ACYL HOMOSERINE LACTONE RECRUITMENT OF BACTERIA INTO BIOFILMS

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ABSTRACT

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Acylated homoserine lactones (acyl HSLs) are membrane permeant, extracellular signaling molecules that are important in biofilm physiology and quorum sensing. As acyl HSL analogues have been shown to block adhesion, I investigated whether acyl HSLs would promote adhesion. In this study, acyl HSLs were isolated from *Agrobacterium tumefaciens* which produces 3-oxo-C8-HSL and *Chromobacterium violaceum* which produces C6-HSLs. Non acyl HSL-producing strains of these species were used as controls. Live cultures of these strains as well as non bacterial controls were placed in semipermeable dialysis tubing in the San Marcos River and assayed for biofilm growth. Biofilm colonization on the tubes was compared using standard plate counts. Each biofilm was analyzed with molecular techniques to identify the different bacterial species present and to obtain an overall community diversity of each biofilm. While the cell numbers for each biofilm did not differ significantly, the microbial diversity profiles varied.

INTRODUCTION

Biofilm formation

Bacteria in the environment commonly exist as biofilms. Biofilms are bacterial populations enclosed in a matrix that are adherent to a vast array of surfaces and interfaces (Costerton et al, 1995). Biofilms are highly organized multicellular systems formed through regulated developmental processes. Their specific functional and structural design influence the biofilms' response to available nutrients, predation, metabolic processes, and resistance to antimicrobial agents (Lawrence et al, 1991). The bacteria within biofilms show variant physiological properties from planktonic cells which may account for their increased resistance to antibiotics and desiccation (Batchelor et al, 1997). Planktonic cells interact with a surface in response to various signals or to the nutrient availability of that environment (O'Toole and Kolter, 1998). In response to favorable conditions, cells will attach to a surface, divide by binary fission forming microcolonies, and then produce an exopolymer to develop a mature biofilm that is protected from adverse conditions (Costerton et al, 1995). Surface colonization by microorganisms seems to take place through an ordered series of recruitment processes starting with a pioneer species that responds to environmental signals (Loo et al, 2000). Pioneer organisms modify the surface by making it suitable for further colonizers. Growth and reproduction of the

primary colonizers contributes to biofilm accumulation as does recruitment of new organisms into the biofilm community (Dang and Lovell, 2000).

During biofilm development simple cone shaped microcolonies are formed which are attached to a surface separated by water filled spaces or channels dispersed throughout the structure. This primitive circulatory system alongside development of mushroom shaped colonies throughout the biofilm allows for nutrient flow and argues for some form of cell-to-cell communication within biofilms (Costerton et al, 1995). Therefore, a biofilm can be considered a highly differentiated community which is able to use intercellular communications to facilitate their adjustment to changes in the environment.

Quorum sensing

It has been shown that diverse gram-negative bacteria release various N-acyl homoserine lactones (acyl HSLs) also referred to as autoinducers (Fuqua et al, 1994). These autoinducers regulate several traits including bioluminescence, production of virulence factors, conjugal transfer in *Agrobacterium tumefaciens*, and pigment production in *Chromobacterium violaceum.* Acyl HSLs are membrane-permeant signaling molecules that allows bacteria to monitor the density of cells within their population (Fuqua and Greenberg, 1998). In this process, known as quorum sensing, each cell will produce a low level of acyl HSL through the activity of an acyl HSL synthase (usually a Lux I homolog) and as the population density increases so does the amount of acyl HSL in the surrounding environment (Fuqua and Greenberg, 1998). This mechanism of autoinduction was first observed in the early 1970s with a

marine bacterium *Vibrio fischeri* in which bioluminescence was controlled with the synthesis of the first autoinducer, N-3-oxohexanoyl-L-HSL (Fuqua et al, 1994). The cell membrane is permeable to this autoinducer, therefore it accumulates inside the cell and in the surrounding environment in equal concentrations (Fuqua et al, 1996). At a certain threshold level, the accumulated acyl HSLs will interact with a receptor protein (usually a Lux R homolog) which can activate specific acyl HSL dependent genes (Fuqua and Greenberg, 1998). Therefore quorum sensing allows the bacteria to take a census of their population and regulate specific gene expression when needed. Several other genes similar to LuxR and LuxI have been identified in various Gram-negative bacteria. *A. tumefaciens*, which also produces N-3-oxooctanoyl-L-HSL, uses the TraR and TraI proteins (both LuxR and LuxI homologs) for regulation of conjugal transfer of Ti plasmid (Fuqua et al, 1996). LasR and LasI proteins regulate virulence factors in *Pseudomonas aeruginosa* (Fuqua et al, 1996).

The structure of acyl HSLs are usually conserved among various bacteria with differences occurring only in the length and degree of saturation in the acyl side chain (4-14 carbons), side chain substituents at 3-position, or the occurrence of double bonds (Teplitski et al, 2000). These signals are generated by an enzyme which uses S-adenosylmethionine and acyl carrier proteins as substrates (Moré et al, 1996). The same acyl HSL signaling molecule may be produced by several bacteria but they regulate the expression of different properties (Swift et al, 1996). Conversely, bacteria can produce multiple acyl HSLs that each have different phenotypic effects which may allow them to regulate genes based on their specific population (Swift et al, 1996). In the rhizosphere, there is also evidence that acyl HSLs produced by one

species may induce density-dependent responses in other species which may be significant in the formation of bacterial communities (Teplitski et al, 2000). Acyl HSL seem to have an important role in the actual development of biofilms. A study using a mutant strain of *P. aeruginosa* unable to produce autoinducers showed that they produced atypical biofilms without water channels or usual microcolonies as compared to a wild type biofilm (Davies et al, 1998). In *P. aeruginosa,* Las I mutants which are unable to produce 3-oxo-C12-HSL can form the characteristic microcolonies but biofilm development is stopped before maturation begins (Parsek and Greenberg, 2000). This result demonstrates that without the autoinducer present early biofilm colonization is normal with acyl HSLs only playing an important role when it is time for biofilm differentiation. It may be possible that free living cells do not have the means to recognize acyl HSLs because different phenotypic traits are being expressed in this state.

Some organisms, such as *P. aeruginosa*, also secrete cyclic dipeptides that act as acyl HSL mimic compounds (Holden et al, 1999). Some of these mimic substances act positively on bacterial colonization while some act negatively. Organisms are producing other signaling molecules that may play a role in biofilm formation.

Even though acyl HSLs are the most studied of the intercellular signalling molecules, other means of bacterial communication have been identified. For example, *Ralstonia solanacearum* uses a 3-hydroxypalmitic acid methyl ester, a non acyl HSL signal, in combination with acyl HSLs to allow pathogenicity (Holden et al, 1999). Gram-positive bacteria also regulate their behaviors based on population

density. In *Streptomyces* antibiotic production, morphogenesis, and sporulation are controlled by diffusible quorum-sensing molecules, γ-butyrolactones , that are structurally similar to acyl HSLs (Holden et al, 1999). Communication within biofilms may also include certain extracellular signaling molecules that influence physical contact, gene expression, competition and production of antimicrobial products (Davey and O'Toole, 2000).

