OCS Study
MMS 2001-094

Coastal Marine Institute

Survival of a Hydrogen-Utilizing Bacterium when Introduced into Native and Foreign Environments

MMS U.S. Department of the Interior
Minerals Management Service
Gulf of Mexico OCS Region

Cooperative Agreement
Coastal Marine Institute
Louisiana State University

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Survival of a Hydrogen-Utilizing Bacterium when Introduced into Native and Foreign Environments

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December 2001

Prepared under MMS Contract
14-35-0001-30660-19921
by
Department of Oceanography and Coastal Sciences
School of the Coast and Environment
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Baton Rouge, Louisiana 70803

Published by

U.S. Department of the Interior
Minerals Management Service
Gulf of Mexico OCS Region

Cooperative Agreement
Coastal Marine Institute
Louisiana State University

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CITATION

LaRock, P.A. and L.S. Donovan. 2001. Survival of a hydrocarbon-utilizing bacterium when introduced into native and foreign environments. US Dept. of the Interior, Minerals Management Service, Gulf of Mexico OCS Region, New Orleans, LA. OCS Study MMS 2001-094. 52pp.

PREFACE

As the pressure and need for waste cleanup increases, bioremediation is emerging as an attractive means to restore inaccessible or environmentally sensitive areas. The degradation of spilled hydrocarbons is one application where the seeding of bacteria is particularly attractive since it is the microbes who ultimately consume and remove these materials, whether they occur naturally (as in marsh plant cuticles) or as an anthropogenic input. Typically, one would select for an organism capable of performing the requisite remediation task, overlooking the fact that success under one set of conditions does not guarantee success elsewhere. In other words, competitive pressures may well prevent the introduced microbes from becoming established and affecting the job they were chosen for.

In order to assess the fate of an introduced species of microbe, you must have a means of specifically identifying and tracking that species, whether it becomes the dominant or minority microbe in a consortium. You must also have a way of collecting enough material for analysis without disrupting the community or the experiment. To resolve these technical difficulties we borrowed methods used by the molecular biologist to, 1) quantitatively amplify the DNA of target species, and 2) develop molecular probes that would unequivocally and rapidly identify the test species.

Initially, a suite of hydrocarbon utilizing bacteria consisting of *Pseudomonas aeruginosa, Xanthomonas maltophilia* and *Serratia marsescens* was used to develop a multiplex or competitive PCR primer and probe set for the estimation of the relative proportion of the total microbial community that any one of these species occupied in an environmental matrix. The probes not only served as PCR primers, but when labeled with a fluorescent marker, they also would specifically identify the test organism.

Coastal wetlands are a particular concern in the event of an oil spill and so our early efforts at field experiments focused on the microbial consortia associated with this environment. Our test species did not fare well and we consistently observed a new, and as yet unidentified, species of *Acinetobacter* as the dominant microbe. Our attentions then focused on this organism for all of the subsequent competition experiments and we developed a molecular probe for its identification.

This body of this report focuses on the competitive studies performed on the *Acinetobacter* sp., which actually was the second half of the project. The development of the molecular tools that were used is presented as a reprint in Appendix A since it was published before the microbial studies ended.

The upshot of these efforts was the development of a methodology that may be used to assess changes in any microbial assemblage as a result of changes in the ecosystem, or for tracking introduced microbial species during bioremediation efforts.

ACKNOWLEDGMENTS

The support and efforts of a number of people is gratefully acknowledged. Brent Tatford, who is now in medical school, undertook the formidable task of developing the multiplex PCR methodology which was his M.S thesis done under the supervision of Vince Wilson in the Department of Environmental Sciences, LSU. Matt Mahler diligently provided technical assistance in the initial phases of the project and was responsible for the isolation of the *Acinetobacter* culture used throughout. Fred Rainey and Naomi Ward-Rainey of the Department of Biological Sciences undertook the identification of the *Acinetobacter* sp. and construction of the molecular probe that was used to track its persistence in the competition experiments that were done by Lisa Donovan in fulfillment of her requirements for the M.S. degree. Susan Meiers contributed to the success of this work through her efforts in the field aspects and by training Lisa in the use of molecular probes, as well as keeping things on track in the daily operations of a laboratory. Kim Nguyen aided in sorting out the numerous details associated with aseptic techniques and general microbiological methodology associated with the project.

Special thanks are extended to Pat Roscigno of the Minerals Management Service, New Orleans district for his support and understanding throughout the delays and difficulties encountered during this project.

Paul LaRock

ABSTRACT

Increasingly we are seeing technological developments in which fluids of diverse origins are brought together, either by design or by accident. In Florida, for example, surface waters are being pumped into groundwater aquifers to increase drinking water supplies for the southern metropolitan areas. There is great concern, however, that coliform bacteria or other disease-causing microbes that are prevalent in surface waters, might proliferate when introduced into the subsurface regime. In a similar vein we are seeing renewed interest in the use of bioremediation to detoxify contaminated ground water, and in particular this approach has been proposed for the remediation of spilled oil. In general, bioremediation consists of either seeding bacteria that have been adapted in the laboratory to rapidly degrade the material in question, or modification of the environment with "fertilizers" to promote the development of natural microbial assemblages that are associated with the contaminant. The seeded bacteria might begin the degradation process immediately and more efficiently, whereas fertilizer addition might entail a relatively long lag period before effects are seen. The critical question, however, is whether the seeded microbes can compete and survive against native species in a foreign environment.

A new species of *Acinetobacter* that is capable of using hexadecane as its sole carbon source was isolated from coastal wetlands in Louisiana. When laboratory grown cultures of the *Acinetobacter* were re-introduced to an experimental flow-through marsh environment and hexadecane added, the microbe quickly displaced all other bacteria and represented essentially 99% of the microbial community. When the *Acinetobacter* was added to Mississippi River water supplemented with hexadecane in a chemostat, it was completely displaced after six days, at which time an indigenous microflora developed that reached a community density of 2×10^6 CFU per ml. One of the controls in this experiment was simply the addition of hexadecane to a chemostat that had only river water passed through it, and a microbial community of approximately 10^6 developed right from the start. The conclusions are that, 1) seed bacteria may readily be introduced back into the environment from which they originated, 2) Seed bacteria do not necessarily survive in foreign environments, and 3) seeded bacteria may actually inhibit the development of normal microflora capable of utilizing the contaminating material.

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INTRODUCTION

In oil producing and handling areas, considerable attention revolves around cleanup and containment practices in the event of a release to the environment. Over the decades we have seen an evolution in oil-spill remediation, but unfortunately the conditions under which spills occur and the predominating weather factors often prevent the successful application of any physical cleanup procedure. Experience in Prince William Sound, Alaska, revealed just how futile existing efforts can be when confronted by a large-scale oil spill. In fact, the National Academy of Science Select Review Panel concluded that it would have been better to leave the cleanup to natural processes rather than to affect measures that in no way could restore the environment. Over one million dollars per day were spent on a physical cleanup effort whose success was highly questionable. Because of the extraordinary cost of spill amelioration, interest surged bioremediation technology after the *Exxon Valdez* accident (Beardsley, 1989).

One of the side effects of the *Valdez* incident was that it afforded scientists the opportunity to apply some bioremediation efforts, mainly the application of bacterial "fertilizers" (mixtures of nitrogen and phosphorus compounds) to enhance the activity of oil-degrading microbes already present in the contaminated area. The results were very encouraging, suggesting that the bioremediation accelerated oil degradation when compared to untreated areas, although the rates of hydrocarbon mineralization could not be assessed (Fox, 1994; Lindstrom et al., 1991). The key questions to be answered, however, are, 1) how beneficial wold seeded bacteria be in accelerating hydrocarbon decomposition relative to natural processes, and 2) If bacteria were seeded into an oil spill, how well would the introduced species compete for survival against the native microbial community?

The variables involved, and their interaction, in maintaining the structural balance in a microbial consortium appears almost limitless and includes such factors as interspecies competition, succession of species, environmental factors (temperature, salinity, and light), physiological properties of the individual species and the production of antagonistic chemicals. For an introduced bacterial species to successfully colonize a new or foreign environment, it must have a strategy to deal with these external pressures, and one potential factor governed by the invader itself may be the rate at which ATP is produced. Bacteria with a high rate but a lower yield of ATP production, may have a selective advantage in establishing itself when resources are limited and shared (Pfeiffer, et al., 2001). In a series of soil experiments in which *Pseudomonas fluorescens* was initially introduced, the addition of a *Pseudomonas putida* strain proved unsuccessful in establishing itself. In all cases, when one culture was introduced into an environment which the other had already colonized, and when the two were grown in co-culture, *P. fluorescens* either completely impeded or significantly reduced colonization of *P. putida* (Alachi, et al., 1991). These observations suggested that both bacteria were competing for similar niches or resources within the soil environment and that one bacterium, *P. putida*, lacked the ability to compete against *P. fluorescens* in this specific setting.

The density or size of a native bacterial community also impacts upon the ability of a seeded species to establish a niche. The bacterium, *Pseudomonas fluorescens,* was able to inhibit the growth of the fish pathogen *Vibrio anguillarum* when the initial cell densities of *P. fluorescens* were 100 to 1000 times greater than those of *V. anguillarum* (Gram, et al., 1999). The same effect was not observed, however, when initial cell densities were similar between the two organisms, suggesting that a culture introduced into a community saturated with another organism(s) may have difficulty in establishing a foothold

There are a variety of biochemical mechanisms by which microbes are able to assume a position of superiority in a consortium. Some bacteria are known to produce agents that are 1

antagonistic to competing bacteria. Lactic acid bacteria, when grown in the presence of *E. coli*, *Listeria monocytogenes*, and *S. aureus*, inhibited these pathogens (Kot, et al, 2000). A study of marine epibiotic bacteria showed some of these microbes, which do not normally produce antibiotics, could be encouraged to produce antimicrobial compounds by exposure to other cultures or chemicals (Burgess, et al, 1999). Similar findings were obtained in a series of laboratory experiments in which the marine bacterium *Streptomyces tenjimariensis* was exposed to 53 bacterial isolates grown in co-culture with the result that 22.6% of these competing bacteria elicited antibiotic production by *S. tenjimariensis* to the extent that the competitors could not develop colonies on nutrient medium (Slattery et al., 2001). In other words, the presence of an introduced organism induced the production of an antibacterial agent by the native species, resulting in the elimination of the competitor.

The production of probiotics is yet another means by which bacteria can enhance their survival chances. Probiotics are compounds that foster colonization of the producing strain while inhibiting the growth of competitors. The presence of a probiotic producer often has synergistic effects when one is found in the human gastrointestinal tract. Certain resident microflora (typically lactic acid bacteria) have been shown to be antagonistic to potentially pathogenic and inflammatory microbes (Dunne, et al, 1999). Probiotic-producing bacteria have been shown to out-compete other bacteria for nutrients and for adhesion sites, as well as inhibit colonization by opportunistic species (Gatesoupe, 1999).

In addition to probiotics and antibiotics, bacteria can also produce bacteriocins, toxins that are highly specific and can act upon competitors of the same species, but are diverse within each species. These proteins are encoded on a plasmid, as are immunity determinants that protect the toxin producer itself from the lethal effects of the bacteriocins (Perry and Staley, 1997). Competition for resources is key in the survival of bacteriocin-producing bacteria where limited nutrients promotes a keen struggle (Frank, 1994). Conversely, an excess of nutrients may also be a determinant in the colonization of a specific microbe. In experiments that compared the population dynamics of oligotrophs and copiotrophs relative to nutrient availability, it was found that abundant resources encouraged growth of copiotrophs while inhibiting colonization of oligotrophs in natural soils (Hu, et al, 1999). The ability to adapt to changing nutritional regimes also enters into the picture. Bacteria that had been grown in two distinct environments, one of which was nutrient rich, and the other nutrient poor, were combined and their abundance over time followed. It was expected that the bacteria grown at high nutrient levels would not be able to compete in a nutrient deficient environment, however, the opposite was shown to be the case. The bacteria were, in most instances, able to adapt to the new environment that contrasted to that in which they were initially grown, suggesting that resource availability may not play a major role in colonization by seeded bacteria (Velicer and Lenski, 1999).

In microcosm experiments designed to model oil spill bioremediation (Tagger, et al, 1983), a mixed culture of marine hydrocarbon-degrading bacteria were introduced to a spill situation to accelerate decomposition of the oil. All of the added bacteria were displaced in favor of the indigenous species which were better suited to the prevailing conditions after only a few days. Because this experiment was carried out *in situ*, wind and tidal dispersal of the seeded organisms would limit their effectiveness since dispersal would also remove the hydrocarbon substrate and any added nutrients that were essential for the seeded microbes, further reducing their ability to proliferate. Native microflora, on the other hand, could continue to repopulate as conditions essential for their proliferation would remain essentially unchanged.

The present work was undertaken to eliminate as many variables as possible and to look specifically at competition between introduced and native microflora under carefully controlled conditions. Temperature, turn-over time, and nutrient availability were held constant and the

hydrocarbon substrate, hexadecane (C16), was added in excess throughout the experiments. Dispersal of organisms and C16 were non-issues and exogenous carbon sources were found not to interfere. The success or displacement of the introduced *Acinetobacter* sp. would, therefore, be a function of competition between microbial populations resulting from interspecies effects or factors peculiar to a specific environmental.

MATERIALS AND METHODS

Media

Two media were used during these experiments. The first medium (ASW) consisted of diluted artificial sea water (instant ocean) prepared so the final salinity was 1.5% to which 2.5 g/L peptone (Difco Laboratories) and 0.5 g/L yeast extract (Difco Laboratories) were added. The second medium (BH) consisted of half-strength Bushnell-Hass salts (Bushnell and Hass, 1941) adjusted to a final salinity of 1.5% using NaCl. Peptone and yeast extract (PYe) were added in the same concentrations as used in the ASW medium. For solid media, agar was added at a final concentration of 1.5%. The hydrocarbon source in all experiments was hexadecane (C16) (Acros Organics, Fair Lawn, New Jersey) which was added directly to maintenance slants, and was added to the covers off the petri dishes in plating experiments. In the chemostat experiments, Triton X-100 (Sigma-Aldrich, St. Louis, MO) was added to facilitate dispersion of the C16 such that its reservoir concentration was 0.008%. For the chemostat experiments, the C16 was added in 1 ml volumes directly to the growth vessel and was replenished daily as it was flushed out in the overflow. The C16 was filter sterilized by passage through a 0.4 um membrane filter (Millipore Corp., Bedford, MA)

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Seed Culture

A dominant organism to be used in the seeding experiments was isolated from a sample collected from the marshes off the Louisiana coast using a chemostat enrichment process. The medium pumped through the chemostat was half strength BH adjusted to a salinity of 1.5% with a turnover time of one day. Hexadecane (C16) was added directly to the chemostat vessel and was replenished as needed. Multiple chemostats were run for up to one week, but in all cases the same dominant organism was isolated and has been tentatively identified as a new Acinetobacter species, a species herein referred to as A-84.

Fluorescent Probe

In order to specifically and unequivocally identify this organism, a rhodamine labeled 18 mer oligonucleide probe was developed that was used to follow the fate of this organism when it was reintroduced into the environmental samples. Hybridization of the fluorescently-labeled probe was done according to the methods of Amann et al., (1991).

Chemostat Experimental Design

The chemostats used in this experiment all contained a growth volume of 200 ml. Media was pumped into the vessels at a rate of 200 ml per day using a peristaltic pump (Harvard Apparatus, Boston, MA) and spent medium was remove through an overflow tube in the side of the vessel. Samples were withdrawn and C16 added through a sampling port in the top of the vessel. Because of the design of the chemostat, C16 was lost from the system through the overflow and was replenished as needed or on a daily basis using 1 ml aliquots.

For those experiments in which a culture, A-84, was used alone to assess the effects of organic carbon additions, cell number was followed by direct counting under phase objectives. When dealing with environmental materials, plate counting was used to follow cell numbers because of the interfering debris. In order to follow the fate of A-84 when reintroduced to the environment from which it was isolated, a marsh microcosm was established in the laboratory. Marsh plants were dug up and placed in a 0.2 m^2 container and marsh water was pumped into the system from a 20 L reservoir. Then, using a second pump, the marsh water was pumped through three separate chemostat vessels (with a turnover time of one day) such that vessel one contained marsh water alone, vessel two contained the marsh water plus C16, and vessel three contained marsh water, C16, and culture A-84 (cell density at the start of the experiment in the growth vessel was 10^7 cell/ml).

In order to follow the fate of culture A-84 when introduced into a foreign environment, in this case Mississippi River water, three chemostats were again established. River water was pumped from a 20 L reservoir (turnover time was one day) into three chemostats using the same experimental treatments as noted above, i.e., river water alone, river water with C16, and river water with C16 and culture A-84. In this instance NaCl was added to give a final salinity of 1.5% so that salt effects would not interfere with the experimental observations.

RESULTS

Of primary concern to coastal Louisiana are oil spills and their effects on the coastal marsh system. Microbes are the ultimate clean-up device, thus the purpose of this effort was to determine the survival capabilities of seeded microbes which might be used to accelerate biodegradation. Our first efforts were directed at selecting a key organism from the wetlands that might serve as a suitable seed for oil spill remediation, not only in a wetland setting, but in other aquatic environments as well. To do this chemostats were established using plant debris and sediments that had been inoculated with C16. In all instances one organism repeatedly became dominant and has been identified as a unique *Acinetobacter* species (Fig. 1). As seen in the figure, this organism occupies a singular space in the species tree suggesting that while it has common traits with other *Acinetobacter*, it is different from these other species and appears to occupy its own niche. Furthermore, the *Acinetobacter* sp., herein referred to as A-84, was chosen as our primary test organism because it was readily and repeatedly isolated from the Louisiana marsh environment, degraded C16 readily and required no growth factors as is characteristic of the genus in general (Juni, 1984). Organisms of this genus are found in water, soil, and sewage, making *Acinetobacter* sp. an ideal candidate for seeding, as it is appears to normally exist in many environments. The waxy cuticle found in marsh grasses serves as a one natural hydrocarbon source that *Acinetobacter* would routinely be expose to, and so for the biodegradation and competition studies, this organism would be expected to offer great promise. In order to follow the passage of this specific bacterium through subsequent mixed culture experiments, a fluorescently labeled probe was prepared that enabled us to precisely, rapidly and accurately identify this organism in the presence of competitors.

