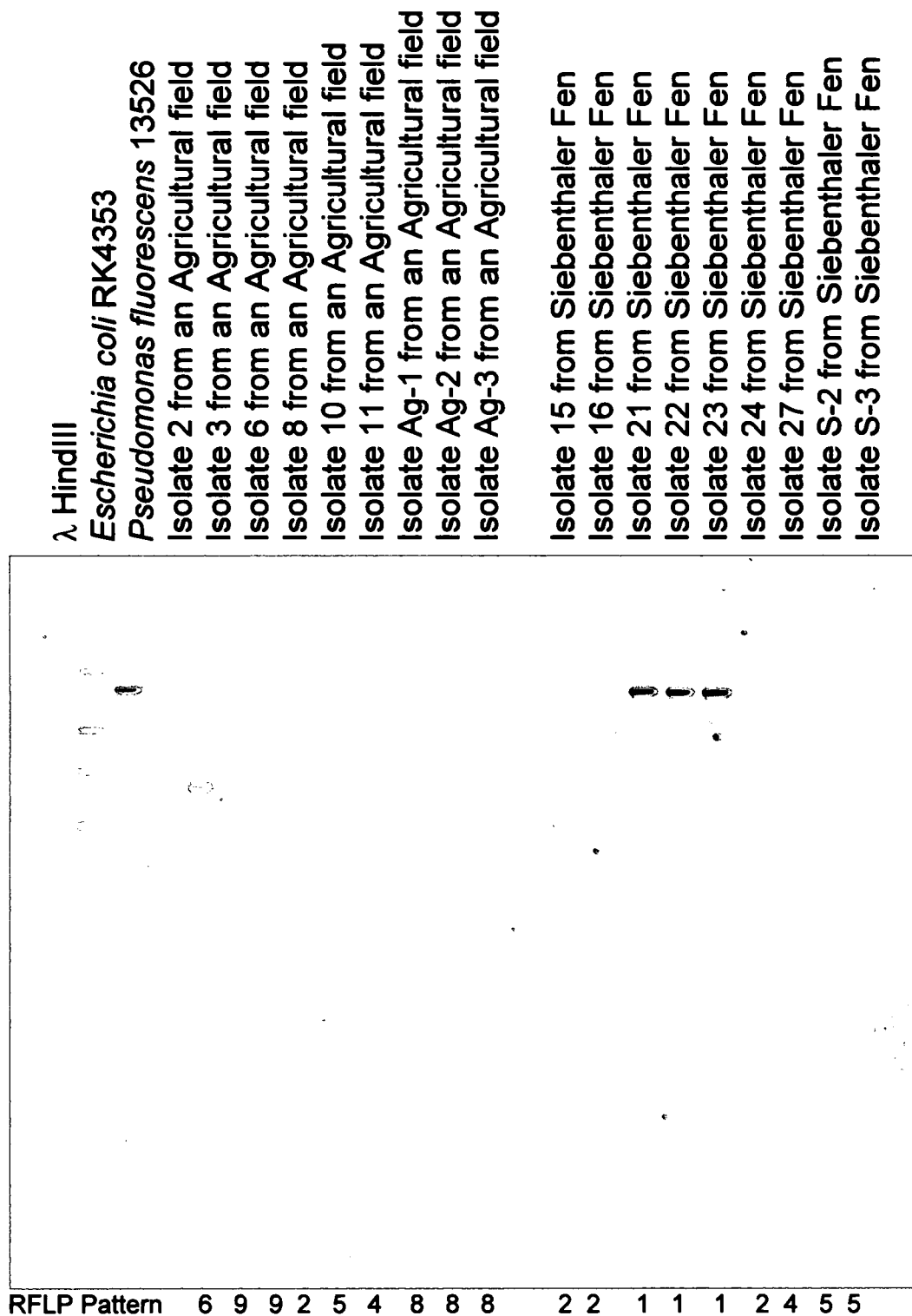


Figure 4. Southern Blot analysis of genomic DNA isolated from environmental isolates probed with *narG* (Probe B 2.1 kb) from *E. coli*.

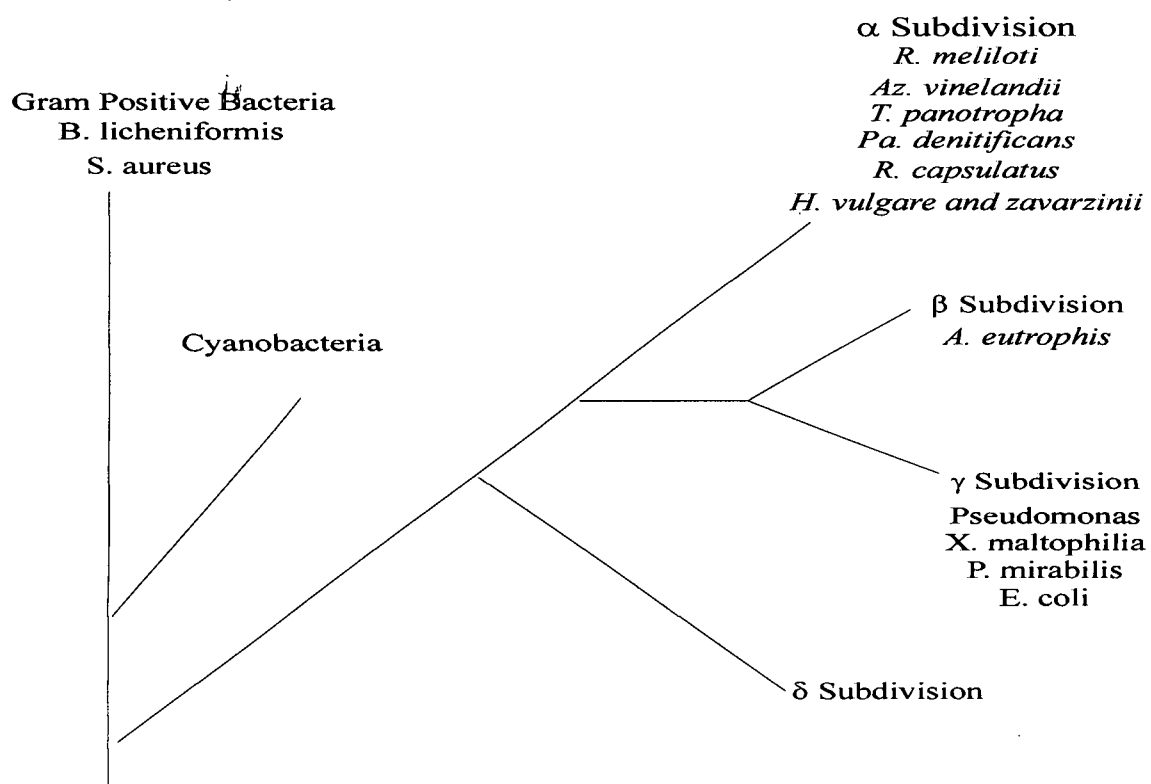


Figure 5. Phylogenetic tree depicting the relationships between different groups of bacteria.