Furanones

Because these processes are fundamental to the interaction of bacteria, it is not surprising that certain organisms have evolved strategies to interfere with bacterial signaling. Other intercellular signaling molecules such as cyclic dipeptides and quinolones have been identified (de Nys et. al., 1998). Such signals have been shown to cross-talk with acyl HSL signals or interfere with communication between bacteria (de Nys et. al., 1998). Acyl HSL regulated behaviors have been shown to be disrupted by halogenated furanones produced by the marine red alga *Delisea pulchra* (Givskov et. al., 1996). Furanones are structually similar to acyl HSLs and may bind competitively at acyl HSL receptor sites causing inhibition of the acyl HSL regulated behavior (Givskov et al, 1996). These acyl HSL mimics are proposed to have a role in the inhibition of bacterial colonization on marine organisms (de Nys et al, 1998). When furanones were added to *Serratia liquefaciens* swarm plates, swarming motility, which is an acyl HSL regulated process, was slowed or inhibited (Givskov et al, 1996). Non-acyl-HSL regulated phenotypes like swimming motility and production of flagella were unaffected (Givskov et al, 1996). Within biofilms some

members of the genus *Cytophaga* also produce an extracellular glycoprotein which inhibits adhesion to and gliding on a substrata (Burchard et al, 1998).

As these acyl HSL analogues have been shown to block adhesion of biofilms, is it possible that acyl HSLs might promote bacterial adhesion? Since quorum sensing is dependent on population density it would be expected that acyl HSLs do not participate in initial biofilm formation but only in biofilm differentiation (Davies et al, 1998). However, if acyl HSLs are present at the time of initial biofilm organization could biofilm formation be enhanced or increased?

Chemotaxis

It is now known that bacterial species use signals to communicate with each other. Attraction of bacteria into biofilms could possibly be seen as a form of chemotaxis. In natural environments, motility in prokaryotic organisms has evolved as a way to respond to temporal changes in the surrounding environment and migrate to the most favorable conditions. Swimming motility is advantageous for those organisms residing in aqueous environments, but in areas with low water content like biofilms, soil and microbial mats movement by gliding may be more feasible (Spormann, 1999). Swimming is described as a switch between two modes of active translocation, gliding and twitching (Spormann, 1999). Most motility is derived from the use of a flagellum, but all swimming bacteria are not flagellated (Spormann, 1999). This difference is exhibited in bacteria that are able to swarm in a few layers of water molecules which would be present in biofilms (Spormann, 1999). Many bacteria who

are defective in flagellum-mediated motility appear to be blocked in the initial stages of biofilm formation (O'Toole and Kolter, 1998).

Swarming motility is also reported to be controlled through acyl HSL signaling molecules. Swarming which is seen in *Chromobacterium, Proteus, Bacillus, Vibrio,* and recently in *Escherichia coli* is likely to play a role in bacterial virulence and colonization of submerged surfaces or biofilm formation (Eberl et al, 1996). Several studies have also reported that motility is required for both pathogenesis and biofilm formation on abiotic and biotic surfaces (Korber et al, 1994). Physical and chemical gradients are commonly encountered in nature, and motility enables the cell to respond positively by moving toward an attractant or negatively by moving away from a repellant. This directed movement in response to extracellular chemicals is referred to as chemotaxis which might be a possible mechanism to explain the attraction or recruitment of bacteria into biofilms.

In the absence of a gradient, the cell undergoes a random walk through the environment consisting of swimming in a straight line followed by frequent episodes of tumbling where the cell stops and reorients itself to swim in a new random direction. Both swimming and tumbling are accomplished by rotation of the flagellar motor and a chemotactic response depends on the relative probabilities of counterclockwise movement (swimming) or clockwise movement (tumbling) of the motor (Neidhardt, 1987). However, if a chemical gradient exists, the probabilities of swimming and tumbling are altered and the cells' motility now becomes biased towards an attractant or away from a repellant. When the concentration of the

attractant becomes higher, the swimming intervals become longer and the tumbling becomes less frequent.

Membrane sensory proteins, also known as chemoreceptors, sense these gradients and interact with cytoplasmic proteins that affect the movement of the flagella therefore determining the ratio of swimming to tumbling (Spiro et al, 1997). Transmembrane receptors or methyl-accepting chemotaxis proteins detect external stimuli and recognize them as attractants like amino acids, oligopeptides, and sugars or repellants like metal ions or extreme pH (Burkart et al, 1998).

Bacteria have signaling pathways that are used to detect and monitor their internal physiology and the external environment. In prokaryotes, intracellular signaling is dominated by a pathway consisting of a receptor, histidine kinase, and asparate kinase elements (Falke et al, 1997). These two-component systems control such diverse functions as cell division, antibiotic resistance, virulence, response to environmental stress, and taxis (Falke et al, 1997). The chemosensory system in *E. coli* and *Salmonella typhimurium* have been extensively studied to understand the molecular mechanisms of bacterial chemotaxis. Several genes in *E. coli* have been identified that are involved with the communication between the receptors and the motors to determine whether the cell will swim or tumble. These include several *che* genes that regulate rotation of the flagella and are necessary for proper chemotactic function (Neidhardt, 1987). In both *E. coli* and *S. typhimurium*, swarming is eradicated completely when there are mutations in all known *che* genes (Burkart et al, 1998). Similar genes may be present in other organisms that allow the cells to respond to signaling molecules.

Biofilm community analysis

Bacteria in the environment are not well characterized because it is difficult to obtain pure cultures that represent natural populations. Biofilms develop to form diverse communities containing several types of organisms that interact and coexist with each other. Traditional methods of cultivation have limited the ability to determine and compare microbial diversity within biofilm communities or natural samples. It is estimated that most bacteria (usually ≥95%) cannot be cultivated by traditional techniques (Amann et al, 1995).

Recently developed molecular techniques have allowed for the genetic characterization of the 16S rRNA genes that are present in all prokaryotic cells. These genes contain sufficient size and sequence variation which allows us to identify unculturable organisms, determine genetic diversity within specific communities, and infer phylogenetic relationships between dominant microorganisms (Muyzer et al, 1993). Many rRNA sequences have already been discovered and analyzed which have allowed microbiologists to sequence unknowns and compare them with a known sequence to identify the most similar species. Polymerase chain reaction (PCR) allows selective amplification of a specific gene of interest. In this study, sequencing of PCR products allowed a two way data analysis. Similarities between known rRNA sequences and our unknowns were compared and the phylogenetic linkages between various aquatic organisms were determined. Genetic fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) of PCR amplified 16S rDNA fragments can also be used for microbial community analysis (Muyzer et al, 1993).