One of the primary characteristics of wetland existence is the very high level of organic substrates that are present, which conceivably can serve as a competitive substrate rather than the introduced oil. In order to determine the effects of organic levels on A-84, a series of chemostat experiments were performed. The first such experiment (Fig. 2) followed the temporal changes of the bacterial population within the chemostats at 0.1 and 0.5% organic loadings in a mineral salts menstruum. In the earlier part of the experiment the population declined, particularly at the lower organic substrate, but began to level off at about 420 hr. At 420 hr the original volume of C16 was nearly depleted, as the oil was flushed out through the overflow, so C16 was routinely added every other day, which stabilized the low organic (0.1% PYe) chemostat at 4×10^7 cell/ml. At 740 hrs the organic level was raised to 0.5%, resulting in an increase in cell density to approximately 2×10^8 until the end of the experiment. By comparison, the high organic chemostat (0.5% PYe) sustained a population of $2\text{-}3 \times 10^8$ cell/ml after equilibrating at 420 hr., but the addition of C16 at 420 hr prompted an increase in the cell density up to 750 hrs after which the equilibrium population of 3×10^8 cell/ml was obtained. The results indicate that supplemental organic material over and above the C16 serves to increase the numbers of A-84, but whether this added substrate competed or negatively influenced the rate of C16 decomposition is uncertain. The C16 addition to the 0.1% PYe chemostat at 420 hr did, however, induce an increase in the population suggesting cometabolism of the substrates.

A second set of experiments was initiated at a low level of organics (0.1% PYe) in order to better define the effects of the added C16 as a second substrate which, if both substrates are used, should be manifest as an increase in A-84 cell density in the presence of C16 relative to a medium without C16. The results of the earlier stages of this experiment (Fig. 3) showed that initially the populations in the chemostats slowly decline, but the density in the presence of C16 is approximately three times that of the vessel lacking C16. The long term results (Fig. 4) showed an increase of approximately two to three times the number of bacteria in the chemostat vessel containing C16, indicating that both substrates are being used. It is apparent that at the lower organic levels C16 doubles the population, suggesting that it is used along with the PYe.

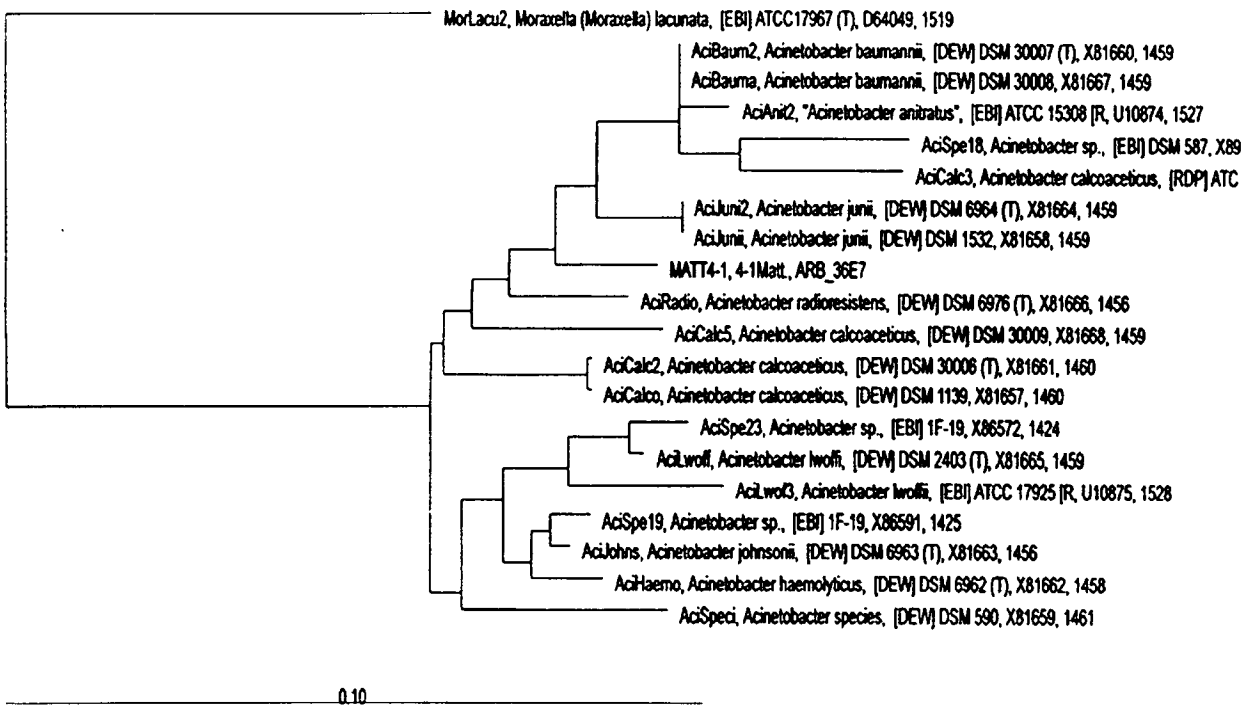

Fig. 1. *Acinetobacter* species tree. Notice that A-84 is distinctly different from all other *Acinetobacter* species such that it occupies its own space in the diagram and is designated by the code MATT4-1, 4-1Matt, ARB_36E7.

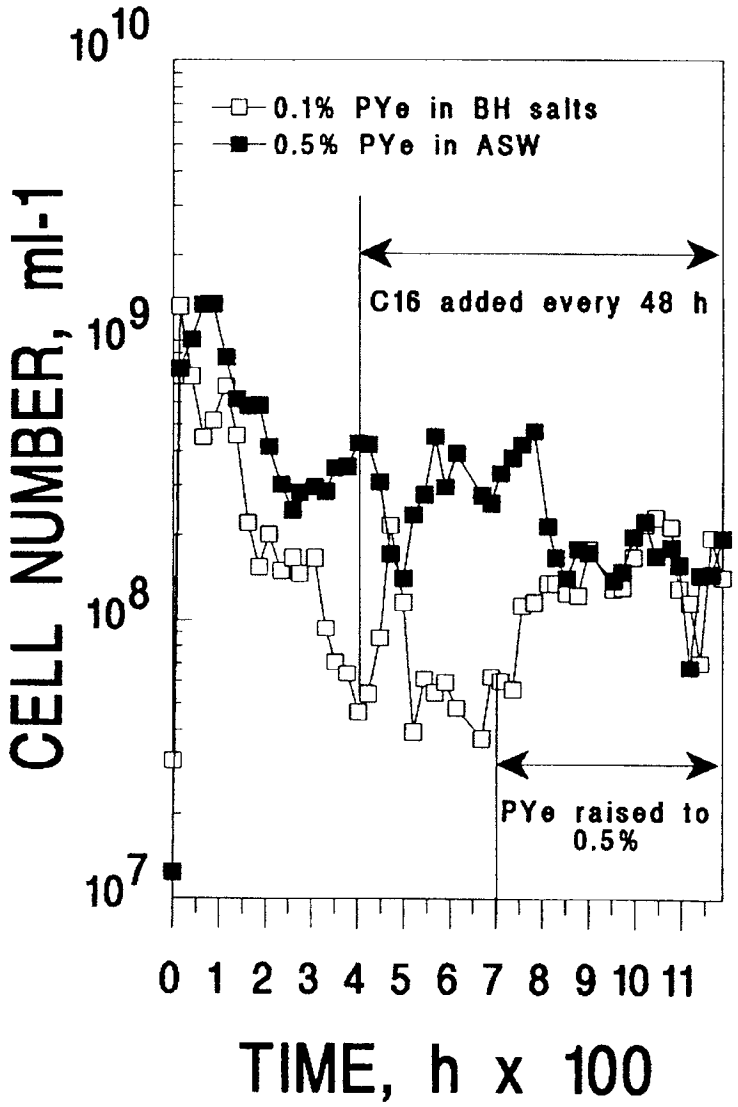

Fig. 2. Maintenance of culture A-84 at varied supplemental organic levels in the presence of hexadecane. Open square (□) represents cell number in 0.1% organic content BH salts media. Solid square (■) represents cell number in 0.5% organic content ASW media. Hexadecane was added at the start of the experiment and not added again until time 400 hr. Because the hydrocarbon was being flushed out of the vessel, it was necessary to add it every other day, which ultimately stabilized the 0.1% chemostat at a density of 5 x 10^7 bacteria ml^{-1}. The organic content of both chemostats was brought to 0.5% with a reservoir change at 750 hr.

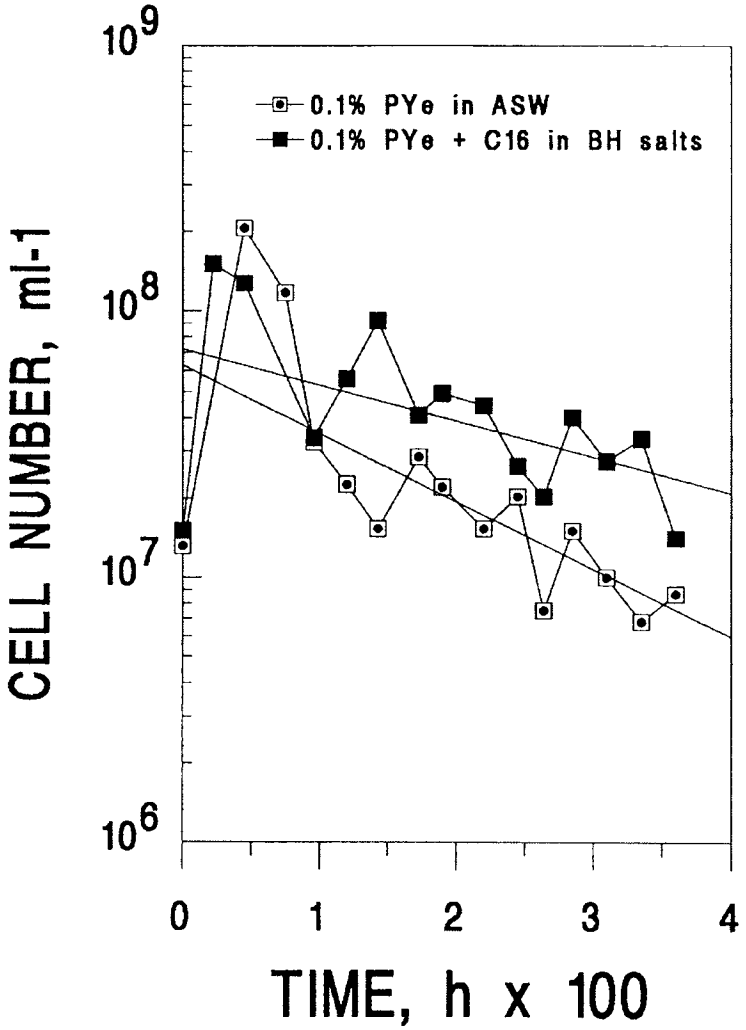

Fig. 3. The increased biomass of culture A-84 by the addition of hexadecane to a medium containing 0.1% peptone-yeast extract. Solid square (■) represents 0.1% PYe medium plus C16. Open square (□) represents 0.1% PYe medium without the addition of C16. The duration of this experiment was 380 hours, and represents the initial stages of the long-term experiment of Fig. 4.

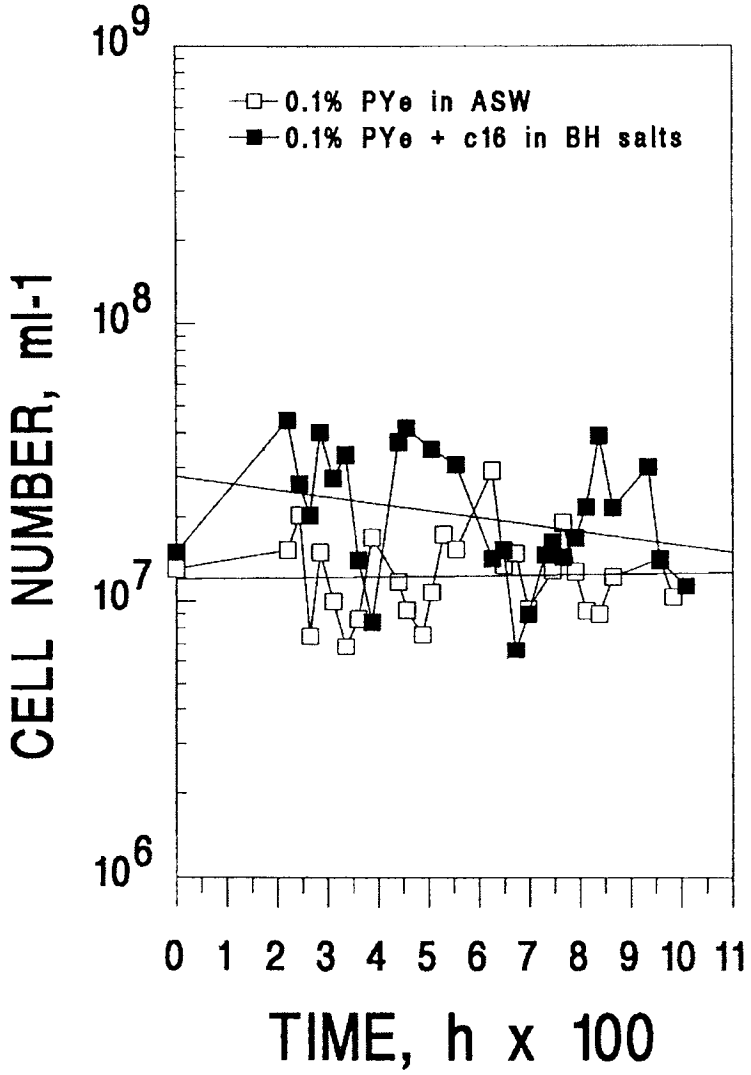

Fig. 4. The increased biomass of culture A-84 by the addition of hexadecane to a chemostat containing 0.1% peptone-yeast extract . Over the 41 days that this experiment ran the hexadecane increased the biomass by an average of 300% indicating that both substrates can be used simultaneously. Solid square (■) represents 0.1% organic medium plus C16. Open square (□) represents 0.1% organic medium without addition of C16.

An artificial marsh was established (Fig. 5) in order to simulate the effects of releasing oil to a wetland environment and the selective pressure this released oil would exert on the indigenous microflora. Marsh water was pumped from a reservoir into the marsh, from which it was passed through three chemostats: one containing raw water alone, the second containing raw water to which C16 had been added, and the third having raw water with C16 to which A- 84 had been added. The results (Fig. 6) revealed that the chemostat through which the raw water alone passed reached an equilibrium cell density of approximately 4 x 10^4 cell/ml by day 10 of the experiment. In marked contrast, the two chemostat vessels containing C16 showed a nearly identical increase in cell number over the course of the experiment with a cell density of approximately 3 x 10^6 cell/ml or roughly a fifty-fold increase over the control vessel lacking C16. Of greater interest, however, was the finding that A-84 emerged as the dominant organism in the chemostat containing raw water plus C16, and that it also represented virtually 100% of the population in the chemostat to which it had been introduced. Identification and confirmation A-84 as the dominant organism was done by fluorescent probe analysis. In other words, under the selective pressure of C16 addition, A-84 appeared to be preferentially selected and was able to survive when re-seeded into the environment from which it had been isolated.

The previous experiments suggested that A-84 is able to use C16 quite readily and that it emerged or was maintained as the dominant organism, whether introduced by seeding or selected for by C16 addition in a wetland environment. The main question to be resolved now is whether this organism can compete and survive in a foreign environment. The Mississippi River carries an enormous amount of maritime traffic, and like the wetlands, is an area of great concern regarding the potential for oil spillage. To examine the survival and competitive characteristics of A-84 in a Mississippi River environment, a set of three chemostats was established through which unammended river water was passed through the first chemostat, raw river water plus C16 through the second chemostat, and raw river water plus C16 and inoculated with A-84 in the third chemostat. This experiment was carried out over a 29-day period and the results are shown in Fig. 7. The vessel containing the raw river water alone reached an equilibrium value of 1.5 x 10^5 cell/ml by day seven, which was maintained throughout the duration of the experiment. The chemostat containing raw river water plus C16 eventually reached an equilibrium bacterial density of 2 to 3 x 10^6 cell/ml. The vessel to which A-84 had been introduced showed a marked decline from an initial cell density of 2 x 10^7 cell/ml, which were predominantly A-84, down to 2 x 10^5 cell/ml by day seven after which it increased and eventually reached a final cell density of 2 x 10^6 cell/ml at the end of the experiment. Interestingly, A-84 had been completely displaced after seven days, as determined by fluorescent probing, by a new community representing microflora indigenous to the Mississippi River environment. In other words, A-84 could not compete with native microflora even though the selective agent, C16, was the same as in experiments in the marsh environment.