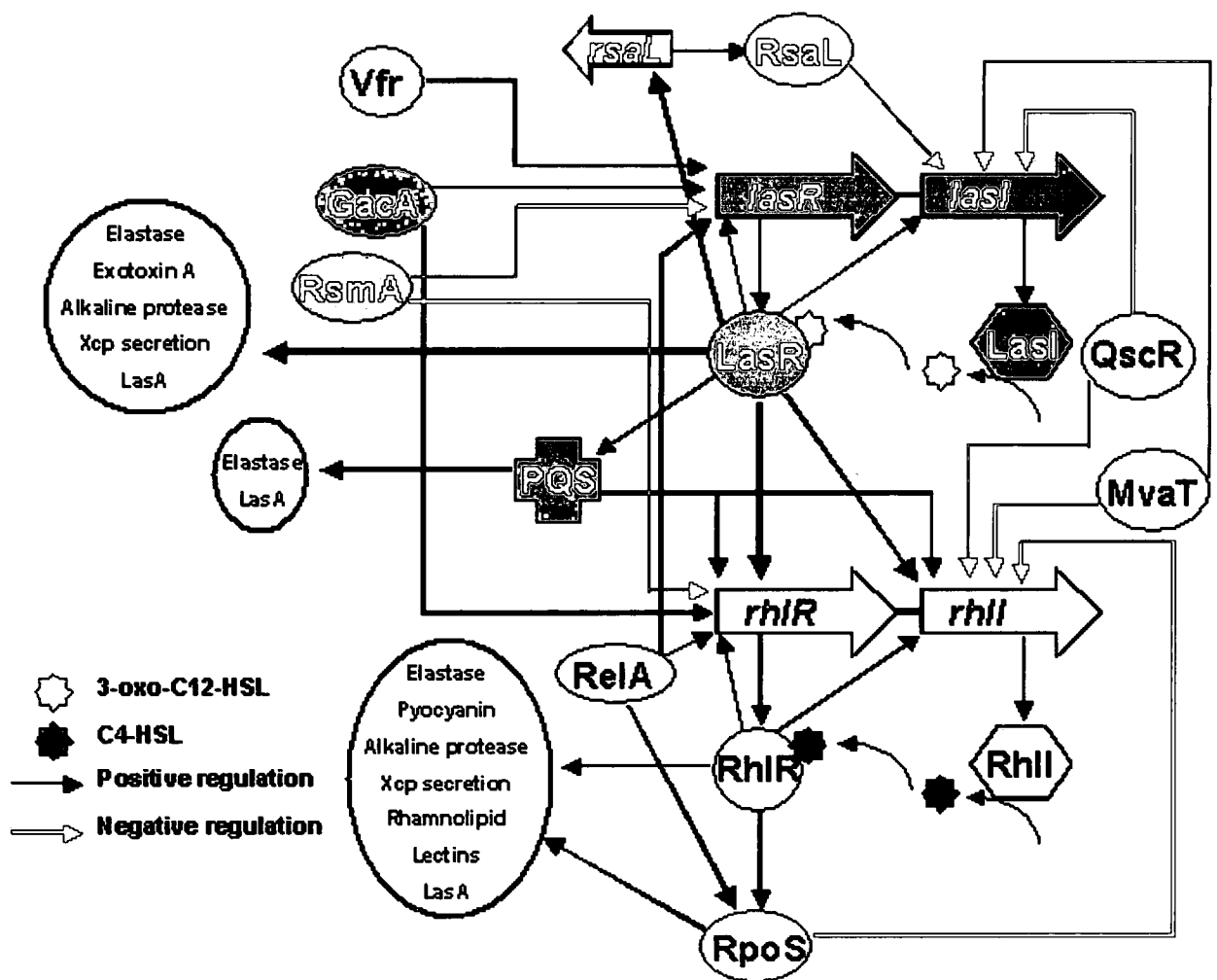


Figure 6. Model of quorum sensing in *Pseudomonas aeruginosa* (Williams, 2005).