In DGGE, DNA fragments with different sequences are separated despite shared lengths. During PCR a high temperature melting GC-clamp needs to be added to one of the primers to allow for almost 100% of all base changes to be detected and to keep the double stranded DNA from becoming completely denatured (Sheffield et al, 1989).

Separation is accomplished on a linear gradient of denaturants that includes both urea and formamide. DNA fragments melt in discrete melting domains, and as the lowest melting temperature is reached double stranded molecules become partially melted and branched resulting in decreased mobility in the gel. Complete melting is prevented by the presence of the aforementioned GC-clamp added during PCR. Fragments which differ by as little as a single-base substitution in the lower temperature domains will melt at slightly different denaturant concentrations causing the fragments to separate from each other (Sheffield et al, 1989). This separation allows determination of sequence variation and therefore differences within the biofilm communities.

DGGE is not without limitations, and there are several assumptions that should not be made. The intensity of the bands within the gel does not necessarily refer to the predominance of that organism in the community (Gillan et al, 1998). The number of bands within the gel also does not necessarily correlate with the number of individuals within a population due to heterogeneity of 16S rRNA which may be present in multiple copies (Dahllöf et al, 2000). Only after each individual band is excised, reamplified, and sequenced can the identity and number of organisms within your community be determined. Another possibility is that bands at identical

positions in a gel are not necessarily derived from the same species but potential error source can be improved by using narrower denaturing gradients to provide higher resolution of the banding patterns (Muyzer et al, 1993). Despite such limitations DGGE remains highly sensitive because a species that composes less than 1% of a total mixture can be detected (Muyzer et al, 1993).

As acyl HSL analogues have been shown to block adhesion of biofilms, the purpose of this study was to determine if acyl HSLs would promote bacterial adhesion and if differences exist between acyl HSL signal molecules. My hypothesis is that dialysis tubing containing the signaling molecules produced by *A. tumefaciens* and *C. violaceum* would attract greater biofilm populations than the bacterial and non-bacterial controls.

To determine whether these signaling molecules attract biofilms, acyl HSLs were isolated from two strains of *A. tumefaciens* which act as 3-oxo-C8-HSL overproducers and one strain of *C. violaceum* that produces C6-HSLs. Non acyl HSL producing strains of these species were used as controls (Table 1).

The goals of this research were to determine whether acyl HSLs could be used to recruit or attract biofilm populations and to gain a further understanding of the biology of biofilms. Since different bacterial species produce various acyl HSLs, it was also determined by DGGE and bacterial identification if different signaling molecules might attract different bacterial populations or if each autoinducer is species-specific. I predicted that the composition of aquatic biofilm communities can be altered in response to the presence of individual acyl HSLs, and that these changes can be detected by DGGE.

This research is significant in areas such as water recycling or in processes where specific bacterial populations could be attracted to outcompete harmful bacterial populations. In newborn animals, biofilms may serve as beneficial "barrier" populations that may prevent susceptibility to bacterial infections (Costerton et al, 1981). Since biofilms cause problems in medicine and industry, a further understanding of signaling within biofilms might help combat some of the trouble they cause in these fields. Biofilm bacteria have been found to be up to 1,000-fold more resistant to antibiotics than their planktonic counterparts (Davey and O'Toole, 2000). Because of their increasing resistance to antibiotics and antimicrobials, biofilms in medical environments are difficult if not impossible to eradicate.

MATERIALS AND METHODS

Strains and growth conditions. Strains are listed in Table 1. *A. tumefaciens* KYC6 was used as an endogenous 3-oxo-C8-HSL overproducer. *A. tumefaciens* A136 was used as an indicator strain for exogenous AHL autoinducers in cross feeding assays. *A. tumefaciens* R10 (pcF218) was used as a 3-oxo-C8-HSL overproducer. *A. tumefaciens* WCF47 (pcF218) was used because it has no 3-oxo-C8-HSL production. *C. violaceum* ATCC 31532 was used as an overproducer of C6-HSLs). *C. violaceum* 026, which is a mini-Tn5 mutant of ATCC 31532, was used because it has no C6-HSL production (see table 1 for strain designations). Previously prepared stocks of *E. coli* DS291, *P. aeruginosa* 10145, and *C. violaceum* 31532 PCR products were used as DGGE standards. For long term storage, cultures were suspended in a mixture of Luria Bertani (LB) broth, glycerol and appropriate antibiotics and frozen at -80°C. Prior to use the frozen cultures were incubated on LB agar (strains KYC6, 31532, and CV026), LB agar supplemented with tetracycline (strains R10 and WCF47), or LB agar supplemented with tetracycline and spectinomycin (strain A136). Tetracycline was used at a concentration of 4.5μg/ml and spectinomycin was used at a concentration of 50μg/ml. All cultures were incubated at 30°C. For baiting experiment cultures were grown in 20 mls of LB broth with shaking.

Cross feeding assay for AHL detection. For cross feeding assays, ATGN or LB medium was covered with 50 μg of X-gal solution (20mg/ml 5-Bromo-4-chloro-3-indolyl-β-D-galactoside in dimethyl formamide). The AHL reporter strain, *A. tumefaciens* A136, was streaked on the plate and the culture or supernatant to be tested was streaked 1cm away. If AHLs are present they will diffuse through the agar and activate the *tra*I-*lac*Z fusion in the reporter strain (McLean et al, 1997). All 6 strains were tested for AHL production.

Sample collection and processing. Cultures were grown in 20 ml LB broth in 30°C incubator with shaking for 25 hours along with an uninoculated broth tube. The broth was transferred to centrifuge tubes and the extracellular material was removed by centrifugation at 2400 rpm for 5 minutes. 9 mls of supernatant containing a live inoculum was then pipetted into a sterile 4-inch piece of semipermeable dialysis tubing (Spectra/Por, MWCO 1000, 24mm diameter) which was clamped at both ends and placed in sterile water to be taken to the San Marcos River. Two controls consisting of a piece of tubing with no supernatant and tubing containing 9 mls of river water were prepared at the river. Each tube was placed in the river and left for various intervals (1, 5, 18 or 24 hours). After this time, the tubes were placed in separate sterile water carriers and transported back to the lab where they were cut into 1 inch pieces, placed into scintillation vials containing 10ml sterile water, and shaken ten times to remove any planktonic bacteria. The piece of tubing was then transferred to another scintillation vial containing 10mls of sterile water and was sonicated for 8 minutes to remove any adherent bacteria. Serial dilutions of the sonicate were performed to determine the amount of colonization on each tube. After incubation

individual colonies were transferred to R_2A plates for identification purposes by Biolog or fatty acid analysis (Microcheck, Northfield, VT). The sonicate and 10^{-1} dilution plates were used for DGGE to determine community profiles as described below. Comparison of biofilm populations (CFU/6.45 cm^2) was analyzed with a one-way ANOVA.

Microscopy. To verify bacterial attachment, the dialysis tubing was observed using scanning confocal laser microscopy (SCLM). The tubing was stained using Live/Dead Baclight viability stain (Adams and McLean, 1999).