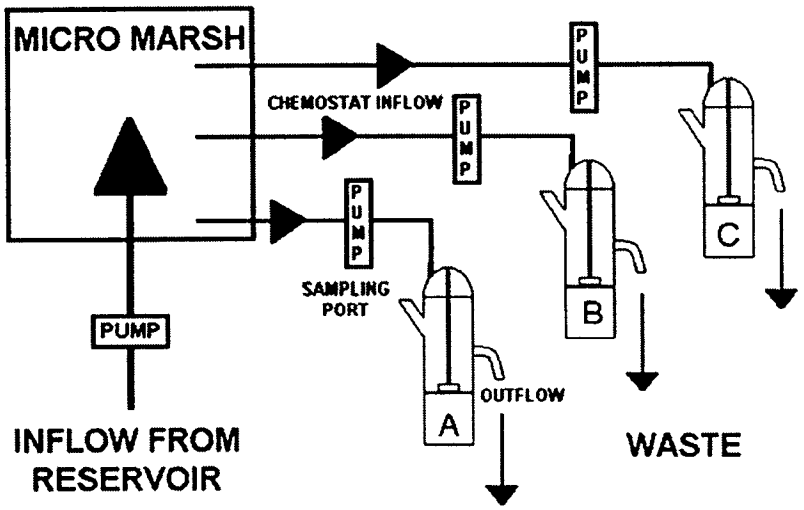

Fig. 5. Schematic representation of a microcosm to feed three experimental chemostats to assess the success of A-84 survival. The experimental conditions used were, (A) raw water directly from the marsh and no suplements, (B) raw water with C16 added to the chemostat daily, and (C) raw water with C16 added daily and A-84 inoculated into chemostat at the start of the experiment

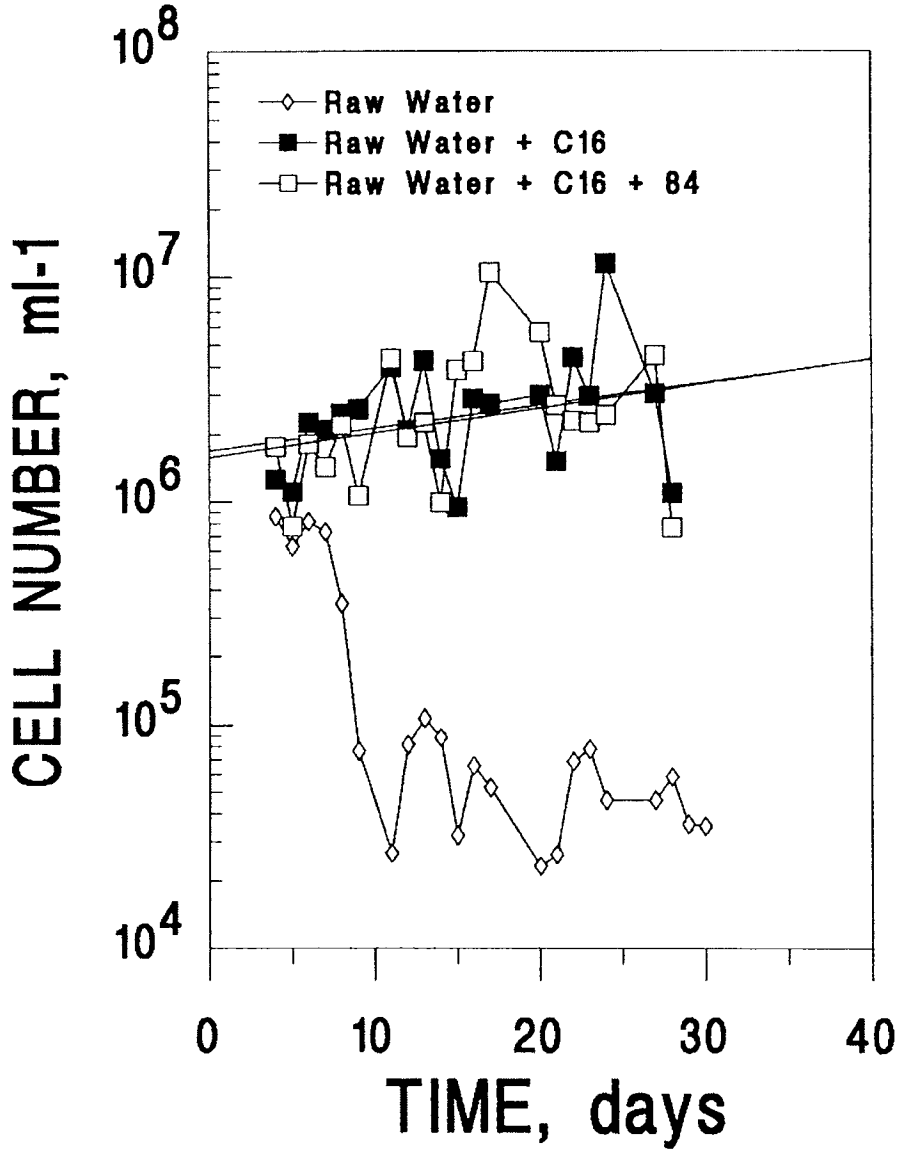

Fig. 6. The successful survival of A-84 when reintroduced into a marsh environment. Open diamond (◇) represents cell number observed in raw marsh water. Open square (□) represents cell number observed in raw marsh water with C16 added daily. Solid square (■) represents raw marsh water with C16 added daily, which was inoculated with A-84 at the start of the experiment.

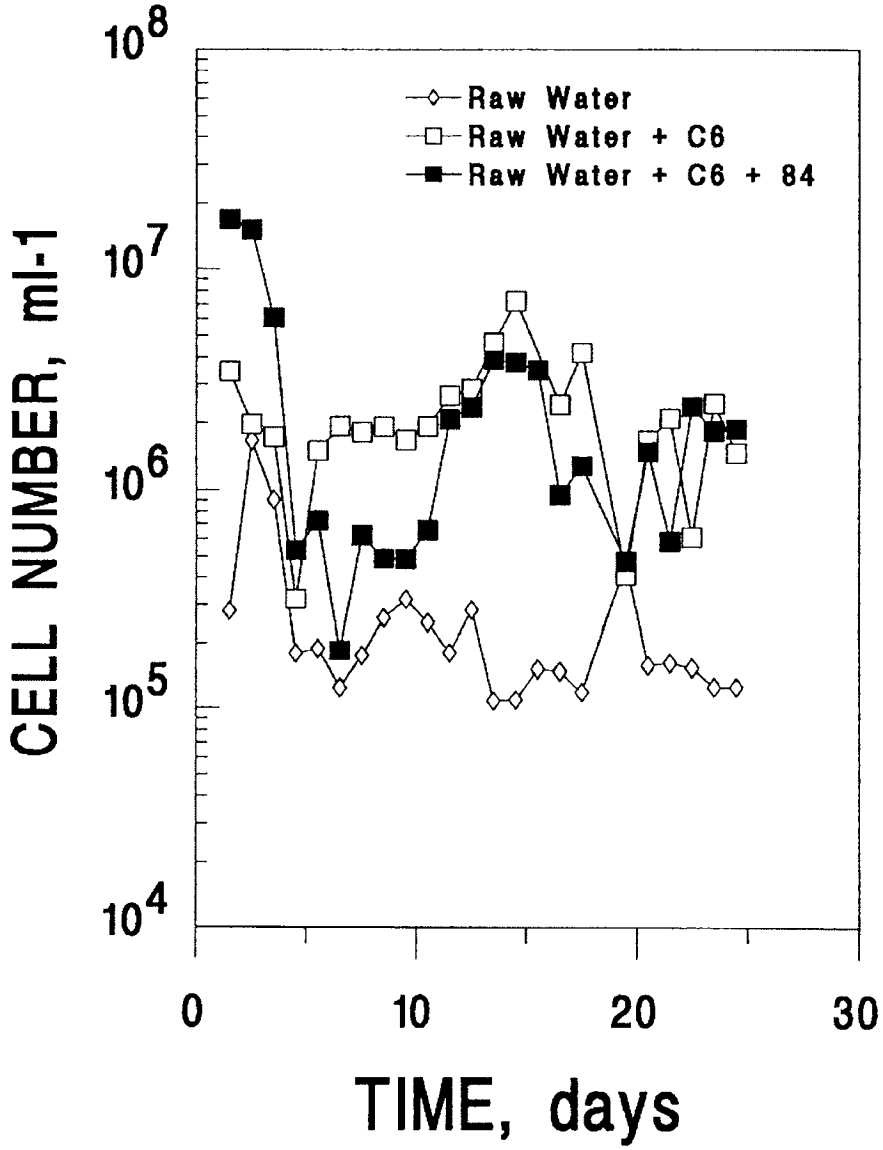

Fig. 7. The unsuccessful competition of A-84 when introduced into a foreign environment (Mississippi River water). Open diamond (◇) represents cell number observed in raw Mississippi River water. Open square (□) represents cell number observed in raw river water with C16 added daily. Solid square (■) represents raw river water with C16 added daily, which was inoculated with A-84 at the start of the experiment.

DISCUSSION

In oil producing and handling areas, considerable attention revolves around cleanup and containment practices in the event of releases to the environment. Because of the visibility and awareness of spilled oil, remediation practices are generally of a physical or chemical nature with the intent of reducing the readily seen material. Bacteria are the ultimate remediation tool, however, although their rate of progress is too slow to suit the immediate political needs of the situation. In order to expedite microbial clean-up operations, in concept one can either seed bacteria that have a unique ability for hydrocarbon utilization into the released material, or augment native communities with appropriate nutrients (usually N and P compounds) to foster their development. Bioaugmentation has gained more favor for remediation application for a variety of technical reasons, particularly in subsurface and soil situations where bacteria cannot easily be introduced. It is also argued that indigenous microbes would have developed the appropriate enzyme systems required to degrade the prevalent contaminant, and are already adapted to surviving and successfully competing in the particular environment. In fact, survival and competitive displacement are probably the greater issues than is the ability to utilize a particular compound.

In order to specifically address the survival of introduced bacteria, we used a system that would essentially define growth parameters and environmental conditions, as well as eliminate dispersal of both the seed bacterium and the critical nutrients. We also experimented with a variety of hydrocarbon-utilizing microbes generously provided by colleagues, but resorted to isolating bacteria from coastal wetlands, as this would also provide a native environment to which we could re-introduce laboratory grown cultures and follow the success or failure of their survival. As it turned out, the dominant organism, one that consistently overran all others, was an *Acinetobacter* that had sufficiently unique characteristics to suggest it is a new species, which leads us to believe that microbes may alter their physiology in response to their environment. The genus *Acinetobacter* is almost ideal for this particular project as it readily uses C16 (Stewart et al., 1959) as well as other hydrocarbons (Baumann et al., 1968), can use inorganic nitrogen sources, (Jyssum and Joner, 1965) grows well on all complex media, with most strains able to grow on defined media containing a single carbon and energy source, and does not have any growth factor requirements (Juni, 1984). It is also a common, but not significant, inhabitant of soil, water and sewage (Juni, 1984). In our experiments culture A-84 grew exceptionally well, with doubling times well under an hour in the C16 chemostats.

The key findings are; 1, when C16 was added to a complex medium, the population in the chemostat more than doubled (Figs. 3 and 4), 2, when A-84 was re-introduced to a wetland environment it proliferated and emerged as the dominant organism in the uninnoculated, C16-containing control as well (Fig. 6), and 3, when A-84 was introduced into a foreign environment (Mississippi River water) it was eliminated from the chemostat, and a native community then developed in its place (Fig. 7).

The experiments involving a mixture of organic compounds (peptone and yeast extract) and C16 were done to determine whether exogenous substrates might be used in preference to the hydrocarbon, thus limiting the efficacy of seeded microbes. Lindley and Heydeman, (1986) showed that there was a sequential degradation of alkanes, one selectively being metabolized before the next was used. Klecka and Maier (1988) demonstrated that in enrichment cultures, the rate of pentachlorophenol metabolism was reduced when phenol was added and used preferentially. The reverse is also true where the addition of a second substrate enhances decomposition of n-alkanes. Sveum et al., (1994) reported that the addition of organic carbon, in the form of fish meal, was a more effective means of delivering N and P fertilizers, and that this organic addition enhanced the decomposition of n-alkanes as determined by a reduction in the n-C17/pristane ratio. In our experiments organics (PYe) were added to maintain the population density of A-84 under the imposed conditions largely because of the relatively high amount of

organic material associated with wetlands (Mitch and Goselink, 1993). We did not measure C16 utilization (such a task could not be done under the experimental conditions because of the large quantity added to the chemostats in order to assure an excess and compensate for loss through the overflow), but instead looked at differences in the size of the populations of A-84 that had been grown on PYe alone and PYe supplemented with C16. The findings (Figs. 3 and 4) showed that the presence of C16 more than doubled the population of A-84, and leads us to conclude that both substrates are being used simultaneously. We cannot draw any conclusions about changes in efficiency with which C16 is utilized, but significantly, the population of A-84 is maintained.

The next point of concern was whether A-84 could survive when re-introduced to the environment from which it had been isolated. Using a marsh microcosm it was readily apparent that A-84 easily proliferated, and in fact emerged as the dominant bacterium in the control chemostat to which C16 had been added (Fig. 6). The addition of C16 to the marsh samples further resulted in bacterial populations that were 100 times greater than the raw water alone, and indicated that exogenous organic carbon exerted no competitive effect against the decomposition of the C16.

The picture changes, however, when A-84 was introduced into a foreign environment, which in this case was Mississippi River water. Culture A-84 was initially dominant, but disappeared at a rate that essentially matched the flow rate through the chemostat. In other words, it didn't grow at all and was flushed out of the vessel by day seven. After elimination of A-84 a native community developed, that at least in terms of cell number, paralleled the growth pattern of the control sample to which only C16 had been added. Again the C16 vessels had microbial communities that were significantly larger than the raw water alone, but in this case the difference varied by only a factor of 10 to 20 fold. Using our molecular probe, we verified that A-84 was undetectable after day seven.

Our findings strengthen the argument for bioaugmentation of an existing microbial community by adding critical nutrients rather than seeding with a bacterial strain selected for its degradation capabilities. Clearly, culture A-84 excelled at growing on C16, but was unable to survive in another environment. In the Mississippi River competition experiment (Fig. 7), A-84 was added in a five fold excess over the native bacterial community, but yet it was unable to grow, presumably because of some factor in the river water itself. Bacteriocins, probiotics, or other anti-microbial agents may have prevented A-84 from establishing itself in the new environment, but of greater interest is the observation that the native community did not emerge until after A-84 had essentially been displaced, and what ever the inhibitory agent was toward A-84, it had no effect on the indigenous bacteria. Our experiments did not cover a broad permutation of nutritional and environmental factors that might have affected growth, but suffice it to say that within the boundary conditions of our experimental protocol, the seeded bacteria were unable to establish a foothold and ultimately provide any functional benefit.

Bioaugmentation of a system is not without drawbacks. Characteristically, one adds nutrients such as nitrogen, phosphorus and possibly an electron acceptor to the contaminated area to stimulate the native bacteria (Sutherson, 1996). The difficulty arises in trying to maintain adequate nutrient concentrations to accelerate biodegradation. In most open surface water systems such additions, whether nutrients or seeded bacteria, would readily disperse depending on weather and tidal conditions. Subsurface environments, on the other hand, would be more successfully treated by bioaugumentation because of the relatively slow motion of ground water and the limited dispersal in such systems (Sutherson, 1996).

Bioremediation systems that rely on using reactors or controlled environments, in which the waste is pumped to the bioreactor are logical applications for using specialized microbes. Under these conditions essential parameters can be consistently maintained and tailored to specific remediation requirements. In a broad sense, sewage treatment and sludge digestion

facilities are examples of using specialized microbes. Such microbes have been successful in their application, however, biroemediation is not a one size fits all process. As seen in our experiments, a single organism can not be expected to perform effectively invaried locations, and the fact that A 84 may be a unique species of Acinetobacter further implies that adaptation to a specific environment may be the most critical factor affecting bioremediation.

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Appendix A

Species-specific detection of hydrocarbon-utilizing bacteria.

Wilson, V., B. Tatford, X. Yin, S. Rajki, M. Walsh and P. LaRock

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FCR/jj/Apr01/220
19 April 2001

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Journal of Microbiological Methods 39 (1999) 59–78

Journal of Microbiological Methods

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Species-specific detection of hydrocarbon-utilizing bacteria

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Received 6 May 1999; accepted 30 July 1999

Abstract

Rapid detection and quantitative assessment of specific microbial species in environmental samples is desirable for monitoring changes in ecosystems and for tracking natural or introduced microbial species during bioremediation of contaminated sites. In the interests of developing rapid tests for hydrocarbon-degrading bacteria, species-specific PCR primer sets have been developed for *Pseudomonas aeruginosa*, *Stentrophomonas* (*Xanthomonas*) *maltophilia*, and *Serratia marsescens*. Highly variable regions of the 16S rRNA gene were used to design these primer sets. The amplification products of these primer sets have been verified and validated with hemi-nested PCR and with ligase chain reaction (LCR) techniques, and have been applied to the analyses of environmental water samples. These species-specific primer sets were also chosen to amplify in conjunction with a universal set of PCR primers chosen from highly conserved neighboring sequences in the same gene. These multiplex or competitive PCR procedures enable testing with an internal marker and/or the quantitative estimation of the relative proportion of the microbial community that any one of these species occupies. In addition, this universal PCR primer set amplified the same size amplicon from a wide spectrum of procaryotic and eucaryotic organisms and may have potential in earth biota analyses.

Keywords: Species-specific detection; *Pseudomonas aeruginosa*; *Stentrophomonas* (*Xanthomonas*) *maltophilia*; *Serratia marsescens*; 16S rRNA gene; Earth biota primers

1. Introduction

The ability to identify and quantify specific microbial species within environmental and clinical settings would be valuable to numerous fields of research. Changes in microbial community structure induced by natural or anthropogenic factors could be monitored. Trends in microbial community composition during bioremediation, whether of indigenous or introduced microbial species, could be determined. The need for species-specific identification of opportunistic bacterial infections also continues to be an issue in human health. However, the techniques available for identifying and quantifying specific microbial species have been of limited usefulness for in situ real world settings due to the necessity of prior culturing of bacteria, inability to quantify microorganisms, or lack of sensitivity (Welsh and McClelland, 1990). More accurate and

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0167-7012/99/$ – see front matter
PII: S0167-7012(99)00098-6

sensitive methods that will offer both species-specific detection and quantification directly in the real world sample are needed, since prior culturing of samples may significantly alter the microbial community structure.