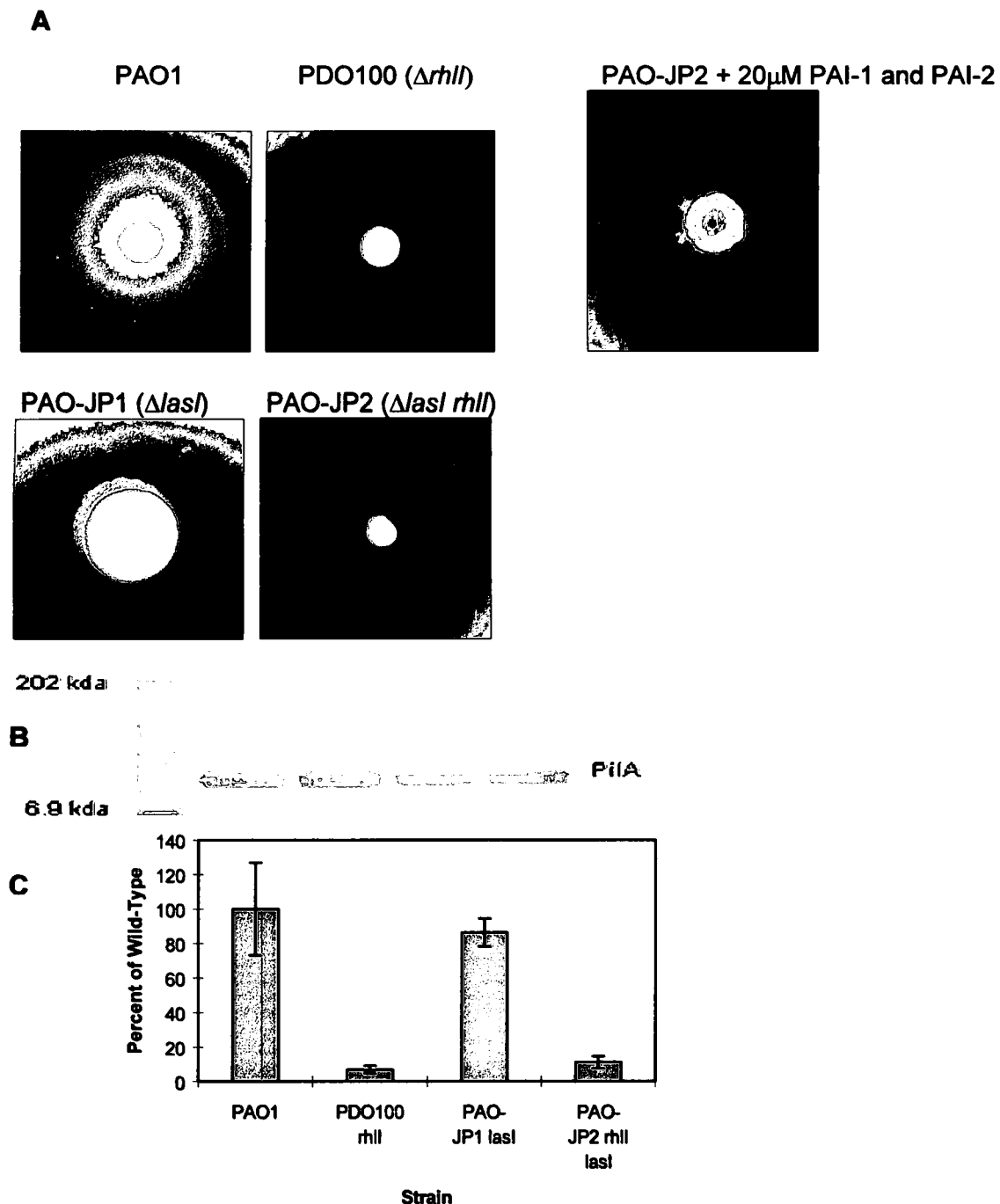


Figure 7. A. Photographs of twitch zones of the *lasI* and *rhII* 'synthase' mutant of *Pseudomonas aeruginosa* PAO1. LB agar plates were stabbed with a needle to the bottom of the plate and incubated for 24 h. at 37° C.

B. Western Blot showing molecular weight markers, wild-type *Pseudomonas aeruginosa* PAO1, PDO100 ($\Delta rhII$), PAO-JP1 ($\Delta lasI$), and PAO-JP2 ($\Delta rhII$ and *lasI*) in lanes. The Western Blot was performed using anti PilA antibodies. (Antibodies courtesy of R. Irvin)

C. The bar graph indicates the relative ability to attach to a surface as compared to the PAO1 strain.

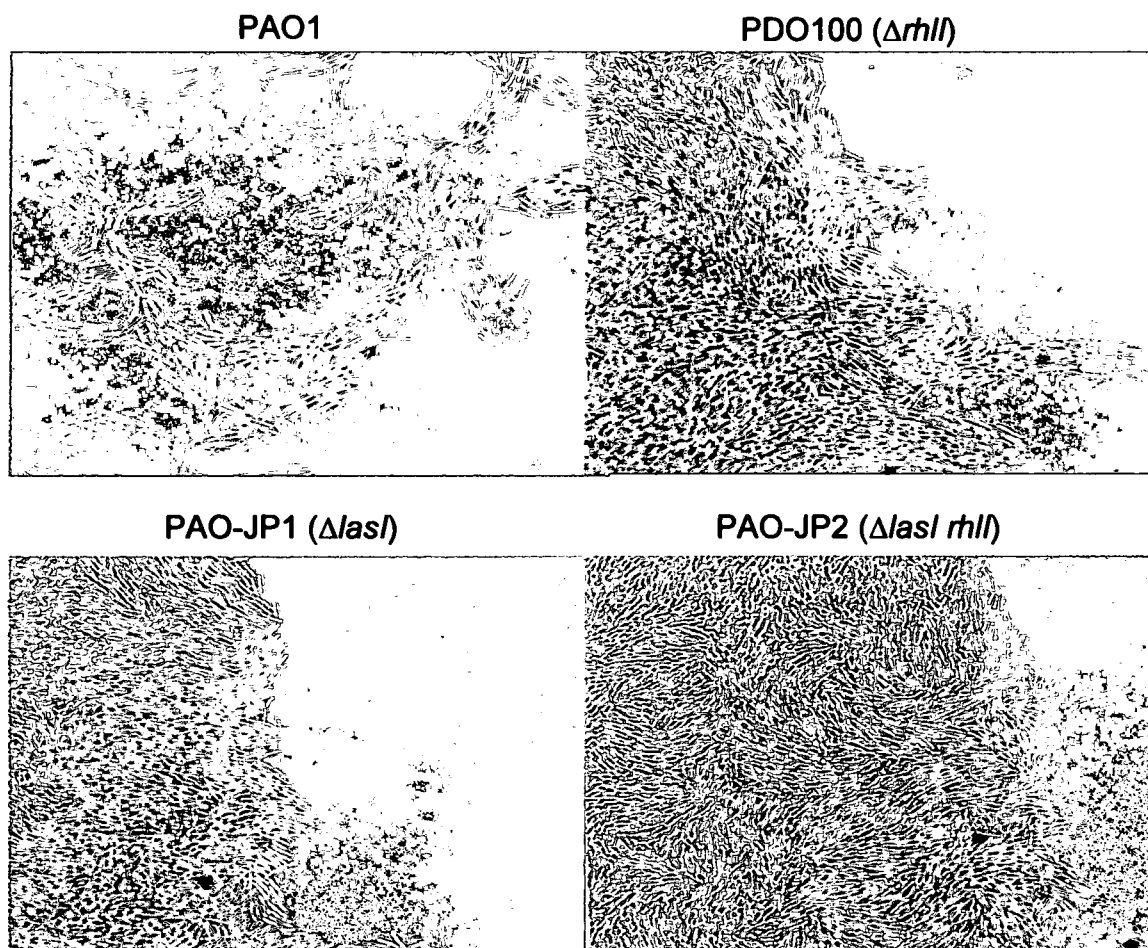
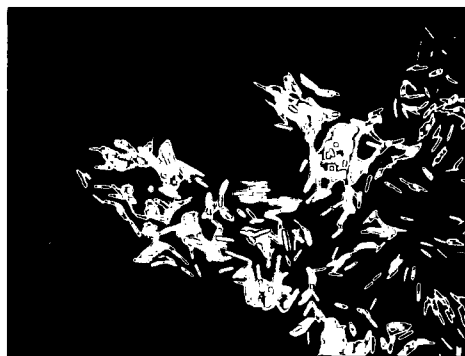
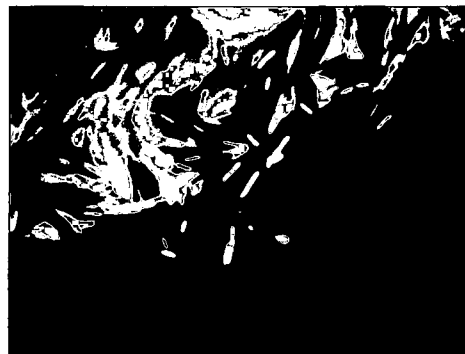
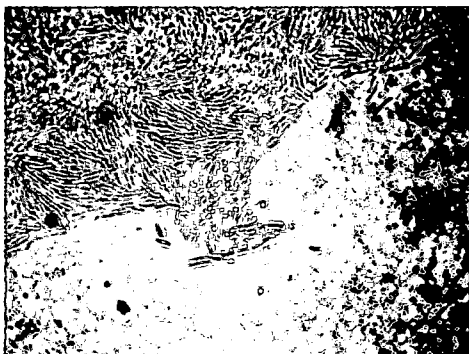


Figure 8. Slide culture assay for twitching motility. The areas in the photomicrographs represent the edges of cell growth (magnification ca. X 500).