DNA Extraction. DNA for sequencing was extracted by using a modification of the Gillan freeze-thaw method (Gillan et al, 1998). When using pure cultures, a loopful of each was suspended in 15µl of TE buffer in a 1.5ml microfuge tube. Physical disruption of the cells was accomplished with ten freeze-thaw cycles of 20 seconds in liquid nitrogen (-196°C) followed by 5 minutes in an 80°C water bath. Each tube was centrifuged for 30 seconds and then stored at -20°C until PCR. For mixed cultures, DNA extraction was performed with the Bactozol™ Kit for DNA isolation. Sonicates were centrifuged to form a pellet which was suspended in TE buffer. Cells were then lysed with Bactozyme which contains activated lysozyme and DNA was isolated from lysate with DNAzol™. After lysis, a 5µl portion was used directly as template for PCR. When using bacteria from dilution plates the entire 10^{-1} plate was swabbed and bacteria was placed into a 1.5 ml microfuge tube containing 1ml of TE buffer. The tubes were centrifuged and the protocol for chemical lysis was followed the same as above.

PCR Amplification. For PCR amplification of 16s rRNA we used the primers GC357F containing a GC-clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3') (Gillan et al, 1998). GC357F corresponds to positions 341-357 of the 16s rRNA of *E. coli* and 518R corresponds to positions 518-534 (Gillan et al, 1998). Reaction mixtures containing 5µl template DNA, each primer at a concentration of 0.5µm, each deoxynucleoside triphosphate at a concentration of 200µm, 3.0 mM $MgCl_2$, 10µl if 10X Buffer II, and 2.5 units of Ampli Taq GOLD Polymerase were adjusted to a final volume of 100µl using 59.5µl of sterile water.

The tubes were first incubated for 10 min at 95°C, followed by 1 cycle of denaturation at 94°C for 15 sec, annealing at 66°C for 30 sec, and primer extension at 72°C for 30 sec. A touchdown PCR was then performed by using 18 cycles consisting of denaturation at 94°C, annealing at 66°C (the temperature was decreased by 0.5°C every cycle until the touchdown temperature of 56°C was reached) for 30 sec, and primer extension at 72°C for 30 sec. The next step was to perform ten cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec , and primer extension at 72°C for 30 sec. The final cycle was 72°C for 5 min. The temperature was then held at 4°C until samples were removed. 2-10µl of amplified products were run on a 4% agarose gel and analyzed after ethidium bromide staining. When using mixed cultures, the cycling parameters were modified to 31 cycles of touchdown PCR to increase the yield of DNA for DGGE. PCR reactions were performed with a Perkin Elmer GeneAmp® PCR System 2400.

Sequencing methods. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) or Life Technologies Rapid PCR purification kit (GIBCO/BRL). We used the ABI Prism Cycle Sequencing kit from PE Applied Biosystems and ABI Prism 377 DNA sequencer. Sequencing reactions were set up using both directions with forward (GC357F) and reverse (518R) primers. Each 10µl reaction mixture contained 1µl of purified PCR product, 4µl of Terminator Ready reaction mixture (contains A,C,G,T-dye terminator; dNTPs; Amplitaq DNA polymerase,FS; $MgCl_2$; Tris-HCL buffer), 0.5µl of primer, and 4.5µl of sterile water. For cycle sequencing the reaction mixtures underwent 30 cycles of: 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. The temperature was held at 4°C until use. The extension products were purified with G-50 Sephadex filtration columns and dried in a vacuum centrifuge and placed at -80°C until use. 3.5µl of loading buffer (5:1 deionized formamide and EDTA with blue dextran) was added to the dried sample and 1.5µl was loaded on a 5% Long Ranger polyacrylamide gel and electrophoresed for 7 hours at 1640V.

Sequence analysis. The sequences obtained were compared to known nucleotide sequences in the National Center for Biotechnology Information (NCBI) database by using a BLAST search. All sequences were aligned in Sequencher and phylogenetic trees were constructed using PAUP 4.0 B2 (Swofford 2000). The 16s rRNA sequences of the following organisms were used for comparison with our unknown samples. They were obtained from GenBank and each nucleotide accession number is in parentheses: α-Proteobacteria: *Agrobacterium tume*faciens (AJ012209), *Sphingomonas adhaesiva* (D84527), *Sphingomonas flava* (UG0173); β-

Proteobacteria: *Bulkholderia cepacia* (AF311972), *Chromobacterium violaceum* (M22510); γ-Proteobacteria: *Acinetobacter johnsonii* (AF188300), *Aeromonas hydrophila* (AB034762), *Pseudoaltermonas* sp. F13 (AF316144), *Pseudomonas fluorescens* ATCC 17573 (AF094730), *Pseudomonas syringae* (AF105390), *Vibrio* sp. (AF218244), *Xanthamonas campestris* (AF290420); Cytophaga-Flexibacter-Bacteroides (CFB): *Cytophaga johnsonae* (MS9051), *Flexibacter flexilis* (M62794); Actinobacteria: *Micrococcus lylae* (AF057290).

DGGE analysis of PCR products. PCR products obtained with primers GC357F and 518R were analyzed by DGGE. 8% acrylamide gels were prepared and run with 0.5 x TAE buffer using the Bio-Rad Dcode system. DGGE gels containing a 30-50% denaturing gradient of urea and formamide which increased in the direction of electrophoresis were prepared with a gradient former. DGGE was run at a constant voltage of 130V at 60°C for 3 hr 30 min. The gels were stained with ethidium bromide (15 min) or SYBR gold nucleic acid stain (15-30 min) and photographed under UV transillumination (UVP, Model TFM-26).

RESULTS

Quantitative studies. During the course of this study, several changes evolved in the experimental protocol as well as technique development. Initially, dialysis tubing with a MWCO of 10,000 was used (Fig. 3-6) but it was decided that the signaling molecules were diffusing out too rapidly. The protocol was modified to MWCO of 1,000 for later experiments (Fig. 7-10). In figures 3-6, broth cultures were grown in flasks for 20 hours and autoclaved supernatants were used which kill the bacteria but retain the acyl HSL activity (McLean et al, 1997). For later experiments (Fig. 7-10), live cultures were used that would constantly produce acyl HSLs, but took this into consideration when enumerating bacteria from the tubing.

Recruitment of bacteria into biofilms was also investigated at various time intervals. Allowing for one hour of colonization permitted very few bacteria to adhere to the tubing. After 18 and 24 hours, there was substantially more growth but after this length of time, attracted bacteria would be dividing and producing their own signaling molecules which would interfere with the acyl HSLs that were initially present. The 5 hour time period was the most extensively sampled because it allowed for substantial attachment without the effect of replication and signaling from outside organisms. Since we were using suspensions containing live organisms, we wanted to make sure that the organisms inside the tubing were not contributing to the

increases in bacterial numbers seen on the dilution plates. The inoculum was determined to contain between 10^8 and 10^9 organisms, which is a sufficient number for acyl HSL production. Before being placed in the river, the suspension was determined to contain 10^6 organisms and after it had been in the river for 5 hours contained between 10^6 and10^7 organisms, indicating some slow growth. Dilutions were then performed on the sonicate from scintillation vial containing the removed planktonic bacteria which also contained 10^6 organisms. So although we were enumerating organisms from inside the tubing their numbers (about 10%) did not contribute significantly to the overall plate counts.