A number of molecular approaches have recently been reported for bacterial species-specific identification. Phospholipid fatty acid profiles have been found to be specific for microbial species, but vary with growth conditions and serve as indicators of changes in community structure rather than identification or quantitation of individual species (Bossio and Scow, 1998; Bossio et al., 1998; Pennanen et al., 1998). Sequencing of genes, such as the rRNA genes, has proven to be valuable in species identification (Wilson and Blitchington, 1996). Reverse sample genome probing has been reported to enable the identification of different species involved in the degradation of hydrocarbons (Shen et al., 1998). Fluorescent and radiolabelled oligonucleotide probes specific for gene sequences in the species of interest have also been useful (DeLong et al., 1985; Braun-Howland et al., 1993). Several polymerase chain reaction (PCR) based procedures have been reported for the identification of bacterial species. Random amplified polymorphic DNA (RAPD-PCR), arbitrarily primed-PCR (AP-PCR), repetitive extragenic palindromic-PCR (REP-PCR), and enterobacterial intragenic consensus sequence-PCR (ERIC-PCR) provide complex banding pattern fingerprints of individual species (Williams et al., 1990; DeBruijn, 1992; Moyer et al., 1994; Chatelut et al., 1995; van Couwenberghe et al., 1995). These species-specific fingerprints are generally not amenable to in situ environmental analyses without prior culturing of samples. All of these approaches either require prior culturing of the bacterial species or provide minimal quantitation capabilities.

A number of PCR primers have been developed for specific bacterial genes including the 16S rRNA gene (Wang et al., 1996; Wheeler et al., 1996). The majority of reported 16S rRNA sequence PCR primers are designed to amplify a wide range of bacterial sequences, which can be subsequently sequenced or subjected to denaturing gradient gel electrophoresis for species identification (Teske et al., 1996; Wheeler et al., 1996; Marchesi et al., 1998). In other cases PCR primers have been designed to be species-specific, such as those reported for in situ PCR and single-cell microscopic identification of *Escherichia coli* and other species (Tani et al., 1998).

In the present study, we have developed species-specific PCR primer sets based on the 16S rRNA gene sequence to be used in multiplex or competitive PCR with a universal primer set within the same gene. The 16S rRNA gene is present throughout the microbial kingdoms, containing both highly conserved sequence regions and highly variable sequence regions (Woese, 1987). Species-specific PCR primer sets have been developed based on highly variable 16S rRNA gene sequences for *Pseudomonas aeruginosa*, *Stentrophomonas* (*Xanthomonas*) *maltophilia*, and *Serratia marsescens*. Pure cultures of these three microbial species were used to develop, standardize, and validate this species-specific PCR detection and potential quantification methodology prior to real world analyses. These microorganisms were chosen for study because all three are environmentally important ubiquitous species known to degrade hydrocarbons (Deziel et al., 1996; Sattler, 1997). *P. aeruginosa* has also been reported to be important in pulmonary infections, especially in cystic fibrosis patients (Davies et al., 1997; Pier et al., 1997). Based on highly conserved 16S rRNA gene sequences, a universal PCR primer set was chosen that appears to be common to most if not all earth biota.

2. Materials and methods

2.1. Standard microbial cultures and DNAs

Pure lyophilized cultures of *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Serratia marcescens* were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and resuspended in 0.5% NaCl. The specific bacterial strains chosen for these studies are listed in Table 1. Nutrient agar plates (Difco, Detroit, MI, USA) were streaked with each rehydrated culture and incubated at 37°C for at least 48 h prior to harvest and/or replating. Bacteria were harvested by suspension in 0.5% NaCl, centrifuged at 10,000 *g* at 4°C for 15 min, and washed twice with phosphate

Table 1
Standard microbial species and strain identifiers

Species	Strain	GenBank accession No.[a]
Escherichia coli	[b]	E05133
Klebsiella pneumoniae	[c]	U33121
Micrococcus lysodeikticus	[b]	AF057289
Pseudomonas aeruginosa	ATCC 25330	X06684
Serratia marcescens	ATCC 27117	M59160
Stenotrophomonas maltophilia (*Xanthomonas maltophilia*)	ATCC 13637	M59158
Vibrio vulnificus	[c]	X76333

[a] Reported 16S rRNA gene sequences.
[b] Microorganism not cultured; isolated DNA purchased (see Materials and methods).
[c] Sequence only used in the present study.

Table 2
Mixed cultures composition

Mixed culture	Species
Not *P. aeruginosa*	*Bacillus pasteuri* *Bacillus racemilactius* *Burkholderia gladioli* *Serratia proteamaculans*
Not *S. maltophilia*	*Bacillus pasteuri* *Bacillus racemilactius* *Burkholderia gladioli* *Serratia proteamaculans*
Not *S. marcescens*	*Bacillus pasteuri* *Bacillus racemilactius* *Burkholderia gladioli*

buffered saline. The final bacterial pellet was resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40) with 1 μg/μl proteinase K (Sigma), incubated at 37°C for 12 h, and the DNA isolated by standard phenol extraction procedures and the purity confirmed by absorbance and restriction enzyme digestion (Marmur, 1961; Wilson et al., 1989). Other DNA isolation methods including cycles of freezing and thawing, cetyltrimethylammonium bromide, or boiling did not improve the DNA isolation efficiency or purity in the present study. Precipitated DNAs were resuspended in TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA).

Mixed microbial cultures lacking *P. aeruginosa*, *S. maltophilia*, and/or *S. marcescens* were inoculated into 500 ml of nutrient broth (Difco) and incubated at 37°C for 24 to 48 h. DNA was isolated from these mixed cultures as Not *P. aeruginosa*, Not *S. maltophilia*, and Not *S. marcescens*, respectively. The composition of each of these mixed cultures is listed in Table 2.

DNAs for *Escherichia coli*, *Micrococcus lysodeikticus*, and salmon sperm were purchased from Sigma (St. Louis, MO, USA). Human genomic DNA was purchased from Boehringer Mannheim (Indianapolis, IN, USA). *Arabidopsis thaliana* DNA specimens were a gift from Dr. John C. Larkin. *Drosophilia melanogaster* DNA was isolated from larvae generously provided by Dr. William R. Lee. *Saccharomyces cerevisiae* was isolated from a package of Fleischmann's bakers yeast purchased at the local grocery store.

2.2. Environmental water samples

Two liters of water were collected from each of two different locations on University Lake, at Louisiana State University, Baton Rouge, LA, on January 17, March 25, and August 3, 1998. One liter was gravity filtered through Watman No. 1 filter paper for removal of debris, then by vacuum suction using a 13 mm 0.2 μm pore size Millipore filter for the collection of bacteria. The filter was then placed in a 1.5 ml microcentrifuge tube, lysis buffer (as noted above) added and the DNA isolated as noted above.

The remaining water was then used to initiate cultures for the possible growth of one of the microbial species of interest. No culture data was available from the January 17 sampling as the sample was neither plated fresh nor properly preserved.

2.2.1. March 25 sampling

Two samples were plated out (0.5 ml on nutrient agar (Difco)). They were incubated for 48 h at 30°C. Twelve colonies from one sample and 10 from the other were picked and streaked for isolation. Isolates were cultured for gram staining. Gram negative isolates (all but two of the 22) were tested by the Biolog™ procedure in an attempt to identify whether any of the three species of interest were present.

Biolog microplates were used for the microbial identification. The 96-well Biolog microplate contains a variety of carbon and nitrogen sources, as well as a redox dye that records increases in metabolic rate (Bochner, 1989). After the microplate was inoculated with the bacterial isolate it was incubated for 24 h. The patterns of positive and negative responses were loaded into a computer through a microplate density reader, and the database scanned for the best statistical match of the substrate utilization profile. The database for the Biolog system is environmentally oriented, unlike many of the other systems, with clinical biases (Bochner, 1989).

2.2.2. August 3 sampling

Samples were plated right after collection onto nutrient agar, Pseudomonas Isolation Agar (Difco), and a selective agar for isolation of *Serratia marcescens* (Farmer et al., 1973). Growth occurred on all plates. Colonies were selected from Pseudomonas Isolation Agar plates and *Serratia marcescens* isolation plates and streaked twice for further purification. Isolates were gram-stained and tested using the Biolog system.

2.3. Enzymes

The *Taq* polymerases (AmpliTaq and AmpliTaq Gold) were purchased from Perkin-Elmer/Applied Biosystems (Foster City, CA, USA). *Pfu* polymerase and *PfuTurbo* polymerase were purchased from Stratagene (La Jolla, CA, USA). The LCR amplification enzyme, *Taq* DNA ligase, and T4 polynucleotide kinase, for end-labeling, were both purchased from New England Biolabs (Beverly, MA, USA).

2.4. Oligonucleotides and radioactive isotopes

PCR primers were chosen with the aid of Oligo v5.0 Primer Analysis software (National Biosciences, Inc., Plymouth, MN, USA) following the alignment of 16S rRNA gene sequences for seven different species (Fig. 1) using MacVector v6.0 (Oxford Molecular Limited Group). Oligonucleotide PCR and LCR primers were custom synthesized by BioServe Biotechnologies, Inc. (Laurel, MD, USA). [γ-^{32}P] ATP (>5000 Ci/mmol) and [γ-^{32}P] ATP Redivue were obtained from Amersham Corporation (Arlington Heights, IL, USA).

2.5. PCR amplification procedures

PCR reactions contained 200 ng of DNA, 2.5 units of either AmpliTaq or AmpliTaq Gold, the manufacturer recommended buffer supplied with the polymerase enzyme, 2.5 mM $MgCl_2$, 200 nM dNTPs and 1 mM of each primer in a total volume of 50 μl. Hot start was composed of either 7 min at 96°C followed by the addition of AmpliTaq and dNTPs, or 1 min at 96°C with AmpliTaq Gold and all other PCR reaction components present. Except as noted in the text, amplification consisted of 35 cycles of 1 min at 96°C, 1 min at the appropriate annealing temperature, and 1 min at 72°C followed by 10 min at 72°C. PCR primer sequences are listed in Table 3 and the annealing temperatures are listed in Table 4. Hemi-nested PCR was performed by the addition of 1 μl of the initial PCR reaction product to a fresh 50 μl PCR reaction containing the appropriate hemi-nested primer set, and the amplification performed as described above.

PCR products were resolved in 4% agarose (3:1 Nuseive/Seakem, Life Technologies, Gaithersburg, MD, USA) minigels, following 45–90 min electrophoresis at 117 V. The PCR fragments were detected by ethidium bromide staining and the gels photographed over UV light. The 1 kb Ladder and Mass Ladder electrophoresis molecular weight standards were purchased from Life Technologies.

Densitometric analyses of gel electrophoresis bands were performed on a Macintosh Quadra 950 computer using the public domain NIH-Image v1.60 program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image). A polaroid photograph of the stained gel was scanned with a Microtek ScanMaker III coupled to the Macintosh computer.

2.6. LCR analyses

These procedures have been well described by Barany (1991) and Wilson et al. (1999). Briefly, 1 μl of the PCR/RE selection solution was subjected to 30 cycles of LCR amplification, using 10–15 U *Taq* DNA ligase, and detection in the presence of

16S rRNA GENE SEQUENCE ALIGNMENT

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Clustal W(1.4) multiple sequence alignment

E.col     1              AA-AT---TGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGG--CCT--A    50

P.aer     1        G----AA--C---TGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCAGCAGGGGCCTTCA    54
S.mal     1              NNTAAGTGAAGAGTTTGATCCTGGCTCAGAGTGAACGCTAGCGGTAGG--CCT--A    52
S.mar     1 GGAAATAACCA-AA-AT---TGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGG--CTT--A    61
M.lys     1 GGAACCCCTTTTAAGATA--T-CAGAGTTTGATCATGGCTCAGGATGAACGCTGGCGGCGTG--CTT--A    63
V.vul     1        GTCT-AT-CC---TAGAGAGTTTGATNNTGGCTCAGATTGAACGCTGGCGGCAGG--CCT--A    52
K.pne     1        GTCT-C---C---TACAGAGNNTGATCCTGACTCAGATTGA-CGCTGGCGGCAGC--CCT--A    49
                              *  ****  ****  ** *****  *** **** ** *      * *  *

E.col    51 ACACATGCAAGTCGAACGGTA-ACAGGAAG-AAG-CTTGCTTCTTTGCT-GACGAGTGGCGGACGGGTGA   116

P.aer    55 ACACATGCAAGTCGAGCT-TATG-A--AGG-GAG-CTTGC--CTTGGAT-T-C-AGCGGCGGACGGGTGA   113
S.mal    53 ACACATGCAAGTCGAACGGCA-GCAC-AGGAGAG-CTTGCT-CTCTG---GANNAGTGGCGGACGGGTGA   113
S.mar    62 ACACATGCAAGTCGAGCGGTA-GCAC-ANGGGAG-CTTGCTCCCTGGGT-GACGAGCGGCGGACGGGTGA   126
M.lys    64 ACACATGCAAGTCGAACGAT--G-A--AGCCCAG-CTTG---CT-GGGTGGATTAGTGGCGAACGGGTGA   123
V.vul    53 ACACATGCAAGTCGAGCGGCA-GCAC-AGA-GAAACTTGTTTCTCGGGT-GGCGAGCGGCGGACGGGTGA   118
K.pne    50 ACACATGCAAGTCGAACGGTA-GCAC-AGA-GAG-CTTGCT-CTCGGGT-GACGAGTGGCGGACGGGTGA   113
            *************** *       *  *    *  ****   *   *       ** **** ********

E.col   117 GTAATGTCTGGG-AAACTGCC--TGATGGAGGGGGATAA-C-TACTGGAAACGGTA-G-CTAATACCTCA   179

P.aer   114 GTAATGCCTAGG-AATCTGCC--TGGTAGTGGGGGATAA-CGT-CCGGAAACGGCC-G-CTAATACCGCA   176
S.mal   114 GGAATACATCGG-AATCTACT--TTTTCGTGGGG-ATAA-CGTAG-GGAAACT-TACG-CTAATACCGCA   175
S.mar   127 GTAATGTCTGGG-AAACTGCC--TGATGGAGGGGGATAA-C-TACTGGAAACGGTA-G-CTAATACCGCA   189
M.lys   124 GTAACACGTGAGTAACCTGCCCTTAACTCTGGG--ATAAGCCTG--GGAAAC--TGGGTCTAATACCGGA   187
V.vul   119 GTAATGCCTGGG-AAATTGCCC-TGATGT-GGGGGATAA-C-CATTGGAAACGATG-G-CTAATACCGCA   181
K.pne   114 GTAATGTCTGGG-AAACTGCC--TGATGGAGGGGGATAA-C-TACTGGAAACGGTA-G-CTAATACCGCA   176
            * **    *  * **  * *   *      ***  **** *     ******     * ********  *

E.col   180 TA--A-C-GTCGCAAGAC-CAAAGAG--GGGGACCTTCG---GGCCTCTTGCCATCGG-ATGTGCCCA-G   237

P.aer   177 T---A-C-GTCCTGAGGG--AGAAAGTCGGGGATCTTCG---GACCTCACGCTATCAG-ATGAGCCTA-G   234
S.mal   176 T---A-C-GACCTACGGG-TGAA-AGCAGGGGATCTTCG---GACCTTGCGCGATTGA-ATGAGCCGA-T   232
S.mar   190 TA--A-C-GTCGCAAGAC-CAAAGAG--GGGGACCTTCG---GGCCTCTTGCCATCAG-ATGTGCCCA-G   247
M.lys   188 TAGGAGC-G-CCT---AC-CGCA-TG--GTGGGTGTTGGAAAGATTTATCGGTTTTGG-ATGGACTCGCG   247
V.vul   182 TG--A-TAGCTTC--GGCTCAAAGAG--GGGGACCTTCG---GGCCTCTCGC-GTCAGGATATGCCCA-G   239
K.pne   177 TA--A-C-GTCGCAAGAC-CAAAGAG--GGGGACCTTCG---GGCCTCTTGCCATCAG-ATGTGCCCA-G   234
            *   *   *              *  *  * **   ** *   *   *   *   *    **   *
```

Fig. 1. Alignment of 16S rRNA gene DNA sequences reported in GenBank for *E. coli* (E.col), *P. aeruginosa* (P.aer), *S. maltophilia* (S.mal), *S. marcescens* (S.mar), *M. lysodeikticus* (M.lys), *V. vulnificus* (V.vul), and *K. pneumoniae* (K.pne). The GenBank accession number for each species is listed in Table 1. The alignment was performed with MacVector then modified to include bases designated as 'N.'