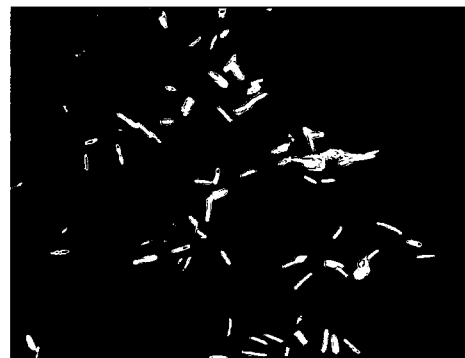
PAO1 mixed
with gfp
tagged PAO1
wild type cells



PDO-100
($\Delta rhII$)
mixed with
gfp tagged
PAO1 wild
type cells



PAO-JP1
($\Delta lasI$)
mixed with gfp
tagged PAO1
wild type cells



PAO-JP2
($\Delta rhII\ lasI$)
mixed with gfp
tagged PAO1
wild type cells



Figure 9. Photomicrographs of the edge of a twitch zone in which 50% of the cells present are gfp marked *Pseudomonas aeruginosa* PAO1 wild-type cells and the other 50% are unmarked *lasI*, *rhII*, or *lasI* and *rhII* 'synthase' mutant cells.

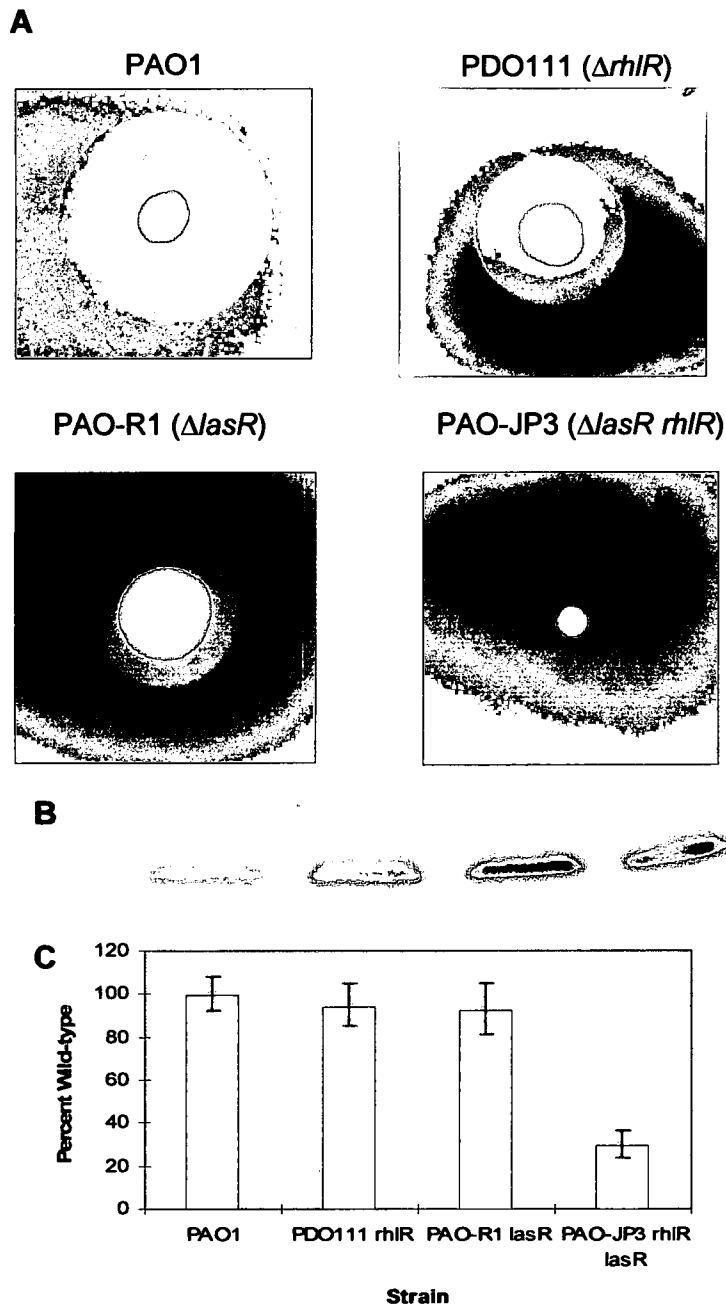
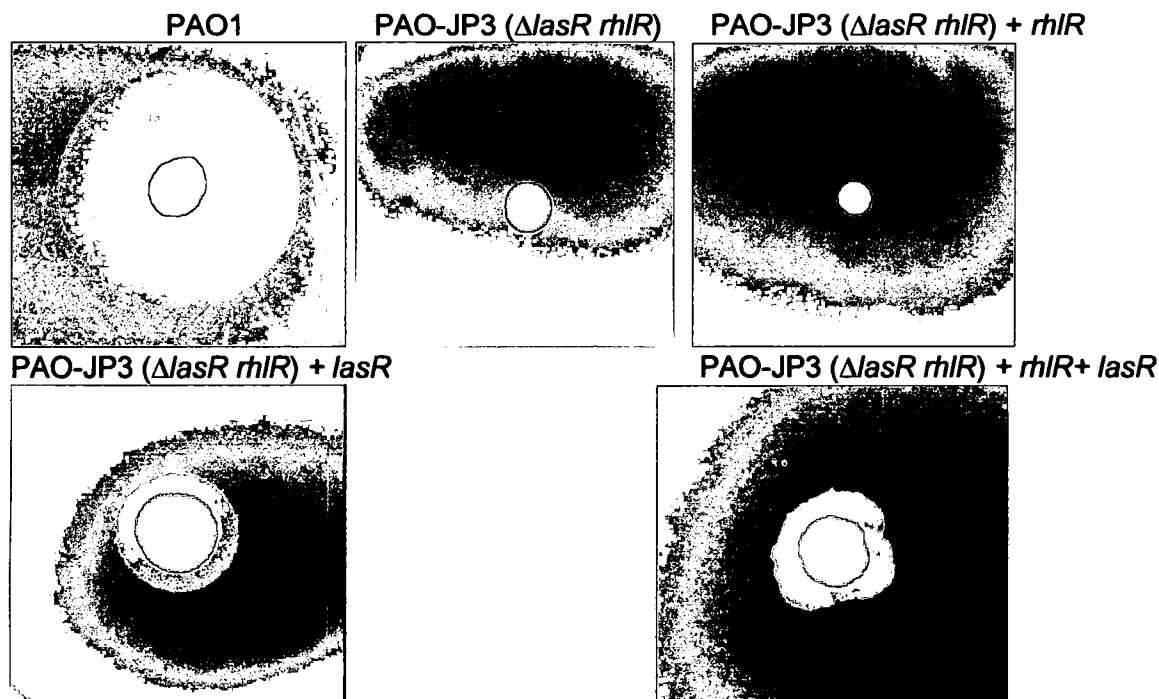


Figure 10. A. Photographs of twitch zones of the *lasR* and/or *rhIR* 'receptor' mutant of *Pseudomonas aeruginosa* PAO1. LB agar plates were stabbed with a needle to the bottom of the plate and incubated for 24 h. at 37° C.