Bacterial attachment to the dialysis tubing was shown with SCLM. Although no significant differences were seen between the amount of attachment to tubes with *A. tumefaciens* R10 and *A. tumefaciens* WCF47, there is clearly less attachment to the control tubing after 2.5 hours in the river (Fig. 11). In figure 12 digital images of dilution plates show that acyl HSL producing strains had more growth than non-acyl HSL producing strains and the controls. The *A. tumefaciens* R10 plate (A) has hundreds of tiny colonies on the plate that cannot be seen in this picture. To give a visual representation of bacterial diversity between the different experimental conditions, digital images were taken of two dilution plates (Fig. 13). The *A. tumefaciens* A136 plate indicates one colony type (orange) while the *C. violaceum* 31532 plate shows 3 distinct colony types: white, orange and purple.

Determining microbial diversity. Since variation in colony morphology was seen between the dilution plates, we wanted to determine if specific organisms were being attracted to different acyl HSLs or if attachment was random. Representative

organisms from various dilution plates (5 hour experiment) that could be isolated in pure culture were identified by various methods. BIOLOG identification was first used to determine common or predominate organisms within these communities (Table 2). There were problems with this method because organisms in the river come from an oligotrophic environment and the media used for identification must be supplemented with sheep blood which was not suitable growth media for most of these organisms. This method did find a predominate organism, *Flavobacterium* sp., which was found on most of the plates (Table 2). Two pure samples which could not be identified with BIOLOG were sent to Microcheck where they were identified by fatty acid analysis (Table 2). All other organisms which could be isolated into pure culture were characterized by DNA sequencing and compared with known 16S rRNA sequences in the NCBI database (Table 3). Unknown C could not be aligned with the other sequences so was not used in subsequent analysis. Phylogenetic trees were constructed in PAUP by the neighbor-joining method rooting the tree with *A. tumefaciens* and *Micrococcus lylae* as outgroups (Fig. 17) as well as rooting at the midpoint (Fig. 18). Figure 19 shows percentage of 1000 bootstrap replicates that support the branching order shown.

DGGE analysis. To determine optimal DGGE conditions for characterization of 16S rRNA fragments from aquatic microbial populations, parallel DGGE with a wide range of denaturants (20-70%) was employed. Using standards (*E. coli* DS291 and *P. aeruginosa* 10145), we found that all the bands were concentrated in an area around the center of the gel. Therefore, we narrowed the gradient to 30-50% for better resolution. Although the denaturing concentration was optimal, we did not observe

the expected banding patterns, seeing only one or two bands per lane with ethidium bromide staining. Since it is possible that DNA extraction by physical means was not giving us an accurate representation of the populations present, we changed to a chemical method of DNA extraction (Bactozol™ kit), but still saw very few bands. These same samples were tested and stained with SYBR gold nucleic acid gel stain which is >10-fold more sensitive than ethidium bromide. These gels revealed the banding patterns seen in figures 20-23. Figures 20 and 21 show the differences in diversity between the tubes containing bacteria and the non-bacterial controls. Arrows in figure 20 highlight the differences in banding patterns between the two groups. From these two replicates, it is obvious that those tubes containing bacteria have a more limited diversity than the nonbacterial control tubes. The DNA from the sonicates and plates were taken from experiments performed on different days, and show different banding patterns. These results showed us that different populations were attaching on different days. Figures 22 and 23 were also performed on a different day but shows the difference between extracting the DNA from sonicates or from plates. Different banding patterns are seen for each showing that a combination of both physical and chemical disruption may be needed to get a representative population profile. It has been shown that a combination of both treatments such as lysis with detergents, bead beating, and freeze-thaw will lyse approximately ninety-six percent of cells including endospores (Head et al, 1998).

A preliminary chemotactic assay (modified version of Weerasuriya et. al., 1998) for C6 and 3-oxo-C8 acyl HSLs was performed using R_2A swarm plates (0.325%

agar) but the *Agrobacterium* and *Chromobacterium* used did not seem to be attracted to it.

DISCUSSION

Biofilm formation is initiated when planktonic bacteria respond to appropriate environmental signals which in some way triggers the transition from the planktonic state of growth to life as an adherent community. Although the cues that cause this transition have not been identified, surface colonization of prokaryotes seems to involve a characteristic assemblage of organisms that are very dynamic (Dang and Lovell, 2000).

It has become clear that many important prokaryotic behaviors are regulated through a population density dependent manner. Although acyl HSLs are produced when bacteria reach a sufficient cell density ("bacterial quorum") and would not be present during the initial stages of biofilm development, we hypothesized that the presence of acyl HSLs in the beginning of biofilm formation would cause increased attachment to tubing containing these molecules. We found that the tubing containing acyl HSLs did not attract significantly more growth than the non-acyl HSL containing tubes in almost all replicates. After one hour though there was significantly more growth on the tubing containing supernatant from *A. tumefaciens* overproducing strains as compared to the *A. tumefaciens* strains that do not produce 3-oxo-C8-HSL. This result suggests that this specific signaling molecule may have an effect only in the very early stages of biofilm formation. The effects of the provided acyl HSLs

decrease over time which would be expected since after several hours in the river the attracted organisms would begin to reproduce and also may produce their own signaling molecules, possibly interfering with the original signaling molecule. We did find in each replicate that tubing containing bacteria had significantly more growth than tubing containing river water and tubing without supernatant. The broth control had consistently higher growth than the other two non-bacterial controls which may be due to the fact that it contains tryptone, yeast extracts, and sodium chloride which are potential nutrient sources for aquatic organisms. Having living organisms inside the tubing significantly changed the surrounding environment and in some way limited the diversity of the colonizers. Although acyl HSLs were not involved in early colonization processes, other signals might play a role. Other intercellular signals such as secreted proteins, bacterial metabolites, genetic material, and undiscovered bacterial products which diffuse away from one cell and into another are important in surface colonization (Watnick and Kolter, 2000). They predict that signaling, whether beneficial or detrimental, is a critical factor in distribution and diversity of bacteria in biofilms. Our study showed a greater diversity of organisms attaching to the non-bacterial controls than the tubes containing bacteria-both acyl HSL producing and non-acyl HSL producing strains, suggesting acyl HSL inhibition.