```
E.col  238 ATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGAC  307

P.aer  235 GTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGAT  304
S.mal  233 GTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCNACGATCCGNAGCTNGTCTGAGAGGATGAT  302
S.mar  248 ATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTNGTCTGAGAGGATGAC  316
M.lys  248 GCCT-ATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGAC  316
V.vul  240 GTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGAT  309
K.pne  235 ATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGAC  304
                ** **** ** ** * ***** ***  *** **** ****     * *    ******** ***


E.col  308 CAGCCACACTGGAACTGAGACACGGTTTAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG  377

P.aer  305 CAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGG  374
S.mal  303 CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCNNNAGTGGGGAATATTGGACAATGG  372
S.mar  317 CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG  386
M.lys  317 CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG  386
V.vul  310 CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG  379
K.pne  305 CAGCCACACTGGAACTGAGAAACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG  374
           * * ******** ******* ****   ****************   ************** *******


E.col  378 GCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAG-CGGG  446

P.aer  375 GCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGG  444
S.mal  373 GCGCAAGCCNGATCCAGCCATACCGNGTGGGTGAAGAAGGCNNNCGGGTTGTAAAGCCCTTTT-GTNGGG  441
S.mar  387 GCGCAAGCCTGATGCAGCCATGCNGNGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAG-CGAG  453
M.lys  387 GCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAG-TAGG  455
V.vul  380 GCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTG  449
K.pne  375 GCGCAAGCCTGATGAAGCCATGCCGCGTGTNTGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAG-CGGG  442
           ********* ***  *** *  * * ***   ** ** **    *** *******   ****  *    *


E.col  447 GAGGAAGG-GAGTAAAGTTAATACCTTTGCTCA--TTG-ACGTTACCCG-CAGAAGAAGCACCGGCTAAC  511

P.aer  445 -AGGAAGGGCAGTAA-GTTAATA-CCTTG-CTGT-TTG-ACGTTAC-CAACAGAATAAGCACCGGCTAAC  507
S.mal  442 AAAGAAATCCAGCTG-GTTAATA-CCCGG-TTGGGATG-ACGGTACCCAA-AGAATAAGCACCGGCTAAC  505
S.mar  454 GAGGAAGG-TGGTGAGCTTAATACGCTCA-TCAA-TTG-ACGTTACTCG-CAGAAGAAGCACCGGCTAAC  518
M.lys  456 GAAGAAG--C-G-------AA-A-----G--------TG-ACGGTACCTG-CAGAAGAAGCACCGGCTAAC  500
V.vul  450 -AGGAAGG-TGGTAGTGTTAATAGCACTA-TCAT-TTG-ACGTTAG-CGACAGAAGAAGCACCGGCTAAC  513
K.pne  443 GAGGAAGG-CGATAAGGTTAATAACCTTG-TCGA-TTGCACGTTACCCG-CAGAAGAAGCACCGGCTAAC  508
           * ***            ** *            ** *** **      **** **************
```

Fig. 1. (*continued*)

200,000–500,000 cpm of ^{32}P end-labeled invariant primers, 1 μM of each wild type or each mutant discriminating primer (or a mixture of all primers, each at 1 μM concentration), 4 μg sheared salmon sperm DNA (or sheared *Micrococcus lysideikticus* DNA), 0.1% Triton X-100, and 10–15 U *Taq* ligase in standard LCR buffer (Barany, 1991; Wilson et al., 1999). The amplification process was a 30 cycle reaction using 94°C for denaturation and 65°C for ligation. The resulting ligation products were resolved on a 7 M urea, 10% acylamide sequencing gel. The gel was dried and exposed to X-ray film.

3. Results

In the interest of developing probes for the study of both the presence of specific microbial species and

```
E.col  512 TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACG  581

P.aer  508 TTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCG  577
S.mal  506 TTCGTGCNAGCAGCCGCGGTAATACGAAGGGTNNAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCG  579
S.mar  519 TCCGTGCCAGCAGCCGCGGTAATACGGAGNGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACG  587
M.lys  501 TACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCG  570
V.vul  514 TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATG  583
K.pne  509 TCCGTGCCAGCAACCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACG  578
           * ***** **** ************** ** **   *******  ******** ********** *   *

E.col  582 CAGGCGGTTTGTT-AAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATA-C-TGG-C  647

P.aer  578 TAAGTGGTTCAGC-AAGCTTGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAAGC-TAC-T  644
S.mal  580 TAGGTNG-TCGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATA-C-TGGAC  648
S.mar  588 CAGGCGGTTTGTT-AAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGNACTGCATTTGAAA-C-TGG-C  652
M.lys  571 TAGGCGGTTTGTC-GCGTCTGTCGTGAAAGTCCGGGGCTTAACCCCGGATCTGCGGTGGGTA-CGCGG-C  637
V.vul  584 CAGGTGGTTTGTT-AAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACTGCATTTGAAA-C-TGG-C  649
K.pne  579 CAGGCGGTCTGTC-AAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAA-C-TGG-C  644
            * *  *         *   *  ******  ** ***** ****  **  ****       * *

E.col  648 AAGCTT-GAGT-CTCGTAGAGGGGGGTAGAATTCCA-GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGG  714

P.aer  645 GAGCTA-GAGTACG-GTAGAGGTGG-TAGAATTTCC-TGTGTAGCGGTGAAATGCGTAGATATAGGAAGG  710
S.mal  649 GA-CTA-GAGTGTG-GTAGAGGGTAGCGGAATTCCT-GGTGTAGCAGTGAAATGCGTAGAGATCAGGAGG  714
S.mar  653 AAGCTA-GAGT-CTCGTAGAGGGGGGTAGAATTCCA-GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGG  719
M.lys  638 AGACTATGAGTGCA-GTAGGGGAGACTGGAATTCCTCGGTGTAGCGGTGGAATGCGCAGATATCAGGAGG  706
V.vul  650 AGACTA-GAGTACT-GTAGAGGGGGGTAGAATTTCA-GGTGTAGCGGTGAAATGCGTAGAGATCTGAAGG  716
K.pne  645 AGGCTG-GAGT-CTTGTAGAGGGGGGTAGAATTCCA-GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGG  711
             **  ****    **** **      ***** *   ******* *** ****** *** **  * ***

E.col  715 AATA-CCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAAC  783

P.aer  711 AACA-CCAGTGGCGAAGGCGACCACCTGGACTG-TACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAAC  778
S.mal  715 AACATCCA-TGGCGAAGGCAGCTACCTGGACCAACACTGACACTGAGGCACNAAAGCGTGGGGAGCAAAC  782
S.mar  720 AATA-CCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCNNAAGCGTGGGGAGCAAAC  786
M.lys  707 AACA-CCGATGGC-AAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAAC  774
V.vul  717 AATA-CCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAAC  785
K.pne  712 AAAA-CCGGTGNCGAAGNCGCCCCCCTGGACAAAGNCTGACNCTCAGGTNCGAAAGCGTGGGGACCAAAC  775
           ** * **  ** * *** *      **** *     ***** ** **   *  **** ****** * ***
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Fig. 1. (*continued*)

their relative importance in community structure, especially in oil bioremediation processes, a search for species identifying DNA sequences was undertaken. Since a large database of bacterial species' 16S rRNA gene sequences is available (Wheeler et al., 1996), the 16S rRNA gene sequence became the focus of this search. This approach began with an alignment of the 16S rRNA gene sequences from *P. aeruginosa*, *S. maltophilia*, and *S. marcescens*, along with *E. coli*, *M. lysodeikticus*,*V. vulnificus*, and *K.*

```
E.col  784 AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGT-CGA-CTTGGAGGTTGTG-CC--CTTGAGGC  848

P.aer  779 AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGT-CGA-CT-AGCCGTTGGGATC--CTTGAG--  841
S.mal  783 AGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATG--CGAACT-GGATGTTGGGTGCAATTTGGCAC  849
S.mar  787 AGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGT-CGA-TTTGGAGGTTGTG-CC--CTTGAGGC  851
M.lys  775 AGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGC-A-CT-AGGTGTGGGGACCA-TTCCACG-  839
V.vul  786 AGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGT-CTA-CTTGGAGGTTGTG-GC--CTTGAGCC  850
K.pne  776 AGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGT-CGA-CTTGGAGGTTGTT-CC--CTTGAGGA  840
           ************************** **  ****** **  * *  *  *  ** *    *   *

E.col  849 GTGGCTTC-CG--G-AGC---TAACGCGTTAAGT-CGACCGTCTGGGGAGTACGGCCGCAAGGTTAAAAC  910

P.aer  842 AT--CTTAGTG--G-CGCACGTAACGCGATAAGT-CGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAAC  905
S.mal  850 GCAGTAT--CG--A-AGC---TAACGCGTTAAGTTCG-CNGCNTNGGGNGTACGGTCGCAANNNNGAAAC  902
S.mar  852 GTGGCTTC-CG--G-AGC---TAACGCGTTAAAT-CGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAAC  913
M.lys  840 GT--TTCCGCGCCGCAGC---TAACGCATTAAGTGCC-CCGCCTGGGGAGTACGGCCGCAAGGCTAAAAC  903
V.vul  851 GTGGCTTT-CG--G-AGC---TAACGCGTTAAGT-AGACCGCCTGGGGAGTACGGTCGCAAGATTAAAAC  912
K.pne  841 GTGGCTTC-CG--G-AGC---TAACGCGTTAAGT-CGACCGCCTGGGGAGTACGGCCNCAAGGTTAAAAC  901
                  *     **   ******  *** *    * *  * *** ** *** * ***     ****

E.col  911 TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACC  980

P.aer  906 TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC  975
S.mal  903 TCAAAGGAATTGNNNNNNNNNNGCACAAGNNNNNNNNNNNNNNNGTTTAATTCGATGCAACGCGAAGAACC  972
S.mar  914 TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACC  983
M.lys  904 TCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACC  973
V.vul  913 TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACC  982
K.pne  902 TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACC  971
           ***** ******          *******               * ********* ***************

E.col  981 TTACCTGGTCTTGACATCCACG-GAAGTTTTCAGAGATGAGAATG---TGCC-TTCGGGAACCGTGA-GA 1044

P.aer  976 TTACCTGGCCTTGACATGCTGA-GAACTTTCCAGAGATG-GATTGG--TGCC-TTCGGGAACAGAGA-CA 1039
S.mal  973 TTACCTGGCCTTGACATGTCGA-GAACTTTCCAGAGATG-GATTGG--TGNC-TTCGGGAACTCGAA-CA 1036
S.mar  984 TTACCTACTCTTGACATCCAGA-GAACTTTCCAGAGATG-GATTGG--TGCC-TTCGGGAACTCTGA-GA 1047
M.lys  974 TTACCAAGGCTTGACATGTTCTCGATCGCCGTAGAGATACGGTT----TCCCCTTTGGGG-C-GGGTTCA 1037
V.vul  983 TTACCTACTCTTGACATCCAGA-GAATCTAGCGGAGACG---CTGGAGTGCC-TTCGGGAACTCTGA-GA 1046
K.pne  972 TTACCTACTCTTGACATCCACA-GAACTTAGCAGAGATG-CTTTGG--TGCC-TTCGGGAACCCTGA-GA 1035
           *****    ********      **        ****      *    *  * ** ***  *       *
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Fig. 1. (*continued*)

pneumoniae (Table 1, Fig. 1 (alignment)). The *E. coli* sequence was also used as a reference for the 16S rRNA gene sequence (Brosius et al., 1981).

Based on this microbial 16S rRNA gene sequence alignment, primers Paer16SH and Paer16SIR were chosen from the variable regions of the 16S rRNA gene in the attempt to develop a *P. aeruginosa* specific PCR primer set (Table 3). Using DNAs isolated from ATCC strains of *P. aeruginosa*, *S. maltophilia*, and *S. marcescens* (Table 1), and DNA isolated from a mixed culture that did not contain *P. aeruginosa* (Not *P. aeruginosa*, Table 2), Paer16SH

```
E.col 1045 CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC 1114

P.aer 1040 CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCC 1109
S.mal 1037 CAGGTGCTGCNTGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC 1106
S.mar 1048 CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC 1117
M.lys 1038 CAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC 1107
V.vul 1047 CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC 1116
K.pne 1036 CAGGTGCTGCATGNCTTTCAACAACTCGTGTTGTGAAATGTTGGGTTAAGTCCCNCCACGAGCGCAACCC 1103
          ****** *** **  * **  ** ******* **** ****************   ************

E.col 1115 TTATCCT-TTGTT-GCCAGCG-GTCC-GGCCGGGAACTCAA-AGGAGACTGCCAGTGATAAAC-TGGAGG 1178

P.aer 1110 TTGTCCT-TAGTT-ACCAGCACCTCG-GGT-GGGCACTCTA-AGGAGACTGCCGGTGACAAAC-CGGAGG 1173
S.mal 1107 TTGTCCT-TAGTT-GCCAGCACGTAATGGT-GGGAACTCTA-AGGAGACCGCCGGTGACAAAC-CGGAGG 1171
S.mar 1118 TTATCCT-TTGTT-GCNAGCNN-TTC-GGCCGGGAACTCAA-AGGAGACTGCCAGTGATAAAC-TGGAGG 1178
M.lys 1108 TCGTTCCAT-GTT-GCCAGCACGTAGTGGT-GGGGACTC-ATGGGAGACTGCCGG-GGTCAACTCGGAGG 1172
V.vul 1117 TTATCCT-T-GTTTGCCAGCGAGTAA-TGTCGGGAACTCCA-GGGAGACTGCCGGTGATAAAC-CGGAGG 1181
K.pne 1104 TTATCCT-TTGTT-TCCAGCG-GTCC-GGCCGGGAACTCAA-AGGAGACTGCCAGTGATAAAC-TGGAGG 1167
          *  * *  * ***  * ***   *    *  *** **** *  ****** *** * *   ***  *****

E.col 1179 AAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGG-CGCAT 1247

P.aer 1174 AAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCG-GT 1242
S.mal 1172 AAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCNAGGGCTACACACGTACTACAATGG-TAGGG 1240
S.mar 1179 AAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGG-CATAT 1247
M.lys 1173 AAGGTGAGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCG-GT 1241
V.vul 1182 AAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGG-CGCAT 1250
K.pne 1168 AAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGG-CATAT 1236
          ****** *** ******** ******** ****** *    ***** *** * * *********

E.col 1248 ACAAAGAGAAGCGA-CCTCGCGAGAGCA-AGCGGACCTCATAAAGTGCG-TCGTAGTCCGGATTGGAGTC 1314

P.aer 1243 ACAAAGGGTTGCCA-AGCCGCGAGTGGG-AGCTAATCCCATAAAAC-CGATCGTAGTCCGGATCGCAGTC 1309
S.mal 1241 ACAGAGGGCTGCAA-GCCGGCGACGGTA-AGCCAATCCCAGAAACCCTA-TCTCAGTCCGGATTGGAGTC 1307
S.mar 1248 ACAAAGAGAAGCGA-CCTCGCGAGAGCA-AGCGGACCTCATAAAGTATG-TCGTAGTCCGGATTGGAGTC 1314
M.lys 1242 ACAATGGGTTGCGATACT-GTGAG-GTGGAGCTAATCCCAAAAAGC-CGGTCTCAGTTCGGATTGGGGTC 1308
V.vul 1251 ACAGAGGGCGGCCA-ACTTGCGAAAGTG-AGCGAATCCCAAAAAGTGCG-TCGTAGTCCGGATTGGAGTC 1317
K.pne 1237 ACAAAGAGAAGCGA-CCTCGCGAGAGCA-AGCGGACCTCATAAAGTATG-TCGTAGTCCGGATTGGAGTC 1303
          ***  * *  ** *     * **  *   ***  * * ** ***      **  *** ***** *  ***
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Fig. 1. (*continued*)

and Paer16SIR were found to amplify only the *P. aeruginosa* DNA, providing the expected 219 bp product fragment (Fig. 2). The annealing temperature and $MgCl_2$ concentrations were varied until the optimal PCR conditions were found for this *P. aeruginosa* primer set (data not shown).

The 162 bp fragment (Fig. 2) represents the PCR product of the universal primer set, UB16SC3 and UB16SDR9, designed from conserved regions of the 16S rRNA gene sequence, based on the *E. coli* reference (Fig. 1). An inosine residue was incorporated into the 14th nucleotide position of the

```
E.col 1315 TGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAG-AAT-GCCACGGTGAATACGTTC 1382

P.aer 1310 TGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAG-AAT-GTCACGGTGAATACGTCC 1377
S.mal 1308 TGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAG-CATTGCTGCGGTGAATACGTTC 1376
S.mar 1315 TGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAG-AAT-GCTACGGTGAATACGTTC 1382
M.lys 1309 TGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAAC-GCTGCGGTGAATACGTTC 1377
V.vul 1318 TGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAG-AAT-GCCACGGTGAATACGTTC 1385
K.pne 1304 TGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAG-AAT-GCCACGGTGAATACGTTC 1371
           ***********  * **********  *************   *****  *  *   ************ *


E.col 1383 CCGGGCCTTG-ACCACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTT 1451

P.aer 1378 CCGGGCCTTGTAC-ACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCGC 1446
S.mal 1377 CCGGGCCTTGTAC-ACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGCAGGTAGCTTAACCTT 1445
S.mar 1383 CCGGGCCTTGTAC-ACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTT 1451
M.lys 1378 CCGGGCCTTGTAC-ACACCGCCCGTCAAGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCT 1446
V.vul 1386 CCGGGCCTTGTAC-ACACCGCCCGTCACACCATGGGAGTGGGCTGCAAAAGAAGTGGGTAGTTTAACCTT 1454
K.pne 1372 CCGGGCCTTGTAC-ACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTT 1440
           ********** ** **************   ** *  ***  *   *    ****  * * *  *****


E.col 1452 CG-GGAGGGCGCT-TACCACTTTGTGATTCA-TGACTGGGGTGAAGTCGTAACAAGGTAAC-C-GTAGGG 1516

P.aer 1447 AA-GGGGGACGGT-TACCACGGAGTGATTCA-TGACTGGGGTGAAGTCGTAACAAGGTAGC-C-GTAGGG 1511
S.mal 1446 CG-GGAGGGCGCT-TNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGTAACAAGNNNNNNNNNNNGAA 1519
S.mar 1452 CG-GGAGGGCGCT-TACCACTTTGTGATTCA-TGACNGGGGNNNNNNNGTAACAAGGTAAC-C-GNNNNN 1503
M.lys 1447 TGTGGGGGGAGCCGT-CGAAGGTGGGAC-CAGCGATTGGGACTAAGTCGTAACAAGGTA---------- 1503
V.vul 1455 CG-GGAGGACGCT-CACCACTTTGTGGTTCA-TGACTGGGGTGAAGTCGTAACAAGGTAGCGCTA--GGG 1519
K.pne 1441 CG-GGAGGGCGCT-TACCACTTNGTGATTCA-TGANTGGGGTGAAGTCGTAACAAGGTA-C-C-A----- 1497
              ** **  *                                        ********


E.col 1517 GAACCTGCGGTTGGATCACCTCCTTA 1542

P.aer 1512 GAACCTGCGGCTGGATCACCTCCTTA 1537
S.mal 1520 GNNNNNNNNN---GATCACCTCCTTT 1545
S.mar 1504 GAACCTGNNNNNNGATCACCTCCTTA 1523
M.lys 1504 G--CC-G--------T-A          1509
V.vul 1520 GAACCTGGCGCTGGATCACCTCCTTT 1545
K.pne 1498 G                          1498
           *
```

Fig. 1. (*continued*)

UB16SDR9 primer sequence, since this base site was not conserved in all species' 16S rRNA gene (Table 3, Fig. 1). The reverse universal primer, UB16SDR9, is similar to but not identical with primer B (or 926r) reported by Lane et al. (1985), while the forward universal primer, UB16SC3, has not been previously reported. This universal primer set, UB16SC3 and UB16SDR9, amplified the appropriate 162 bp fragment in all bacterial DNAs tested, including *P. aeruginosa*, *S. maltophilia*, *S. marcescens*, *M. lysodeikticus*, and *E. coli* (Fig. 3). In addition, DNAs from a plant (*Arabidopsis thaliana*), yeast (*Sac-*

Table 3
PCR primer sequences

Primer	Length	Nucleotide positions[a]	Sequence (5′–3′)
Universal primers			
UB16SC-3	23mer	769–791	GCGTGGGGAGCAAACAGGATTAG
UB16SDR-9	24	930–907	GCCCCCGTCAATTIATTTGAGTTT
Pseudomonas aeruginosa			
Paer16SH	25	449–473	AGGGCAGTAAGTTAATACCTTGCTG
Paer16SIR	25	667–643	CCACCTCTACCGTACTCTAGCTCAG
Stentrophomonas (Xanthomonas) maltophilia			
Xmal16SL	22	452–473	GCTGGTTAATACCTGGTTGGGA
Xmal16SKR	25	671–647	CTACCCTCTACCACACTCTAGTCGT
Serratia marcescens			
Smar16SM	24	462–485	TGGTGAACTTAATACGCTCATCAA
Smar16SNR	21	674–654	CCCCTCTACGAGACTCTAGCT
Smar16ST	27	459–485	AGGTGGTGAACTTAATACGCTCATCAA
Smar16SUR	24	676–653	CCCCCCTCTACGAGACTCTAGCTT
Smar16SV	20	89–108	GGGAGCTTGCTCACTGGGTG
Smar16SWR	29	499–471	GCGAGTAACGTCAGTTGATGAGCGTATTA
Nested primers			
Paer16SX	25	565–589	CGTAAAGCGTGCGTAAGTGGTTCAG
Xmal16SY	17	567–583	CGTGCGTAGGTGGTCGT

[a] Nucleotide positions for the universal primers are based on the reported 16S rRNA gene sequence for *E. coli*, GenBank Accession Number E05133 (Brosius et al., 1981). Nucleotide positions for all other primers are based on the reported 16S rRNA gene sequence for each species (see Table 1). These positions were determined from the microbial 16S rRNA gene sequence alignment (shown in Fig. 1).