B. Western Blot showing wild-type *Pseudomonas aeruginosa* PAO1, PDO111 ($\Delta rhIR$), PAO-R1 ($\Delta lasR$) and PAO-JP3 ($\Delta rhIR$ and *lasR* in lanes. The Western Blot was performed using anti PilA antibodies. (Antibodies courtesy of R. Irvin)

C. The bar graph indicates the relative ability to attach to a surface as compared to the PAO1 strain.

A



B

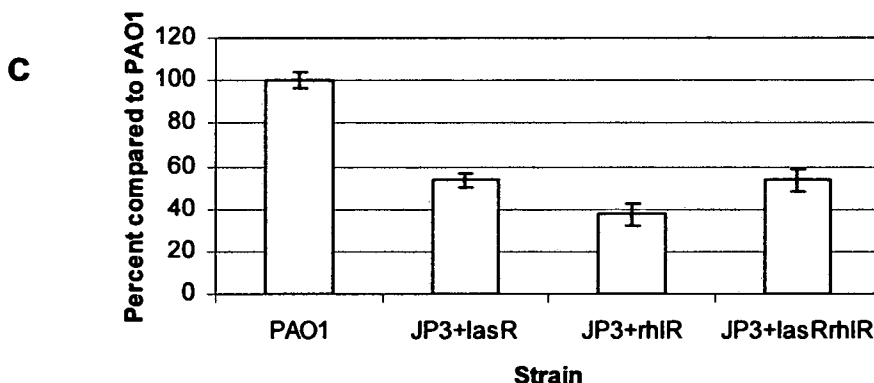


Figure 11. A. Photographs of twitch zones of the *lasR* and *rhIR* 'receptor' mutant of *Pseudomonas aeruginosa* PAO1. Indicated by the + are the gene(s) inserted on a plasmid to complement the mutant. LB agar plates were stabbed with a needle to the bottom of the plate and incubated for 24 h. at 37° C.

B. Western Blot showing molecular weight markers, wild-type *Pseudomonas aeruginosa* PAO1, PAO-JP3 ($\Delta rhIR$ and *lasR*) + *lasR*, PAO-JP3 ($\Delta rhIR$ and *lasR*) + *rhIR*, and PAO-JP3 ($\Delta rhIR$ and *lasR*) + *lasR* + *rhIR* in lanes. The Western Blot was performed using anti PilA antibodies. (Antibodies courtesy of R. Irvin)

C. The bar graph indicates the relative ability to attach to a surface as compared to the PAO1 strain.

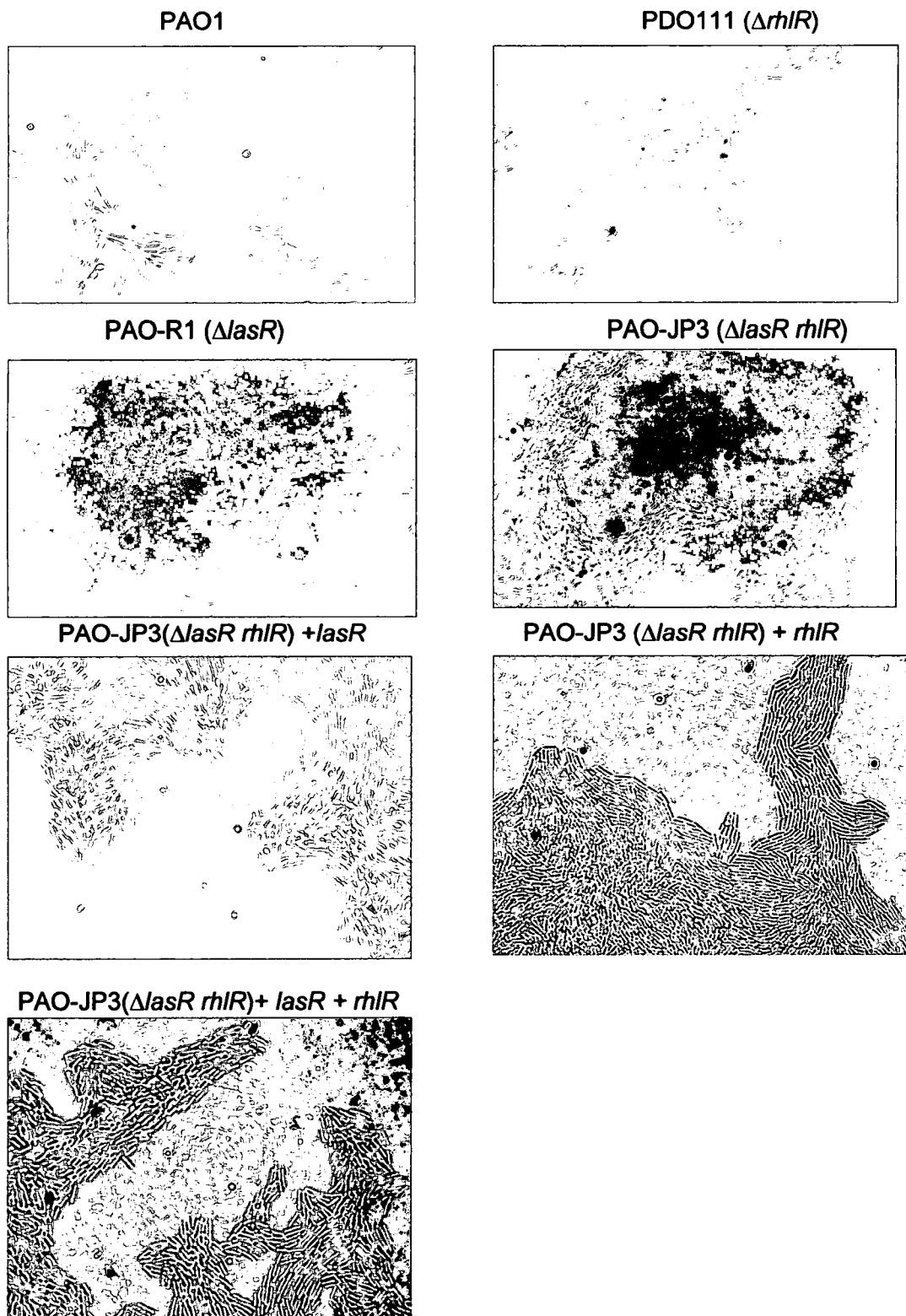
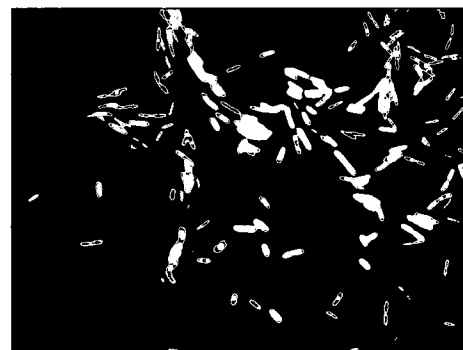
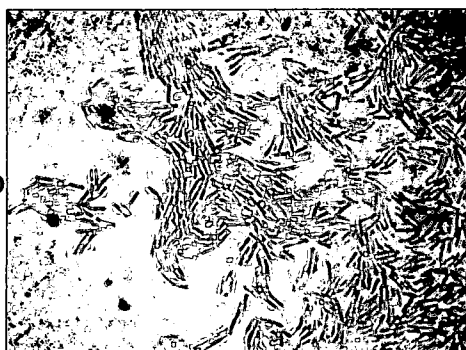