In natural environments, biofilms are consistently highly complex multispecies communities consisting of bacteria that compete and communicate with each other. Quantification and analysis of community diversity are fundamental for examination of both succession and colonization of surfaces. Through amplification and analysis

of 16S rRNA gene segments, we were able to obtain a profile of the microbial communities that adhered to the dialysis tubing. DGGE banding patterns are being used by correlating the number of bands with different environmental factors or by tracing changes in the structure of the community with changes in environmental conditions (Dahllöf et al, 2000). Although individual bands were not identified the banding patterns yielded an overall view of bacterial diversity between the various experimental conditions allowing similarities and differences to be observed. By using a narrow denaturing gradient (30-50%) and highly sensitive nucleic acid stains (SYBR gold) we were able to discern discrete banding patterns within each adherent population that varied between replications, showing that the same organisms do not consistently attach from day to day. DGGE profiles showed that most populations were unique. with the most diversity seen from the three non-bacterial control tubes. There were also common bands between biofilms that may represent common biofilm bacteria or may represent a pioneer species that is needed to prepare the surface for attachment of other organisms. This may be the case with a *Flavobacterium* species that was identified from most of the tubes.

F. johnsoniae (formerly *Cytophaga johnsonae*) is a common aquatic bacterium which is among the fastest of the gliding bacteria (Hunnicutt and McBride, 2000). Because gliding bacteria require temporary adhesion for their motility machinery to work, it is very likely for these organisms to be members of biofilms (Burchard et al, 1998). The *Flavobacterium* sp. and *S. mizutaii* identified with Biolog are all members of the *Cytophaga-Flexibacter-Bacteroides* (CFB) group. *Porphyromonas*, an initial colonizer of dental surfaces, is also a member of this group that may show

some correlation between primary colonization and this group of organisms. Results from several methods of bacterial identification showed a somewhat specialized bacterial community that was dominated by a few related clusters of α and β-*Proteobacteria* as well as *Cytophaga/Flavobacterium*. From phylogenetic analysis of the unknown sequences, *Flavobacterium* sp. was the only organism which could be classified into a known group. The other nine sequences formed two distinct clades with one clade related to *Sphingomonas* and the other clade related to *Bulkholderia cepacia* (also known as *Pseudomonas* and *Ralstonia*). These two distinct monophyletic groups may have specific characteristics that allow them to successfully colonize surfaces such as gliding motility with the CFB group. *Pseudomonas* is commonly found in both soil and aquatic environments and is frequently found in naturally occurring biofilms (Parsek and Greenberg, 2000). *Sphingomonas* is also a commonly found aquatic biofilm organism that is well known for its ability to degrade environmentally hazardous compounds. In many well confined microenvironments, sets of phylogenetically related communities are commonly encountered which may reflect adaptation to selection or evolutionary speciation (Ferris et al, 1996; Gillan et al, 1998; Casamayor et al, 2000). It is likely that these two populations contain novel organisms that are indigenous to the San Marcos River which have yet to be characterized. These organisms may also have not been placed in a group because a closely related group was not chosen for comparison.

In this study, it was evident that acyl HSLs do not increase or enhance biofilm formation after 5, 18 and 24 hour intervals. Of over 20 replicates performed, those

performed at the 1 hour interval with an unautoclaved supernatant were the only ones to show that acyl HSLs have an any effect on initial biofilm formation. For future studies it will be necessary to study this time period more extensively as well as hourly intervals up to 5 hours. Denaturing gels can be used to show changes in colonization patterns over these time intervals. Using purified acyl HSLs in the dialysis tubing would be useful to rule out any other signaling molecules or bacterial products which may interfere with our signaling molecules. Different acyl HSL regulated genes are activated at different acyl HSL concentrations and using purified autoinducers would allow you to control the concentration of signaling molecules being released. We only used two acyl HSL molecules (C6 and 3-oxo-C8), but it would be interesting to see if acyl HSLs from other organisms would have an effect in the early stages of biofilm formation. Some acyl HSL regulated genes require only one type of signal where others require more. N-3-(oxododecanyoyl)-L-homoserine lactone produced by *P. aeruginosa,* which is also a commonly occurring aquatic organism, could be used in future studies. This organisms' quorum sensing system has been well studied, and it has a chemotaxis system very similar to *E. coli.* Certain signals may be more recognizable to specific organisms and have more of an effect on them during biofilm formation.

Future community studies using DGGE and further characterization of the composition and diversity within these communities will require all individual bands to be excised, reamplified, and sequenced. Since band intensity does not directly correspond to the abundance of that organism in the population, methods like *in situ*

hybridization will be needed to determine the predominance of an organism in specific populations (Gillan et al, 1998).

Recent studies with *P. aeruginosa* have shown that chemotaxis is not required within biofilms because nutrients are trapped within the extracellular polysaccharide matrix where they are freely available to all the cells (Déziel et al, 2001). Chemotaxis may not be needed within the mature biofilm, but motility is used when cells leave a community to colonize another surface. In a preliminary chemotactic assay, cells did not appear to be attracted to acyl HSLs. The concentration could have been to low for detection or it could be that bacteria do not respond to these signaling molecules as an attractant. Also since nonmotile bacteria are able to form biofilms other genes besides motility must be involved in biofilm formation suggesting chemotaxis is not the only important mechanism for biofilm formation. From this study, using acyl HSLs as bait for recruiting in other bacteria, I have concluded that acyl HSLs may act as an attractant for some organisms but other signaling molecules and nutrients in the environment may have a more important role in initial biofilm formation.

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Table 1. Genetic designations of bacterial strains used in study.

Organism	Strain	Phenotype/Genotype
Agrobacterium tumefaciens[a]	KYC6 (pcf218)	3-oxo-C8-HSL overproducer/ ▪octapine chromosomal background ▪tra M null mutant plus traR overexpression plasmid
Agrobacterium tumefaciens[a]	A136	no 3-oxo-C8-HSL production/ ▪nopaline chromosomal background ▪no Ti plasmid
Agrobacterium tumefaciens[b]	R10 (pcf218)	3-oxo-C8-HSL overproducer/ ▪octopine chromosomal background ▪traR overexpression plasmid
Agrobacterium tumefaciens[b]	WCF47 (pcf218)	no 3-oxo-C8 HSL production/ ▪octapine chromosomal background ▪R10 with in-frame deletion of traI
Chromobacterium violaceum[c]	ATCC 31532	C6-HSL overproducer
Chromobacterium violaceum[c]	CVO26	no C6-HSL production/ ▪mini Tn5 mutant of ATCC 31532

[a] described by Fuqua et al, 1995

[b] W. C. Fuqua, Indiana University, Bloomington, IN

[c] described by Throup et al, 1995

Cultures grown for 25 hours at 30°C

↓

9mls supernatant (live inoculum) into dialysis tubing

↓

Tubing placed into river for 1, 5, 18 or 24 hours

↘

Scannning confocal laser microscopy

↓

Sonicate tubing to remove adherent bacteria

↙ ↓

DNA extraction — Serial dilutions

↙ ↙ ↓

Polymerase chain reaction — Biolog/ fatty acid analysis — DNA extraction

↙ ↓

Denaturing gradient gel electrophoresis — Polymerase chain reaction

↙ ↓

Sequencing — Denaturing gradient gel electrophoresis

↙ ↓

Phylogenetic analysis — Community profiles

Figure 1. Schematic of acyl HSL baiting experiment.