Table 4
Primer set annealing temperatures and product size

PCR primer set	Annealing temp. (°C)	PCR product (bp)
UB16SC3–UB16SDR9	60–67	162
Paer16SH–Paer16SIR	65	219
Xmal16SL–Xmal16SKR	65	220
Smar16SM–Smar16SNR	65	215
Smar16ST–Smar16SUR	65	220
Smar16SV–Smar16SWR	66	417
Paer16SX–Paer16SIR	66	103
Xmal16SY–Xmal16SKR	65	97

charomyces cerevisiae), fruitfly (*Drosophila melanogaster*), fish (salmon sperm) (*Salmo salar*), and human (*Homo sapien*) were tested (Fig. 3). This universal primer set provided a 162 bp PCR product in every DNA tested.

The universal primer set was designed to be compatible with the Paer16SH and Paer16SIR primer set for the purposes of providing both an internal marker in the analysis of environmental samples and

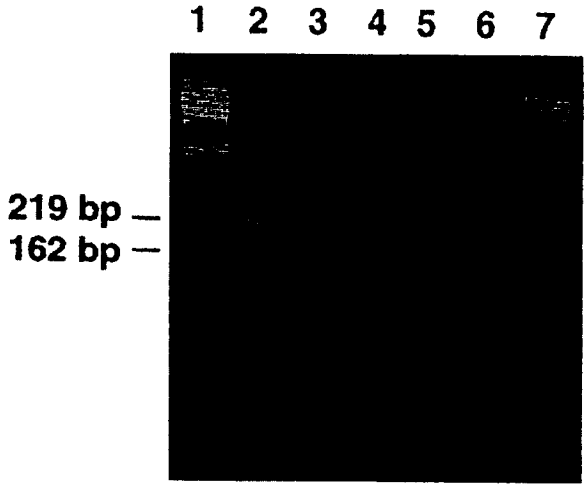

Fig. 2. Specificity of *P. aeruginosa* PCR primers. Ethidium bromide stained 4% agarose gel of the electrophoresed multiplexed PCR products of Paer16SH, Paer16SIR, UB16SC3, and UB16SDR9 primers. Lane 1, Mass Ladder; lane 2, *P. aeruginosa* DNA; lane 3, *S. maltophilia* DNA; lane 4, *S. marcescens* DNA; lane 5, Not *P. aeruginosa* culture mixture DNA; lane 6, No DNA; and lane 7, 1 kb Ladder.

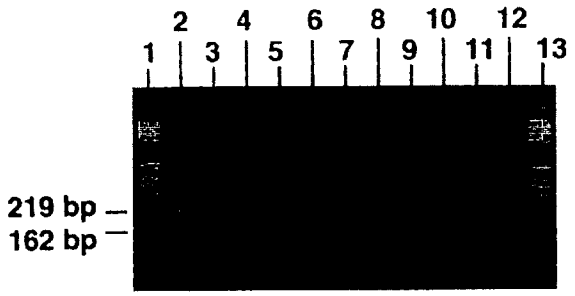

Fig. 3. Universal or Earth Biota primers. Ethidium bromide stained 4% agarose gel of the electrophoresed PCR products of UB16SC3 and UB16SDR9 primers. Lane 1, Mass Ladder; lane 2, *P. aeruginosa* DNA with added Paer16SH and Paer16SIR primers; lane 3, specimen #45 *A. thaliana* DNA; lane 4, specimen #44 *A. thaliana* DNA; lane 5, specimen ts/g13 *A. thaliana* DNA; lane 6, *S. cerevisiae* DNA; lane 7, human genomic DNA; lane 8, *E. coli* DNA; lane 9, *M. lysodeikticus* DNA; lane 10, salmon sperm DNA; lane 11, *D. melanogaster* DNA; lane 12, No DNA; and lane 13, Mass Ladder.

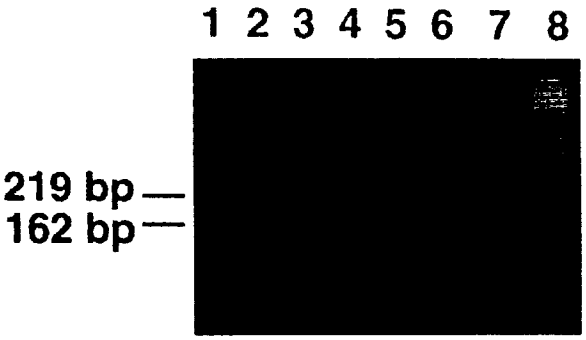

Fig. 4. Competitive PCR analysis of standard mixtures of *P. aeruginosa* and *E. coli* DNAs. Ethidium bromide stained 4% agarose gel of the electrophoresed PCR products of Paer16SH, Paer16SIR, UB16SC3 and UB16SDR9 primers. Lane 1, 50% *P. aeruginosa* DNA; lane 2, 10% *P. aeruginosa* DNA; lane 3, 1.0% *P. aeruginosa* DNA; lane 4, 0.1% *P. aeruginosa* DNA; lane 5, 0.01% *P. aeruginosa* DNA; lane 6, *E. coli* DNA; lane 7, No DNA; lane 8, Mass Ladder.

for potential quantitative PCR. A series of mixtures of *P. aeruginosa* and *E. coli* DNAs were prepared and analyzed with multiplex PCR using the *P. aeruginosa* and the universal primer sets simultaneously. Decreasing proportions of *P. aeruginosa* DNA from 50% down to at least 0.01% of the total bacterial DNA (held constant at 200 ng) provided consistent bands of 219 bp and 162 bp, with decreasing ratio of density of the *P. aeruginosa* specific 162 bp band (Fig. 4). The densitometric analyses of the PCR bands, shown in Fig. 4, provided a linear relationship between the log (DNA ratio) and the density ratio (data not shown). However, 30 cycles of amplification were used in this preliminary quantitative PCR trial and the linear range of amplicon production was not determined.

Primer sets for *S. maltophilia* and *S. marcescens* were chosen from the variable region of the 16S rRNA gene sequence, in the same manner as noted above for the *P. aeruginosa* PCR primer set (Table 3, Fig. 1). Primers Xmal16SL and Xmal16SKR were found to be specific for *S. maltophilia* DNA, providing the expected 220 bp PCR product (Fig. 5). This *S. maltophilia* specific primer set was also compatible with the universal primer set.

The first two primer sets, Smar16SM and Smar16SNR and Smar16ST and Smar16SUR, designed for *S. marcescens* were found to also amplify *S. maltophilia* DNA (data not shown). The third PCR primer set, Smar16SV and Smar16SWR, was found to amplify the appropriate 417 bp fragment in *S. marcescens* DNA, but not in DNAs isolated from *P. aeruginosa*, *S. maltophilia*, and Not *S. marcescens* culture (Table 3, Fig. 6A,B).

The annealing temperature and $MgCl_2$ concentrations were varied to determine the optimal PCR conditions for multiplex PCR of *S. maltophilia* or *S. marcescens* and the universal primer set (data not shown).

Since the environment contains countless microbial species with only a very small percentage having been isolated and the 16S rRNA gene sequence determined, it is entirely possible that these species-specific PCR primer sets might anneal and amplify the same or similar size fragment from an undefined microbial species. Nested PCR primers were designed for *P. aeruginosa* and *S. maltophilia* sequences to enhance specificity. The Paer16SX and Xmal16SY nested PCR primers work in conjunction with Paer16SIR and Xmal16SKR to amplify 103 bp and 97 bp fragments, respectively (Fig. 7). These nested primers failed to amplify other microbial DNAs, or PCR products from Smar16SV and Smar16SWR, or UB16SC3 and UB16SDR9. A 103 bp hemi-nested PCR fragment was observed from UB16SC3 and UB16SDR9 PCR product of *P. aeruginosa*, but this was most likely the result of *P.*

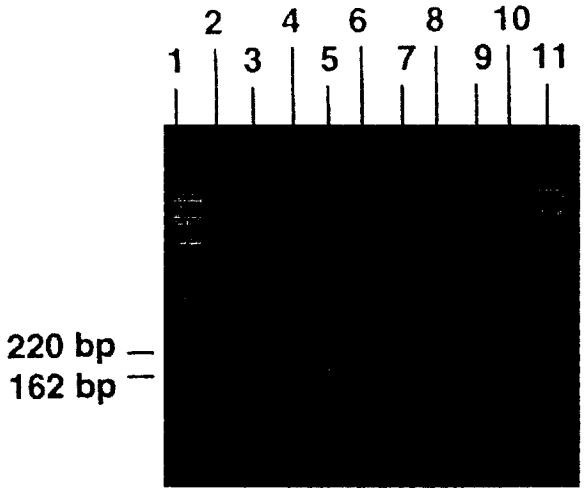

Fig. 5. Specificity of *S. maltophilia* PCR primers. Ethidium bromide stained 4% agarose gel of the electrophoresed PCR reaction products. Lane 1, 1 kb Ladder; lane 2, *S. maltophilia* DNA with Xmal16SL and Xmal16SKR primers; lane 3, *S. maltophilia* DNA with Xmal16SL, Xmal16SKR, UB16SC3, and UB16SDR9 primers; lane 4, *P. aeruginosa* DNA with Xmal16SL and Xmal16SKR primers; lane 5, *P. aeruginosa* DNA with Xmal16SL, Xmal16SKR, UB16SC3, and UB16SDR9 primers; lane 6, No DNA with Xmal16SL, Xmal16SKR, UB16SC3, and UB16SDR9 primers; lane 7, *S. marcescens* DNA with Xmal16SL and Xmal16SKR primers; lane 8, *S. marcescens* DNA with Xmal16SL, Xmal16SKR, UB16SC3, and UB16SDR9 primers; lane 9, Not *S. marcescens* mixed culture DNA with Xmal16SL and Xmal16SKR primers; lane 10, Not *S. marcescens* mixed culture DNA with Xmal16SL, Xmal16SKR, UB16SC3, and UB16SDR9 primers; lane 11, 1 kb Ladder.

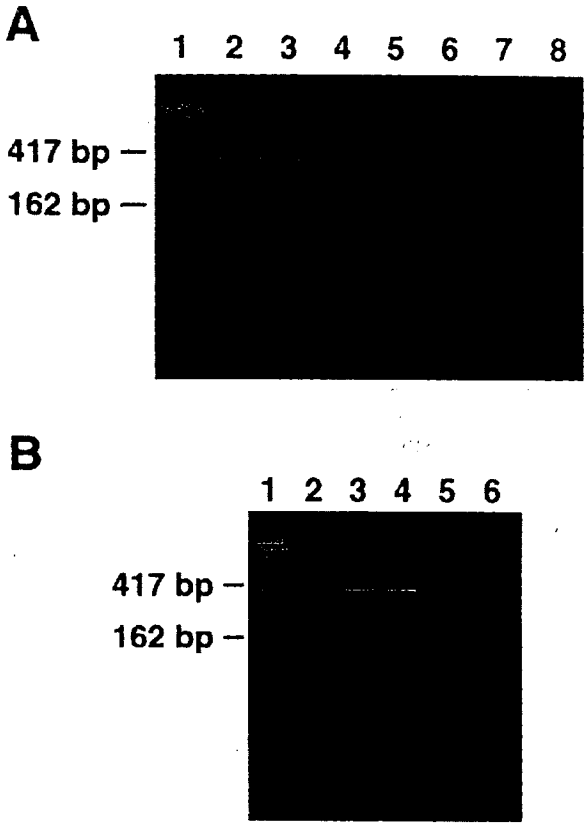

Fig. 6. Specificity of *S. marcescens* PCR primers. Ethidium bromide stained 4% agarose gel of the electrophoresed PCR reaction products. (A) Lane 1, Mass Ladder; lane 2, *S. marcescens* DNA with Smar16SV and Smar16SWR primers; lane 3, *S. marcescens* DNA with Smar16SV, Smar16SWR, UB16SC3, and UB16SDR9 primers; lane 4, *P. aeruginosa* DNA with Smar16SV and Smar16SWR primers; lane 5, *P. aeruginosa* DNA with Smar16SV, Smar16SWR, UB16SC3, and UB16SDR9 primers; lane 6, *S. maltophilia* DNA with Smar16SV and Smar16SWR primers; lane 7, *S. maltophilia* DNA with Smar16SV, Smar16SWR, UB16SC3, and UB16SDR9 primers; lane 8, No DNA. (B) Lane 1, 1 kb Ladder; lane 2, No DNA; lane 3, *S. marcescens* DNA with Smar16SV and Smar16SWR primers; lane 4, *S. marcescens* DNA with Smar16SV, Smar16SWR, UB16SC3, and UB16SDR9 primers; lane 5, Not *S. marcescens* mixed culture DNA with Smar16SV and Smar16SWR primers; lane 6, Not *S. marcescens* mixed culture DNA with Smar16SV, Smar16SWR, UB16SC3, and UB16SDR9 primers.

aeruginosa DNA being carried over in the subsequent hemi-nested PCR reaction (lane 4, Fig. 7).

In addition to hemi-nested PCR analyses, further verification of species-specific PCR amplified sequences was performed with LCR analyses. LCR has been used for the sensitive detection of a specific base at a chosen location within a genomic sequence (Barany, 1991; Wilson et al., 1999). LCR primers are designed based on the sequence surrounding the nucleotide in question and are thus also species-specific sequence dependent. The deoxyguanosine representing nucleotide number 589 of the reported *P. aeruginosa* 16S rRNA gene sequence (Table 1, Fig. 1) was chosen as the focus of the LCR primer design (Fig. 8). The Paer16S589L2 and Paer16S589LR2 primers, listed in Table 5, were 5′ end-labeled with ^{32}P prior to the LCR reaction, providing both the phosphate for ligation and the means to detect the ligated product. The resultant LCR fragments of 56 bp and 60 bp were observed for the synthesized 71mer oligonucleotide duplex standard, and both PCR amplified and nested PCR

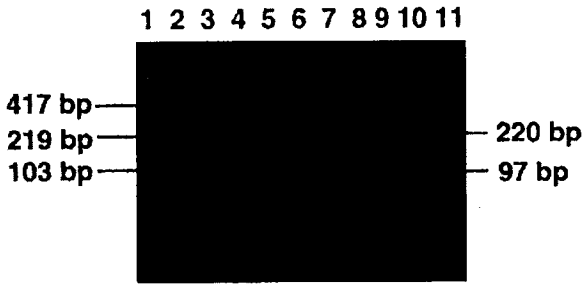

Fig. 7. Hemi-nested *P. aeruginosa* and *S. maltophilia* PCR primers analyses. Lane 1, Mass Ladder; lane 2, PCR product of *P. aeruginosa* DNA amplified with Paer16SH and Paer16SIR; lane 3, PCR product of *S. marcescens* DNA amplified with Smar16SV and Smar16SWR; lane 4, PCR product of *P. aeruginosa* DNA amplified with UB16SC3 and UB16SDR9; lane 5, PCR product of *S. maltophilia* DNA amplified with UB16SC3 and UB16SDR9; lane 6, No DNA; lane 7, PCR product of *S. maltophilia* DNA amplified with Xmal16SL and Xmal16SKR; lane 8, PCR product of *P. aeruginosa* DNA amplified with Paer16SH and Paer16SIR; lane 9, PCR product of *S. marcescens* DNA amplified with Smar16SV and Smar16SWR; lane 10, PCR product of *P. aeruginosa* DNA amplified with UB16SC3 and UB16SDR9; lane 11, 1 kb Ladder. Lanes 2, 3, 4, and 5, samples amplified with Paer16SX and Paer16SIR; lane 6, contained Paer16SX, Paer16SIR, Xmal16SY, and Xmal16SKR primers; lanes 7, 8, 9, and 10, samples amplified with Xmal16SY and Xmal16SKR.

amplified *P. aeruginosa* DNA, but not PCR products of *S. maltophilia* or *S. marcescens* or UB16SC3 and UB16SDR9 PCR product (Fig. 9).