Figure 12. Slide culture assay for twitching motility. The areas in the photomicrographs represent the edges of cell growth (magnification ca. X 375).

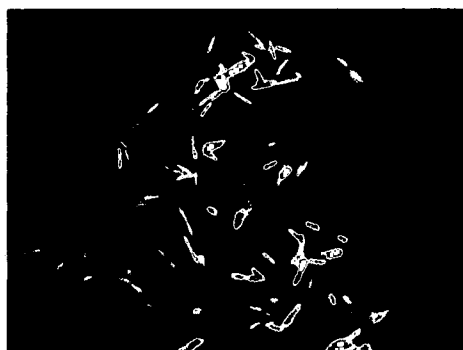
PAO1
mixed with gfp
tagged PAO1
wild type cells



PAO-R1
($\Delta lasR$)
mixed with gfp
tagged PAO1
wild type cells



PDO111
($\Delta rhIR$)
mixed with gfp
tagged PAO1
wild type cells



PAO-JP3
($\Delta lasR rhIR$)
mixed with gfp
tagged PAO1
wild type cells

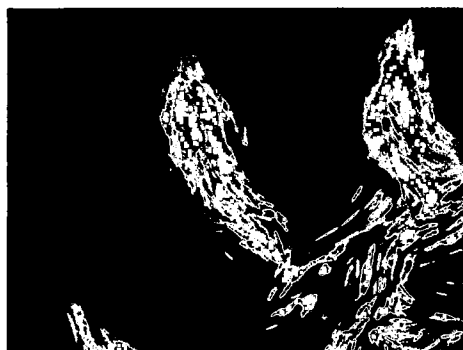
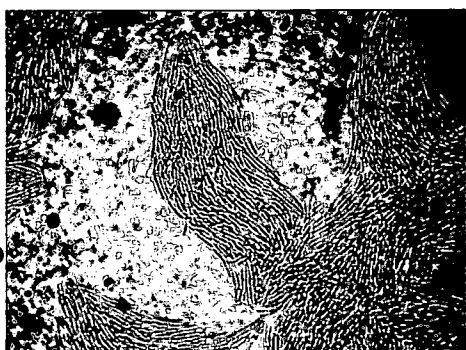
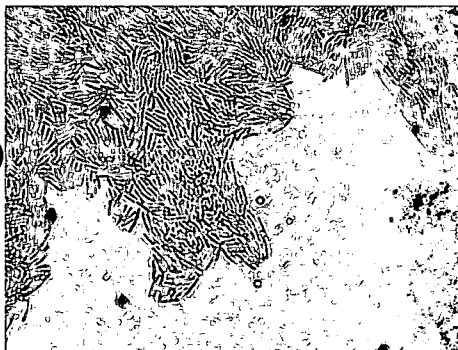
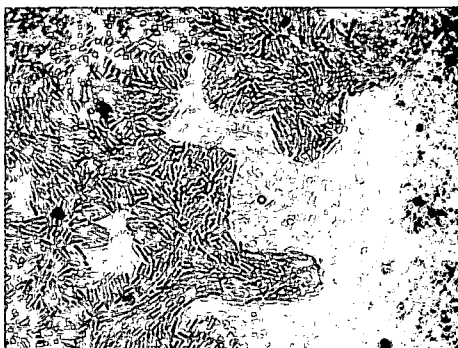


Figure 13. Photomicrographs of the edge of a twitch zone in which 50% of the cells present are gfp marked *Pseudomonas aeruginosa* PAO1 wild-type cells and the other 50% are unmarked *lasR*, *rhIR*, or *lasR* and *rhIR* 'receptor' mutant cells.

PAO-JP3 ($\Delta rhIR lasR$)
+ *lasR* mixed with
gfp tagged PAO1
wild type cells



PAO-JP3($\Delta rhIR lasR$)
+ *rhIR* mixed with
gfp tagged PAO1
wild type cells



PAO-JP3 ($\Delta lasR rhIR$)
+ *lasR rhIR* mixed with
gfp tagged PAO1
wild type cells

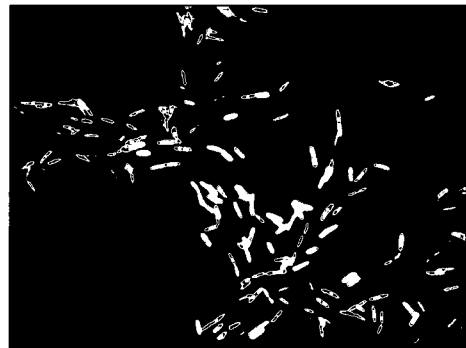
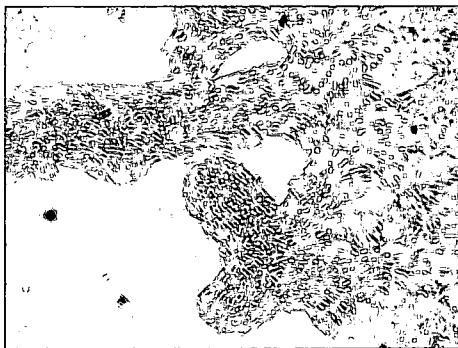


Figure 14. Photomicrographs of the edge of a twitch zone in which 50% of the cells present are gfp marked *Pseudomonas aeruginosa* PAO1 wild-type cells and the other 50% are unmarked *lasRrhIR* 'receptor' mutant with either the *rhIR* gene or the *lasR* gene added back on a plasmid.