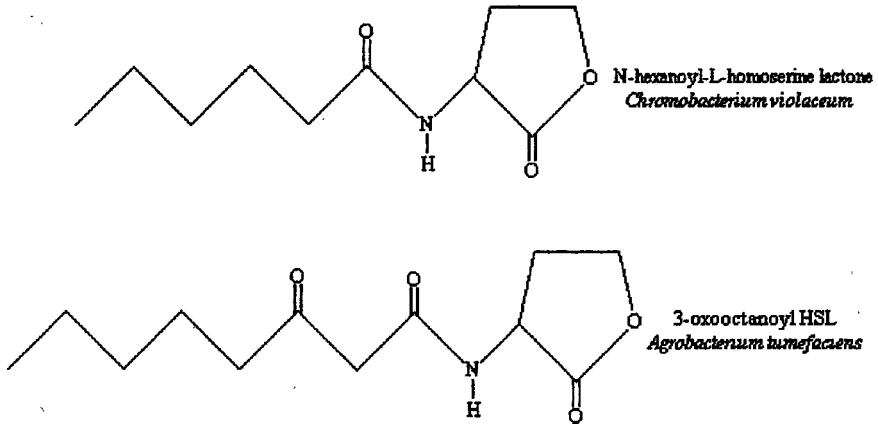

Figure 2. Structure of acyl HSLs used in this study.

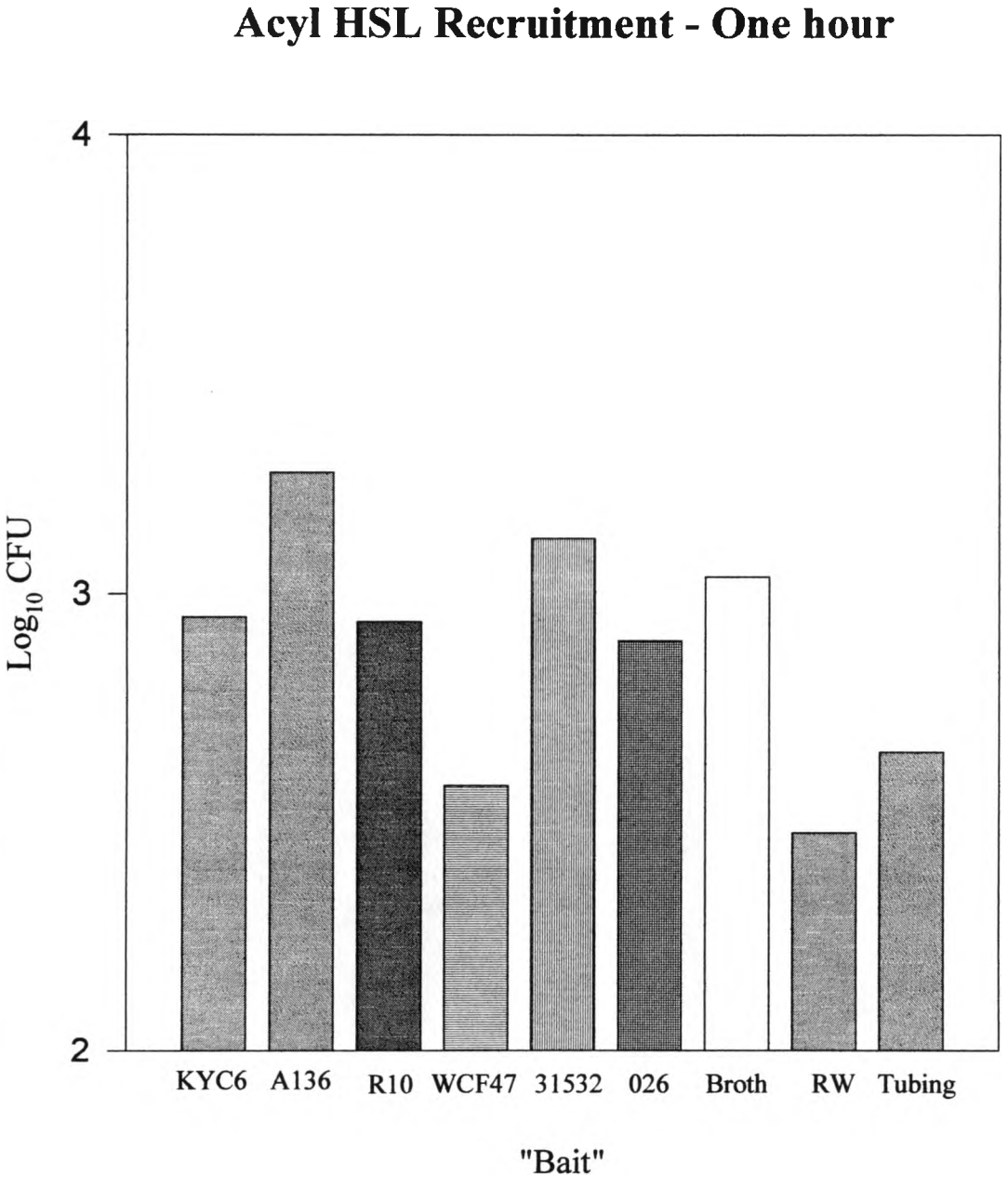

Figure 3. Graph showing effects of acyl HSLs on bacterial attachment to dialysis tubing in the San Marcos River after 1 hour. Supernatant was an autoclaved inoculum. See Table 1 for strain designations. RW corresponds to river water control.