In the interest of determining the usefulness of these species-specific PCR primer sets in the real world, samples of water were obtained from the University Lake, Louisiana State University, Baton Rouge, LA, on three different dates. The microbial community was isolated by microfiltration and DNA isolated for PCR studies, while a second portion was cultured for the three species of bacteria of interest. The first University Lake sample, obtained on January 17, 1998, provided positive results for *P. aeruginosa*, but not for either of the other two microbial species (data not shown). The presence of *P. aeruginosa* was also found in the second and third sampling (March 25 and August 3, 1998). In addition, the second Lake sample, obtained on March 25, displayed positive PCR results for *S. marcescens*, but not for *S. maltophilia* (Fig. 10).

Hemi-nested PCR analyses of the March sample from University Lake provided positive verification of the presence of *P. aeruginosa*, but not *S. maltophilia* (Fig. 11). Instead, an unknown band greater than 400 bp was observed from the *S. maltophilia* nested PCR University Lake sample analysis (Fig. 11). Hemi-nested PCR primers specific for *S. marcescens* have not been developed.

Subsequent LCR analysis of the PCR products of the University Lake samples further validated the presence of *P. aeruginosa* (Fig. 12). Only the PCR amplification product of Paer16SH and Paer16SIR provided distinguishing 56 bp and 60 bp bands in the LCR reaction. The hemi-nested PCR product of Paer16SX and PaerSIR amplified University Lake water also provided positive LCR results for *P. aeruginosa* (data not shown).

Bacterial culture analyses of the University Lake water samples was performed by two different approaches. The first, performed on the March 25 samples, involved the inoculation of nutrient agar for the general growth of microbial species. Following gram negative selection, many of the isolates were not identifiable using the Biolog system. Of those that were identified, none belonged to the genera *Serratia*, *Xanthomonas* or *Pseudomonas*.

Subsequently, a second, more selective bacterial culture method was performed on the August 3 University Lake samples. The water samples were plated directly onto agar plates selective for *Pseudomonas* and *Serratia*. One of the isolates from the *Serratia* selective agar was identified as *P. aeruginosa*, and one was not identified, but the closest species was *P. aeruginosa*, according to the Biolog system. Of the isolates from the *Pseudomonas* agar, one was not identified, but the closest species was *P. aeruginosa*. Another was identified as *Pseudomonas* genus.

PCR analyses of DNAs isolated from colonies taken from the *Pseudomonas* and *Serratia* selection plates of the August 3 water samples displayed both the *P. aeruginosa* (219 bp) and universal (162 bp) bands, but not the *S. maltophilia* or *S. marcescens* PCR product bands (data not shown).

4. Discussion

Rapid PCR based tests that identify specific microorganisms in an environmental sample have been developed and validated. Based on highly

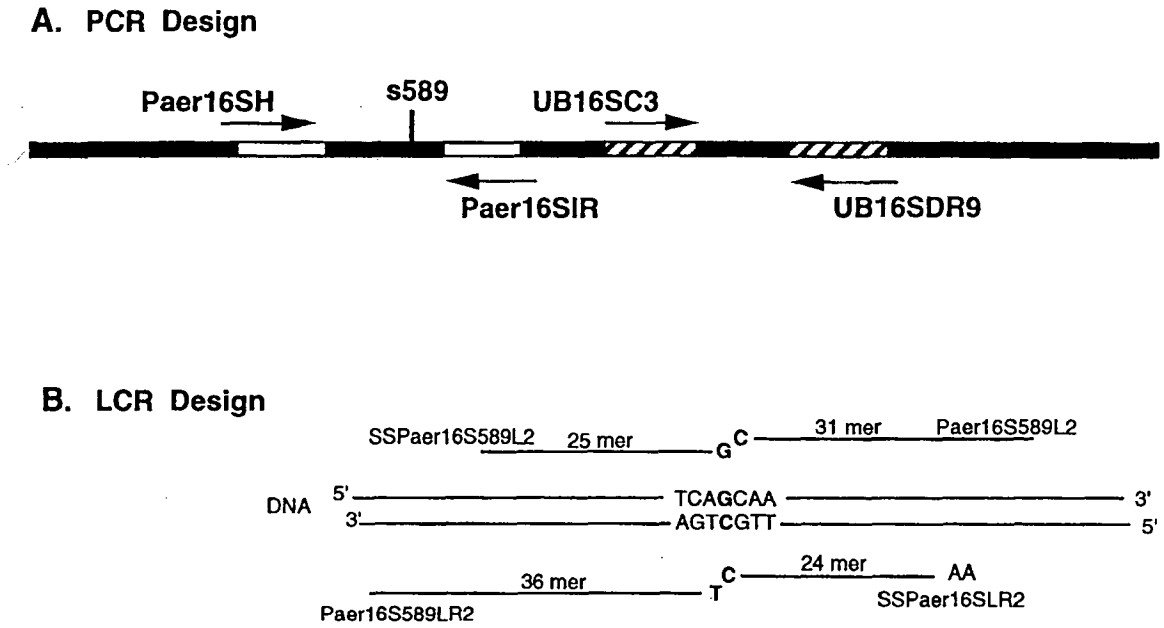

Fig. 8. Diagram of the PCR and LCR primer design for *P. aeruginosa* 16S rRNA gene. (A) The relative positions of the PCR primers. (B) LCR primer design for the detection of a deoxyguanosine residue at position number 589 of the *P. aeruginosa* 16S rRNA gene sequence. The invariant Paer16S589L2 and Paer16S589LR2 primers, as well as the species-specific SSPaer16S589L2 and SSPaer16S589LR2 primers, are complementary to the *P. aeruginosa* sequence. These primers will amplify the appropriate LCR products of 56 bp and 60 bp using the *P. aeruginosa* PCR product template in the LCR reaction. The SSPaer16S589L2 and SSPaer16S589LR2 primers will ligate to the invariant Paer16S589L2 and Paer16S589LR2 primers only in the presence of template DNA containing both the appropriate flanking sequence and a deoxyguanosine at position number 589. See Tables 3 and 5 for sequences of the PCR and LCR primers, respectively.

Table 5
LCR primers

LCR primer	Length	5′–3′ sequence
Paer16S589L2	31mer	CAAGCATGATGTGAAATCCCCAGGCTCAACC
Paer16S589LR2	36mer	TGAACCACTTACGAGCACTTTACGCCCAGTAATTCC
SSPaer16S589L2	25mer	CGTAAAGTGCTCGTAAGTGGTTCAG
SSPaer16S589LR2	24mer	AAGGGGATTTCACATCATGCTTGC
LCR oligonucleotide template standards		
Paer16S589Lstd	71mer	CGGAATTACTGGGCGTAAAGCGCGCGTAAGTGGTTCAG-CAAGCTTGATGTGAAATCCCCGGGCTCAACCTG
Paer16S589LRstd	71mer	CAGGTTGAGCCCGGGGATTTCACATCAAGCTTGCTGAA-CCACTTACGCGCGCTTTACGCCCAGTAATTCCG

variable regions of the 16S rRNA gene sequences, PCR primer sets have been designed that specifically amplify *P. aeruginosa*, *S. maltophilia*, and *S. marcescens* DNA. These species-specific primer sets work in conjunction with a universal primer set that acts as an internal marker and may enable the quantitative estimation of the species of interest by competitive PCR techniques.

These species-specific identification procedures are simpler than previously reported molecular approaches that require sequencing, pattern recognition (fingerprinting by AP-PCR, RAPD-PCR, ERIC-PCR, or REP-PCR), RFLPs, or probing of Southern blots (Williams et al., 1990; DeBruijn, 1992; Moyer et al., 1994; Chatelut et al., 1995; van Couwenberghe et al., 1995; Wilson and Blitchington, 1996). These

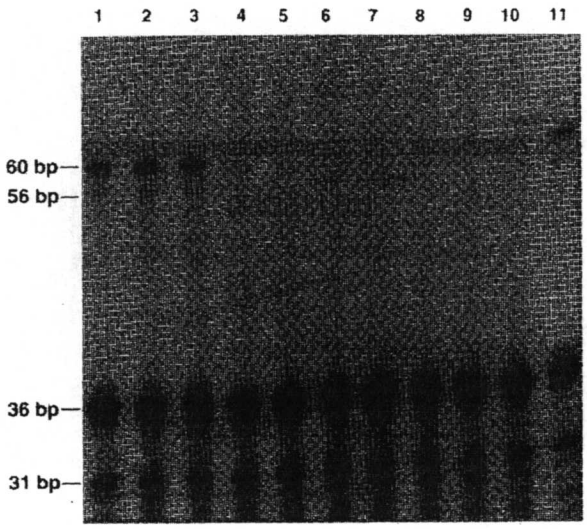

Fig. 9. Specificity and accuracy of LCR primer set. Autoradiograph of LCR analysis of various PCR products of the 16S rRNA sequences of different species, separated on a 7 M urea, 10% polyacrylamide sequencing gel. Duplex DNA of 71 nucleotides in length matching the template region of *P. aeruginosa* 16S rRNA gene were synthesized with a deoxyguanosine in nucleotide position 589. The invariant LCR primers were end-labeled with ^{32}P prior to amplification. The LCR primers were chosen to provide 56 and 60 bp fragment sizes for the sense and the antisense DNA strands (see Fig. 8B). Film was exposed for 30 min. Lanes 1 and 11, synthetic 71mer duplex LCR template DNA (see Table 5); lane 2, PCR product of *P. aeruginosa* DNA amplified with Paer16SH and Paer16SIR; lane 3, hemi-nested PCR product of *P. aeruginosa* DNA amplified with Paer16SX and Paer16SIR; lane 4, PCR product of *S. maltophilia* DNA amplified with Xmal16SL and Xmal16SKR; lane 5, PCR product of *S. marcescens* DNA amplified with Smar16SV and Smar16SWR; lane 6, PCR product of human genomic DNA amplified with Paer16SH and Paer16SIR (PCR negative control); lane 7, PCR product of *P. aeruginosa* DNA amplified with UB16SC3 and UB16SDR9; lane 8, PCR product of Not *P. aeruginosa* mixed culture DNA amplified with Paer16SH and Paer16SIR; lane 9, PCR product of No DNA reacted with Paer16SH and Paer16SIR; lane 10, No DNA.

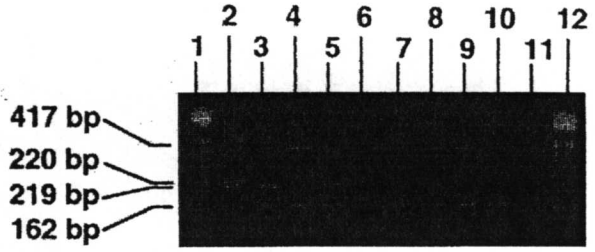

Fig. 10. University Lake water analysis of March 25, 1998, samples. Ethidium bromide stained 4% agarose gel of the electrophoresed PCR reaction products. Lane 1, Mass Ladder; lane 2, *P. aeruginosa* DNA with Paer16SH, Paer16SIR, UB16SC3, and UB16SDR9 primers; lane 3, *S. maltophilia* DNA with Xmal16SL, Xmal16SKR, UB16SC3, and UB16SDR9 primers; lane 4, *S. marcescens* DNA with Smar16SV, Smar16SWR, UB16SC3, and UB16SDR9 primers; lane 5, University Lake sample 1 DNA with Paer16SH, Paer16SIR, UB16SC3, and UB16SDR9 primers; lane 6, University Lake sample 2 DNA with Paer16SH, Paer16SIR, UB16SC3, and UB16SDR9 primers; lane 7, University Lake sample 1 DNA with Xmal16SL, Xmal16SKR, UB16SC3, and UB16SDR9 primers; lane 8, University Lake sample 2 DNA with Xmal16SL, Xmal16SKR, UB16SC3, and UB16SDR9 primers; lane 9, University Lake sample 1 DNA with Smar16SV, Smar16SWR, UB16SC3, and UB16SDR9 primers; lane 10, University Lake sample 2 DNA with Smar16SV, Smar16SWR, UB16SC3, and UB16SDR9 primers; lane 11, No DNA with Paer16SH, Paer16SIR, UB16SC3, and UB16SDR9 primers; lane 12, Mass Ladder.

methods are generally not directly applicable to the analysis of environmental samples due either to the complexity of patterns or the need for culture purification prior to analyses. Reverse sample genome probing may be useful in the bacterial analyses of environmental samples, but the procedures are more complex and technically difficult (Shen et al., 1998). In situ hybridization with fluorescently labeled species-specific probes has the advantage of identification of specific microbial species at the individual cell level (DeLong et al., 1985; Tani et al., 1998). However, the multiplex PCR based procedures described in the present work are amenable to the development of instrumentation for the simple, rapid and routine analyses of numerous real world samples simultaneously (Winn-Deen et al., 1993).

These procedures also enable the rapid verification of the species detected by hemi-nested PCR and/or LCR analyses. Only PCR amplified *P. aeruginosa* and *S. maltophilia* 16S rRNA DNA were amplifiable with these species-specific hemi-nested PCR primer sets, respectively (see Fig. 7). Similarly, only PCR amplified or hemi-nested PCR amplified *P. aeruginosa* DNA provided the appropriate template for LCR product (see Fig. 9). Both hemi-nested PCR and LCR require distinct sequences, thus enabling further sequence verification of the species-specific 16S rRNA gene region under study. Although a single base mismatch depending upon the base change and

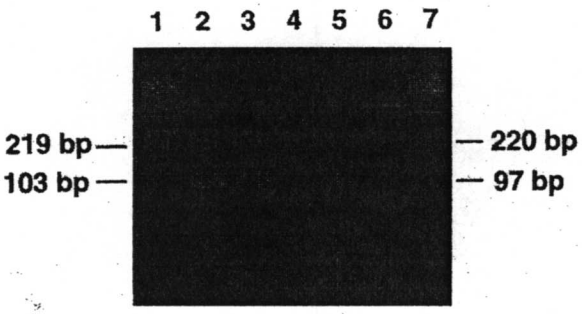

Fig. 11. Hemi-nested PCR analysis of University Lake water samples of March 25, 1998. Lane 1, Mass Ladder; lane 2, PCR product of *P. aeruginosa* DNA amplified with Paer16SH, Paer16SIR, UB16SC3, and UB16SDR9 primers; lane 3, PCR product of University Lake sample 1 DNA amplified with Paer16SH, Paer16SIR, UB16SC3, and UB16SDR9 primers; lane 4, PCR product of No DNA amplified with Paer16SH, Paer16SIR, UB16SC3, and UB16SDR9 primers; lane 5, PCR product of *S. maltophilia* DNA amplified with Xmal16SL, Paer16SKR, UB16SC3, and UB16SDR9 primers; lane 6, PCR product of University Lake sample 1 DNA amplified with Xmal16SL and Xmal16SKR; lane 7, 1 kb Ladder. Lanes 2 and 3, samples amplified with Paer16SX and Paer16SIR; lane 4, contained Paer16SX, Paer16SIR, Xmal16SY, and Xmal16SKR primers; lanes 5 and 6, samples amplified with Xmal16SY and Xmal16SKR.

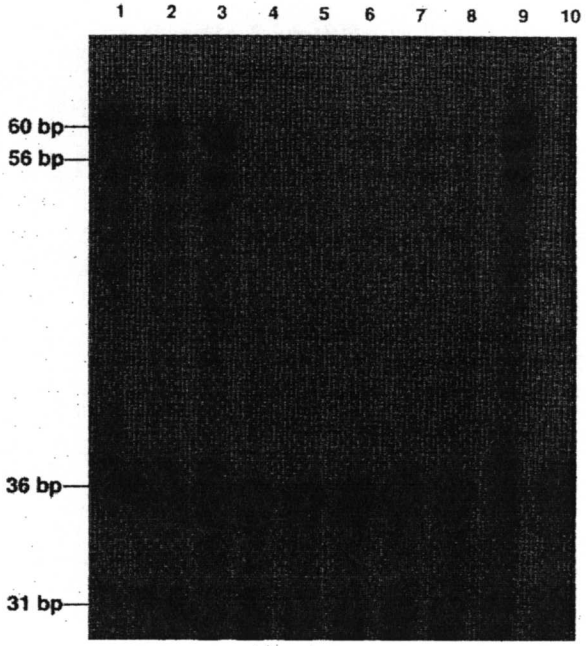

Fig. 12. LCR analysis of University Lake water samples of March 25, 1998. Autoradiograph of LCR analysis of various PCR products of the standard template and University Lake water sample DNAs, separated on a 7 M urea, 10% polyacrylamide sequencing gel. The invariant LCR primers were end-labeled with ^{32}P prior to amplification. The LCR primers were chosen to provide 56 and 60 bp fragment sizes for the sense and the antisense DNA strands (see Fig. 8B). Film was exposed for 45 min. Lanes 1 and 9, synthetic 71mer duplex LCR template DNA (see Table 5); lane 2, PCR product of University Lake sample 1 DNA amplified with Paer16SH, Paer16SIR, UB16SC3, and UB16SDR9; lane 3, PCR product of University Lake sample 2 DNA amplified with Paer16SH, Paer16SIR, UB16SC3, and UB16SDR9; lane 4, PCR product of University Lake sample 1 DNA amplified with Xmal16SL, Xmal16SKR, UB16SC3, and UB16SDR9; lane 5, PCR product of University Lake sample 2 DNA amplified with Xmal16SL, Xmal16SKR, UB16SC3, and UB16SDR9; lane 6, PCR product of University Lake sample 1 DNA amplified with Smar16SV, Smar16SWR, UB16SC3, and UB16SDR9; lane 7, PCR product of University Lake sample 2 DNA amplified with Smar16SV, Smar16SWR, UB16SC3, and UB16SDR9; lane 8, PCR product of No DNA amplified with Paer16SH, Paer16SIR, UB16SC3, and UB16SDR9; lane 10, No DNA.

the location may be tolerable for LCR primers, LCR requires a specific base at the ligation site in order to amplify a ligated product band (Barany, 1991; Wiedmann et al., 1992; Wilson et al., 1999). LCR techniques have been used to detect the single base change observed in sickle cell disease, a specific Listeria species in a mixture, and a single base substitution mutation in human and animal tissues. These ligation procedures are also amenable to multiplex and routine instrumentation analyses (Winn-Deen et al., 1993). Multiple analyses of samples with PCR, hemi-nested PCR, and LCR enable the detection and identification of specific microbial species. Only sequencing would provide higher assurance of the specific species detected.