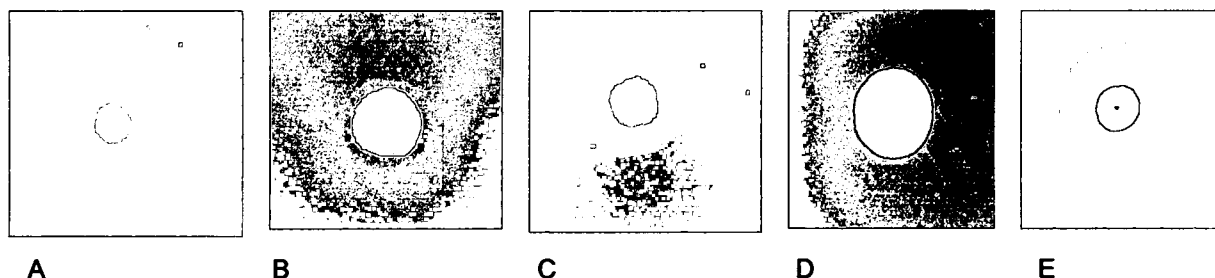


Figure 15a. Macroscopic stab assay for twitching motility. LB (1% agar) plates were stab inoculated to the bottom of the plate and incubated at 37°C for 24 hours. The twitch zone (diffuse zone), at the petri dish/agar interface, is a measure of twitching motility. The center, dense zone is surface colony growth. (A) wild-type PAO1, (B) $\Delta lasB$ mutant, (C) complemented $\Delta lasB$ mutant, (D) $\Delta rhII lasI$ double mutant, (E) complemented $\Delta rhII lasI$ double mutant. Strains courtesy of Dr. Barbara Iglewski, University of Rochester. Assays performed by Dr. Jayne Robinson, University of Dayton.

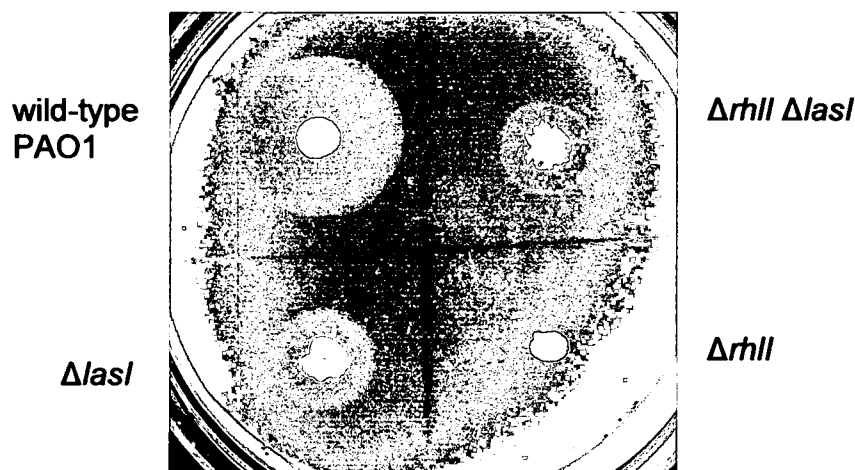


Figure 15b. Macroscopic stab assay for twitching motility. LB (1% agar) plates were stab inoculated to the bottom of the plate and incubated at 37°C for 24 hours. The twitch zone (diffuse zone), at the petri dish/agar interface, is a measure of twitching motility. The center, dense zone is surface colony growth. Strains courtesy of Dr. John Mattick, University of Queensland. Assays performed by Dr. Jayne Robinson, University of Dayton.

Table 1. Various bacterial species, the carbon source used for the Nitrate uptake, rates of nitrate uptake, and homology to the *narG* of *E. coli*.

Data courtesy of Brent Christner for comparison to environmental isolates in Table 2 (Christner 1996).

Strain	Carbon source	Rate of NO ₃ Uptake nmol/min/mg protein	Probe B (2.1 kb HpaI)
α-Purple Bacteria			
<i>Rhizobium meliloti</i>	Mannitol	186 ± 15	(-)
<i>Azotobacter vinelandii</i>			(-)
<i>Thiosphaera pantotropha</i>	Acetate	147 ± 44	(+)
<i>Paracoccus denitrificans</i>	Glycerol	134 ± 21	(-)
<i>Rhodobacter capsulatus</i>	Succinate	243 ± 37	(-)
<i>Hyphomicrobium vulgare</i>	Methanol	146 ± 24	(+)
<i>Hyphomicrobium zavarzinii</i>	Methanol	203 ± 11	(+)
β-Purple Bacteria			
<i>Alcaligenes eutrophus</i>	Glycerol	244 ± 46	(+)
γ-Purple Bacteria			
<i>Pseudomonas fluorescens</i> Biotype A			(-)
<i>Pseudomonas fluorescens</i> Biotype C	Glycerol	151 ± 29	(+)
<i>Pseudomonas aeruginosa</i> PAO1	Glycerol	169 ± 59	(+)
<i>Pseudomonas stutzeri</i> Marine strain	Glycerol	147 ± 36	(-)
<i>Pseudomonas stutzeri</i> Clinical isolate	Glycerol	192 ± 28	(+)
<i>Xanthomonas maltophilia</i>	Glycerol	135 ± 22	(+)
<i>Proteus mirabilis</i>	Glycerol	115 ± 19	(-)
<i>Escherichia coli</i> RK4353	Glycerol	135 ± 5	(+)
M91-3			(-)
Low G+ C Gram Positive Bacteria			
<i>Bacillus licheniformis</i>	Glycerol	158 ± 18	(+)
<i>Staphylococcus aureus</i>	Glycerol	158 ± 57	(-)

Table 2. Various environmental isolates genotype, the location where the isolate was obtained, ability to grow on *Pseudomonas* isolation agar, and rates of nitrate uptake.

Genotype Based on Molecular weight banding pattern	Isolate	Site of origin	Growth on <i>Pseudomonas</i> isolation agar	Rate of Nitrate uptake nmol/min/mg protein
1	21 22 23	Siebenthaler Fen Siebenthaler Fen Siebenthaler Fen	(-) (-) (-)	249.00 \pm 30
2	13 17 15 16 24 8 5	Siebenthaler Fen Siebenthaler Fen Siebenthaler Fen Siebenthaler Fen Siebenthaler Fen Agricultural Field Agricultural Field	(+) (+) (+) (+) (+) (+) (-)	450.59 \pm 60
3	25 9	Siebenthaler Fen Agricultural Field	(+) (+)	192.23 \pm 11
4	11 27	Agricultural Field Siebenthaler Fen	(+) (+)	316.08 \pm 40
5	Str1 Str2 10 S2 S3	Stream Stream Agricultural Field Siebenthaler Fen Siebenthaler Fen	(-) (-) (-) (-) (-)	196.00 \pm 29
6	2	Agricultural Field	(-)	236.01 \pm 30
7	PC1 PC2 PC3	Portland Cement Portland Cement Portland Cement	(-) (-) (-)	291.99 \pm 14
8	Ag1 Ag2 Ag3	Agricultural Field Agricultural Field Agricultural Field	(-) (-) (-)	241.65 \pm 39
9	1 3 6	Agricultural Field Agricultural Field Agricultural Field	(-) (-) (-)	215.35 \pm 23
10 (No homology to <i>narG</i>)	14	Siebenthaler Fen	(-)	108.12 \pm 60
N/A	<i>E. coli</i> RK4353	N/A	(-)	150.97 \pm 1

Table 3. Bacterial strains used the characteristics of the strain and the source.

<i>P. aeruginosa</i> Strain	Relavant Characteristics	Source/Reference
PAO1	Wild-type prototroph	B. Iglewski
PAO-R1	$\Delta lasR$ derivative of PAO1, Tcr	Gambello and Iglewski, 1991
PDO100	$\Delta rhII::Tn501$ derivative of PAO1, Hgr	Brint and Ohman, 1995
PDO111	$rhIR::Tn501$ derivative of PAO1, Hgr	Brint and Ohman, 1995
PAO-JP1	$\Delta lasI$ derivative of PAO1, Tcr	Pearson et al,1997
PAO-JP2	$\Delta lasI$ derivative of PDO100, Hgr Tcr	Pearson et al,1997
PAO-JP3	$\Delta lasR$ derivative of PDO111, Hgr Tcr	Pearson et al,1997

Table 4. Table showing the titer necessary for phage infection on *Pseudomonas aeruginosa* PAO1, PDO100 ($\Delta rhIR$), PAO-R1 ($\Delta lasR$), and PAO-JP3 ($\Delta rhIR lasR$). The piliation or ability to attach to a surface is noted in the second column. All piliation values are relative to the PAO1 wild type.

<i>P. aeruginosa</i> strain	Sensitivity to D3112	Piliation
PAO1	10^3	100
PDO111 ($\Delta rhIR$)	10^5	95 \pm 10
PAO-R1 ($\Delta lasR$)	10^5	93 \pm 12
PAO-JP3 ($\Delta rhIR lasR$)	10^7	30 \pm 6
PAO-JP3 + <i>rhIR</i>	10^3	38 \pm 5
PAO-JP3 + <i>lasR</i>	10^3	54 \pm 5
PAO-JP3 + <i>rhIR</i> + <i>lasR</i>	10^3	53 \pm 5

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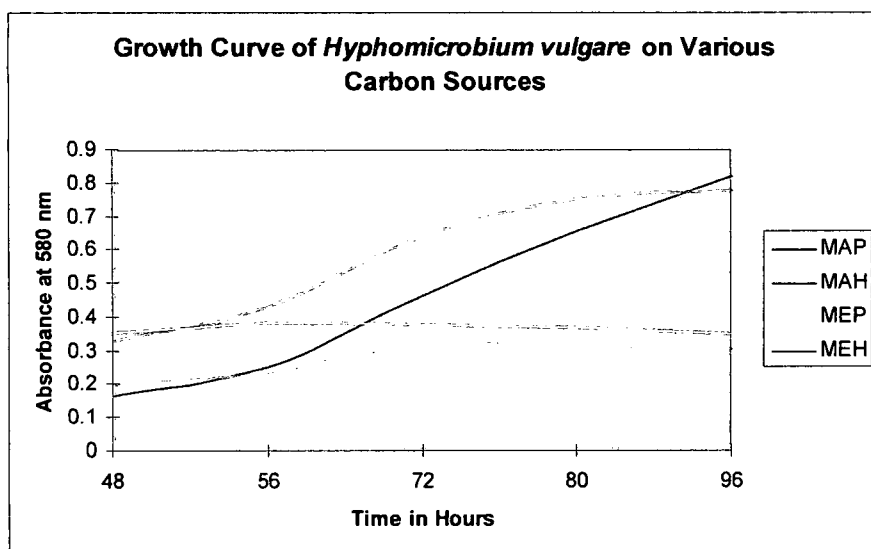
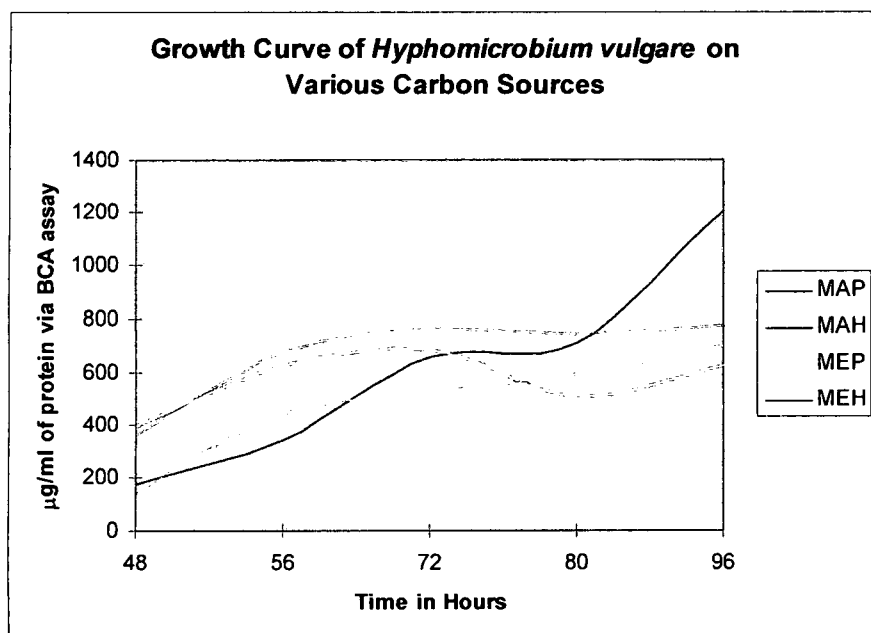
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APPENDIX I.



Appendix 1. Graphs of *Hyphomicrobium vulgare* on various carbon and phosphate sources. MAP=337 media with methylamine as the carbon source and phosphate buffer, MAH=337 media with methylamine as the carbon source and Hepes as the buffer, MEP=337 media with methanol as the carbon source and phosphate as the buffer, and MEH=337 media with methanol as the carbon source and Hepes as the buffer.

Appendix 1. Stalk length in response to starvation of phosphate (PO₄). Average stalk length of *Hyphomicrobium vulgare* is indicated below as a function of time. 2X no phosphate indicates that the culture was removed from phosphate containing media and then subcultured twice into no phosphate media.

	PO ₄	No PO ₄	2x No PO ₄	
Units on scope	2	5.5	7	
	2.5	6	8	
	1.5	6	9	
	2	5		
	1	5		
	1.5	5		
4 days later	2.5	6		
	1.5	5		
	3	5.5		
	4	5		
	2.5	5.5		
	3	6		

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