Environmental water samples were collected from a local lake on three different occasions and subjected to these molecular analyses using PCR and LCR as described above. *P. aeruginosa* was consistently detected in every sample analyzed, while *S. marcescens* was only detected in the second sampling (March, 1998). *S. maltophilia* was not found in any of these samples. These results were further verified with hemi-nested PCR and with LCR procedures as described above. Identification of these microbial species by culturing techniques was difficult. The use of the selective agar procedures enabled the isolation of *P. aeruginosa*. Colonies taken from these plates were analyzed by species-specific PCR primer sets

and only *P. aeruginosa* product was observed on all of the plates.

One of the inherent risks of any molecular sequence specific detection system is the possibility of matching 16S rRNA gene sequences by another species or organism in environmental samples. It has been estimated that only about 1% of all microbial species have been cultured and identified (Hugenholtz and Pace, 1996; Wilson and Blitchington, 1996). Only a small portion of this 1% have been sequenced for the 16S rRNA gene (Olsen et al., 1991; Marchesi et al., 1998). The detection techniques employed need to cover a significant proportion of highly variable regions of the 16S rRNA gene sequence to ensure selection of the microbe in question. However, the possibility of detecting an unknown microbial species with these species-specific PCR primer sets cannot be ruled out. Either sequencing of the PCR amplified fragment, or hemi-nested PCR, nested PCR, and/or LCR should be consistently undertaken in environmental samples or clinical specimens for further validation of the microbial species detected.

Quantitative estimation of the amount of *P. aeruginosa*, *S. maltophilia*, or *S. marcescens* DNA present in a mixed culture or environmental samples may be determinable by competitive PCR procedures with the universal primer set. We have shown preliminary that the proportion of *P. aeruginosa* DNA present in a mixture with *E. coli* DNA can be determined by PCR using the *P. aeruginosa* species-specific PCR primers, Paer16SH and Paer16SIR, and the universal primers, UB16SC3 and UB16SDR9. These procedures are also adaptable for *S. maltophilia* and *S. marcescens* species-specific PCR primers sets. However, there are a number of issues that still need to be addressed in this quantitative competitive PCR assay. The effects of primer set efficiencies, template annealing bias and product inhibition reported by Suzuki and Giovannoni (1996) require the careful determination of the amplification efficiencies of each of the competitive primer sets, for each species. Since these PCR primer efficiencies may vary with low levels of template, the amount of sample DNA analyzed in the PCR amplification should be maintained in a linear PCR reaction range (Suzuki and Giovannoni, 1996). The further development of these correction factors for primer efficiencies should enable the use of these primer sets for the quantitative estimation of these microbial species in real world environmental samples.

The accuracy of such quantitative analyses will, however, still be dependent upon additional variables. The number of copies of the 16S rRNA gene per cell may vary from species to species and perhaps among strains, so that competitive PCR will provide only an estimation of the proportion of an environmental microbial population (Wilson and Blitchington, 1996; Marchesi et al., 1998; Tani et al., 1998). The standard lysis and phenol extraction used in the present study will not efficiently isolate DNA from gram positive bacteria and other species, so that the determined proportion of a species within a microbial community may be biased by these procedures. However, the present work has demonstrated a simple procedure for the linear detection of *P. aeruginosa* DNA down to 0.01% of the total microbial DNA in a prepared standard mixture. This level of sensitivity for quantitative analyses is lower than that reported for PCR-denaturing gradient gel electrophoresis (Muyzer et al., 1993).

Many bacterial species have been noted for their ability to thrive when the carbon source is from anthropogenic compounds, and the bacterial species chosen for these studies are known to degrade hydrocarbons as do a number of microbial species (Deziel et al., 1996; Shen et al., 1998). These species-specific PCR procedures may be useful in the monitoring of natural or bioremediation introduced bacteria in oil spills and other hydrocarbon contaminated sites (Lindstrom et al., 1991). Microbial community structure in environmental settings could also be monitored by the direct detection and quantitative assessment of individual species with these bacterial specific PCR techniques. These procedures could, in principle, be adapted to detect and track organisms for which a 16S rRNA gene sequence has been determined but no culturing method is available. In addition, the detection of specific microbial species is an important issue in the treatment of opportunistic infections in the human clinic (Davies et al., 1997; Pier et al., 1997). Species-specific PCR procedures based on the 16S rRNA sequences may be valuable in the analysis of human and veterinary clinical specimens. Indeed, *P. aeruginosa* is known to be an important opportunistic pulmonary pathogen

in cystic fibrosis (Davies et al., 1997; Pier et al., 1997).

The universal primer set, UB16SC3 and UB16SDR9, appears to amplify the appropriate band fragment size of 162 bp in all single and multicellular organisms tested thus far. The 16S rRNA gene sequence that these universal primers anneal with appears to be not only conserved in the procaryotic kingdoms, but also in eucaryotic organisms, including yeast (*Saccharomyces cerevisiae*), plants (*Arabidopsis thaliana*), fruit fly (*Drosophila melanogaster*), salmon, and human. Presumably, these 16S rRNA gene sequences are also conserved phylogenetically because of their presence in either mitochondria and chloroplast genomes, or pseudogenes, or in genes that evolved from the 16S rRNA sequences (Hu and Thilly, 1994; Liang and Hays, 1996). The matching PCR product fragment size in all species tested suggests an important phylogenetic conservation of this 162 bp or larger portion of the 16S rRNA bacterial gene. The possibility of this UB16SC3 and UB16SDR9 primer set representing an earth biota genetic marker may have value not only in its use as a universal internal standard, but also in the study of life on other planets.

The procedures developed in this work will be of value in monitoring a number of environmental issues, dominated by either natural or anthropogenic factors. Rapid species-specific PCR analyses have been developed for the analyses of environmental specimens, which may be useful in the study of the growth or suppression of individual bacterial species within natural communities. These techniques should be universally applicable to any microbial species of interest, while the universal primer set appears to detect most species and possibly earth biota.

5. Notation

AP-PCR	Arbitrarily primed-PCR
ERIC-PCR	Enterobacterial intragenic consensus sequence-PCR
LCR	Ligase chain reaction
PCR	Polymerase chain reaction
RAPD-PCR	Random amplified polymorphic DNA
REP-PCR	Repetitive extragenic palindromic-PCR

Acknowledgements

This research was supported, in part, by grant R21CA69650 from the National Institutes of Health, National Cancer Institute, grant R825810 from the U.S. Environmental Protection Agency, and grant 14-35-0001-30660/task19921 from the Minerals Management Service, U.S. Department of Interior. We would like to thank Dr. Ralph Portier for providing bacteria isolated from his culture collection, Dr. John C. Larkin for his gift of *Arabidopsis thaliana* DNAs, and Dr. William R. Lee for his gift of *Drosophilia melanogaster* larvae.

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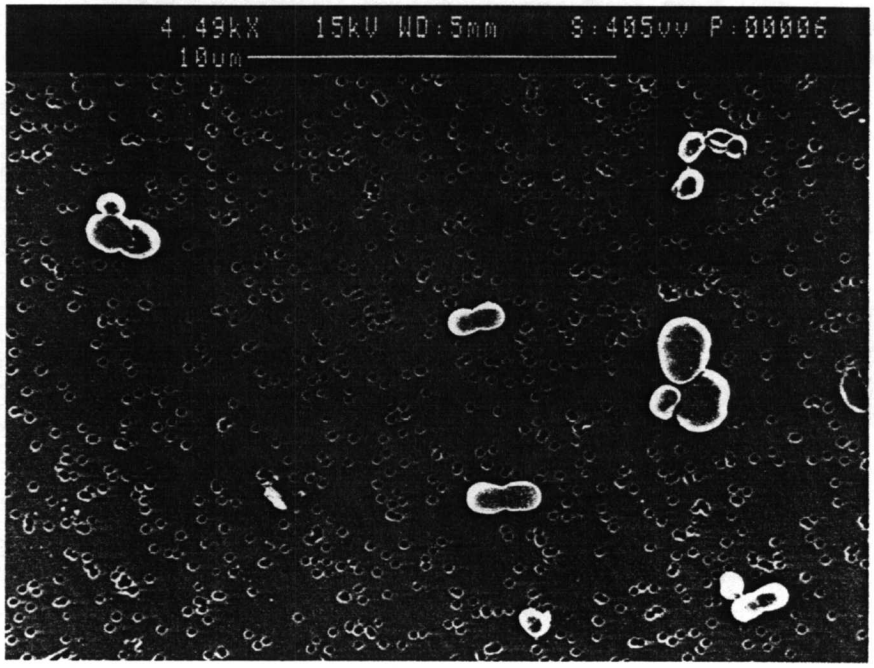
Appendix B

Photographs of *Acinetobacter* A-84 and marsh microcosm.

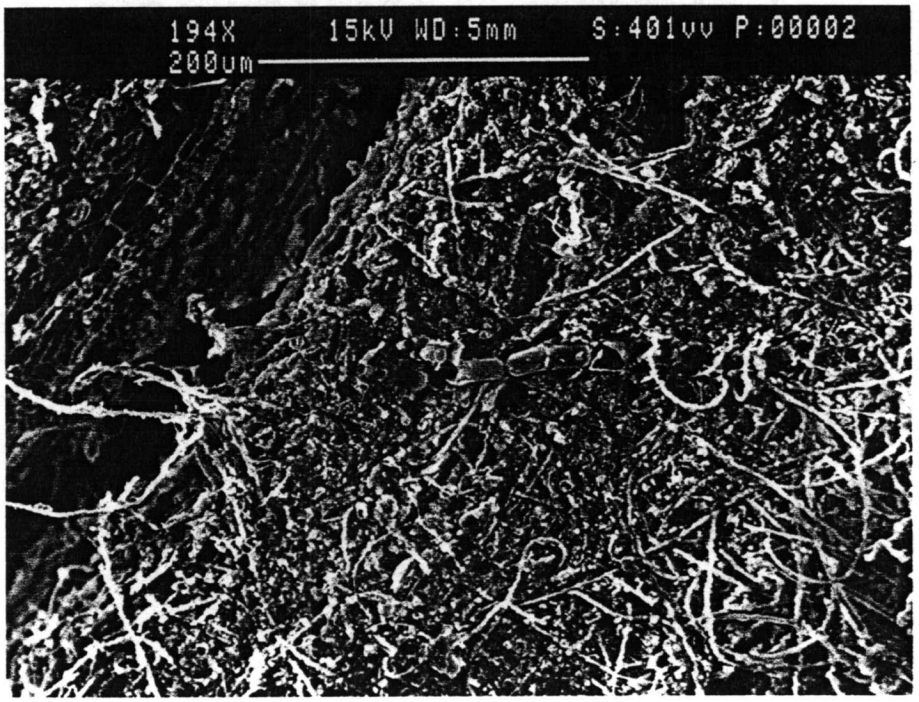
Plate 1. A scanning electron micrograph of Acinetobacter A-84.
Plate 2. A low-power scanning electron micrograph (approximately 200X) showing microbial invasion of decaying marsh vegetation.
Plate 3. The plant marsh microcosm.
Plate 4. An overall view of the marsh microcosm and experimental chemostats.

Plate 1



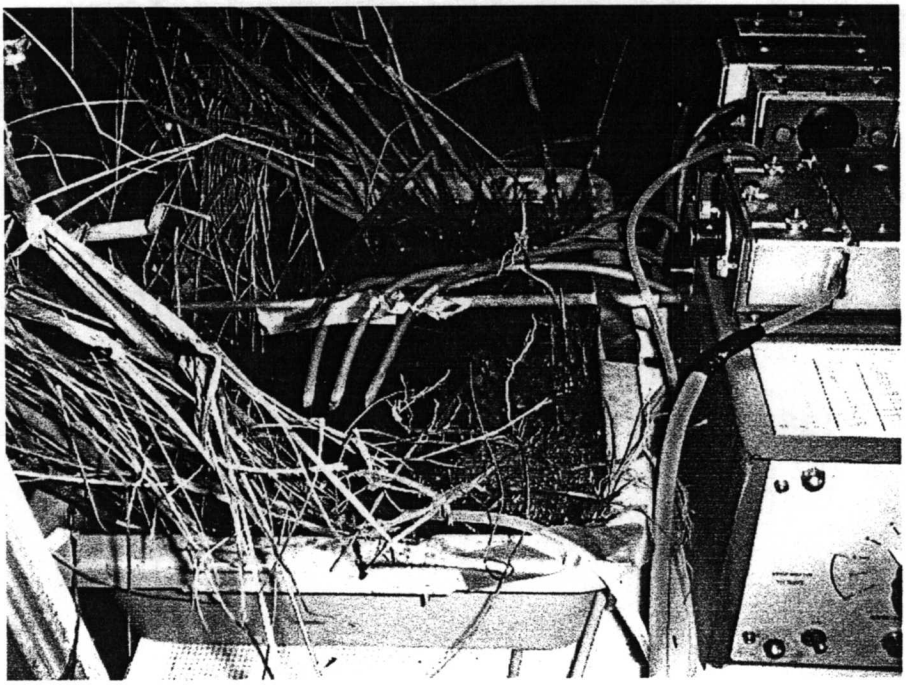
Scanning electron micrograph of *Acinetobacter* A-84. Magnification 4,500x.

Plate 2



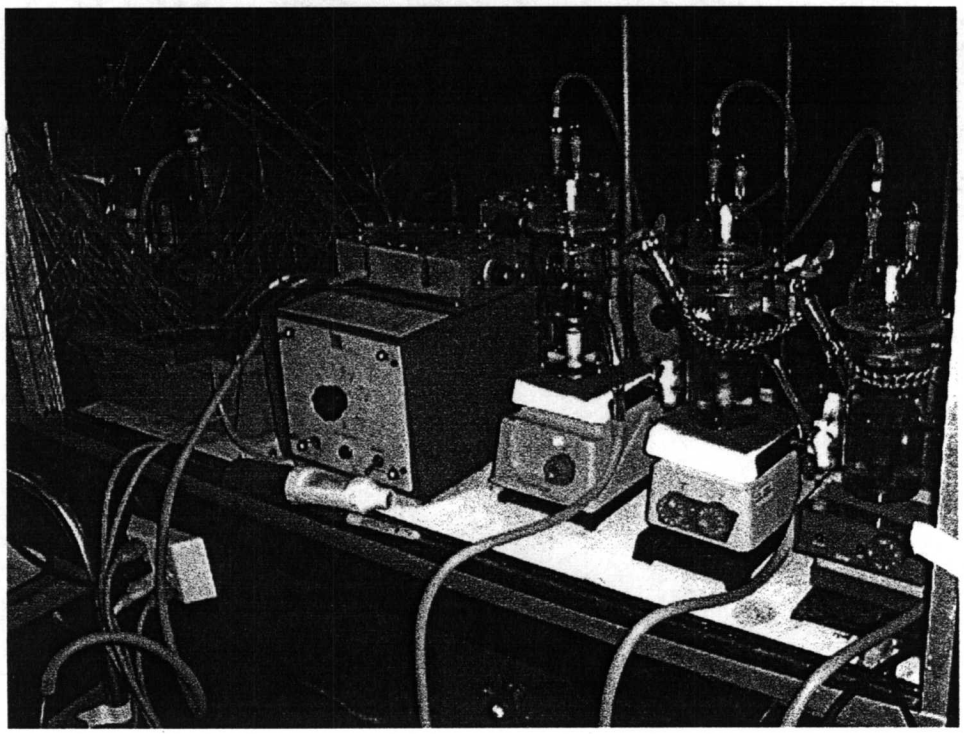
A low-power micrograph (approximately 200X) showing the microbial invasion of decaying marsh plant. Marsh vegetation typically has a waxy cuticle and because of this a substantial fraction of the marsh microflora are capable of utilizing hydrocarbons, and was the reason we used marsh vegetation as a starting point for the isolation of *Acinetobacter* A-84.

Plate 3



The marsh microcosm. Marsh plants were uprooted, replanted in plastic tray and marsh water pumped into the system. From there water was pumped out of the microcosm via the three tubes seen in the center of the photo, and into the experimental chemostats. For the Mississippi River water experiment, the marsh was removed and the water was pumped directly into the chemostats using the peristaltic pump seen on the right side of the photo.

Plate 4



An overall view of the marsh microcosm and the experimental chemostats. The results of these experiments can be seen in Figs. 6 and 7 of this report.

The Department of the Interior Mission

As the Nation's principal conservation agency, the Department of the Interior has responsibility for most of our nationally owned public lands and natural resources. This includes fostering sound use of our land and water resources; protecting our fish, wildlife, and biological diversity; preserving the environmental and cultural values of our national parks and historical places; and providing for the enjoyment of life through outdoor recreation. The Department assesses our energy and mineral resources and works to ensure that their development is in the best interests of all our people by encouraging stewardship and citizen participation in their care. The Department also has a major responsibility for American Indian reservation communities and for people who live in island territories under U.S. administration.

The Minerals Management Service Mission

As a bureau of the Department of the Interior, the Minerals Management Service's (MMS) primary responsibilities are to manage the mineral resources located on the Nation's Outer Continental Shelf (OCS), collect revenue from the Federal OCS and onshore Federal and Indian lands, and distribute those revenues.

Moreover, in working to meet its responsibilities, the **Offshore Minerals Management Program** administers the OCS competitive leasing program and oversees the safe and environmentally sound exploration and production of our Nation's offshore natural gas, oil and other mineral resources. The MMS **Minerals Revenue Management** meets its responsibilities by ensuring the efficient, timely and accurate collection and disbursement of revenue from mineral leasing and production due to Indian tribes and allottees, States and the U.S. Treasury.

The MMS strives to fulfill its responsibilities through the general guiding principles of: (1) being responsive to the public's concerns and interests by maintaining a dialogue with all potentially affected parties and (2) carrying out its programs with an emphasis on working to enhance the quality of life for all Americans by lending MMS assistance and expertise to economic development and environmental protection.