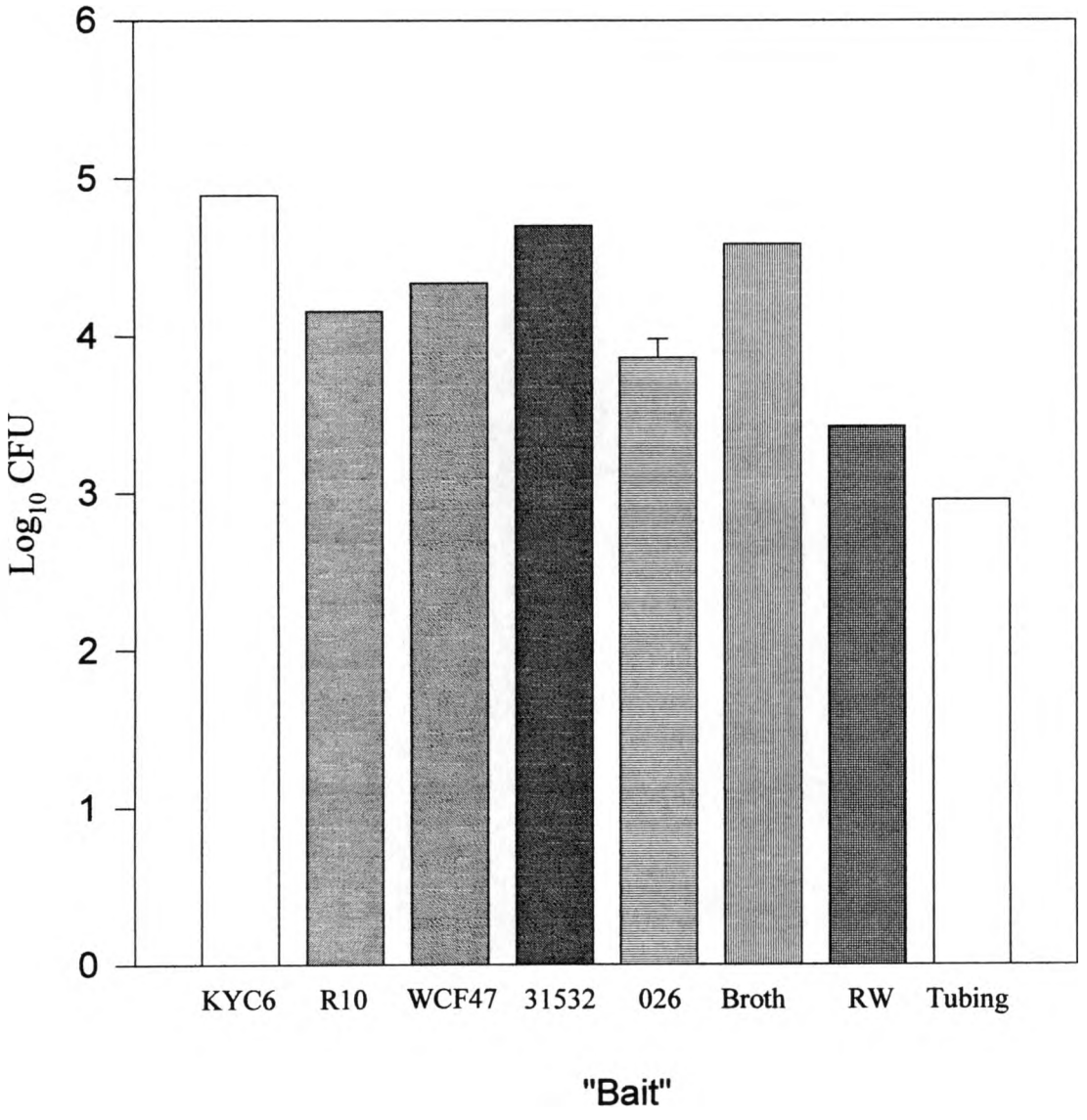

Figure 4. Graph showing effects of acyl HSLs on bacterial attachment in the San Marcos River after 5 hours. Supernatant was an autoclaved inoculum from spent medium from each of these strains.

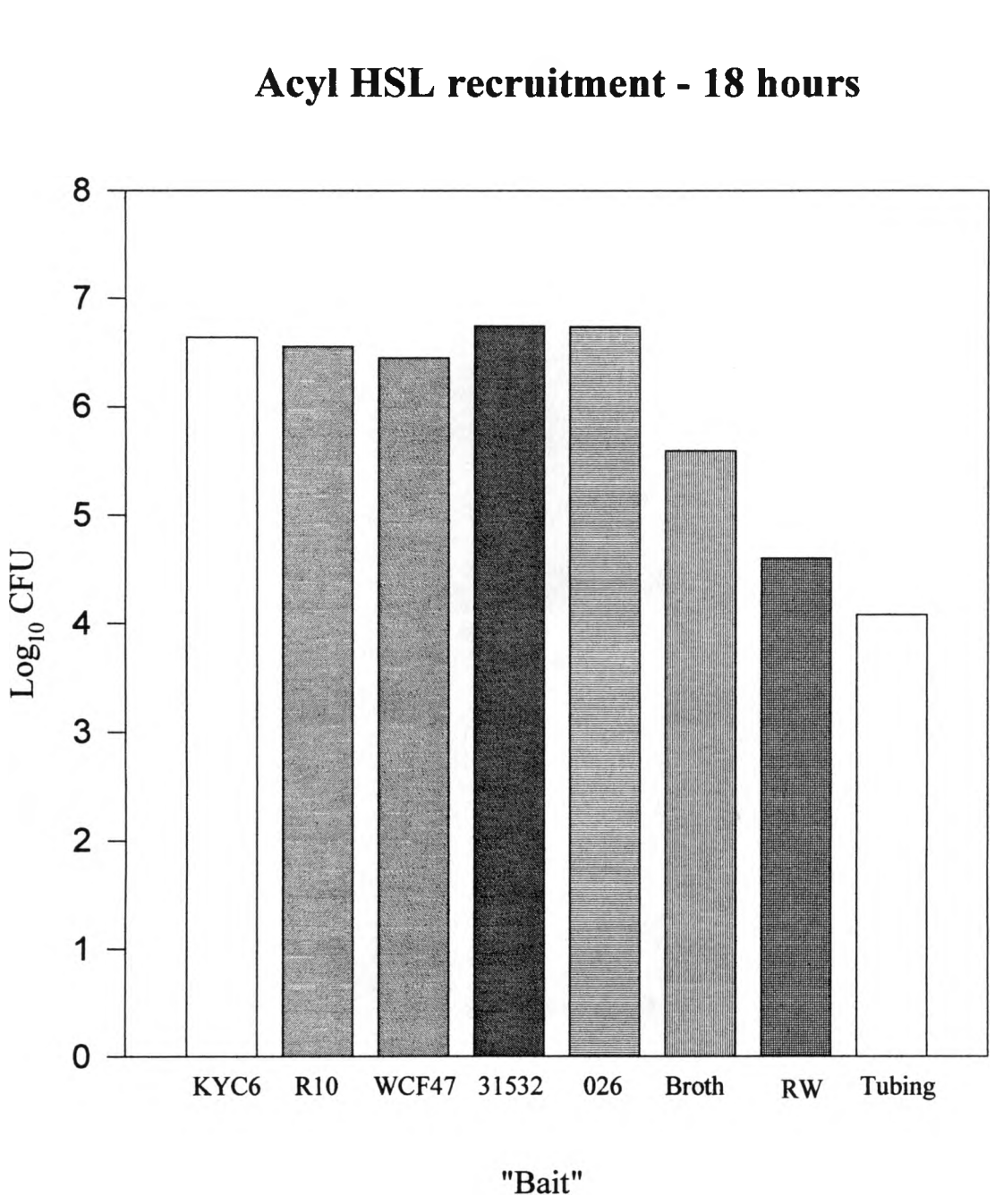

Figure 5. Graph showing effects of acyl HSLs on bacterial attachment in the San Marcos River after 18 hours. Supernatant was an autoclaved inoculum.

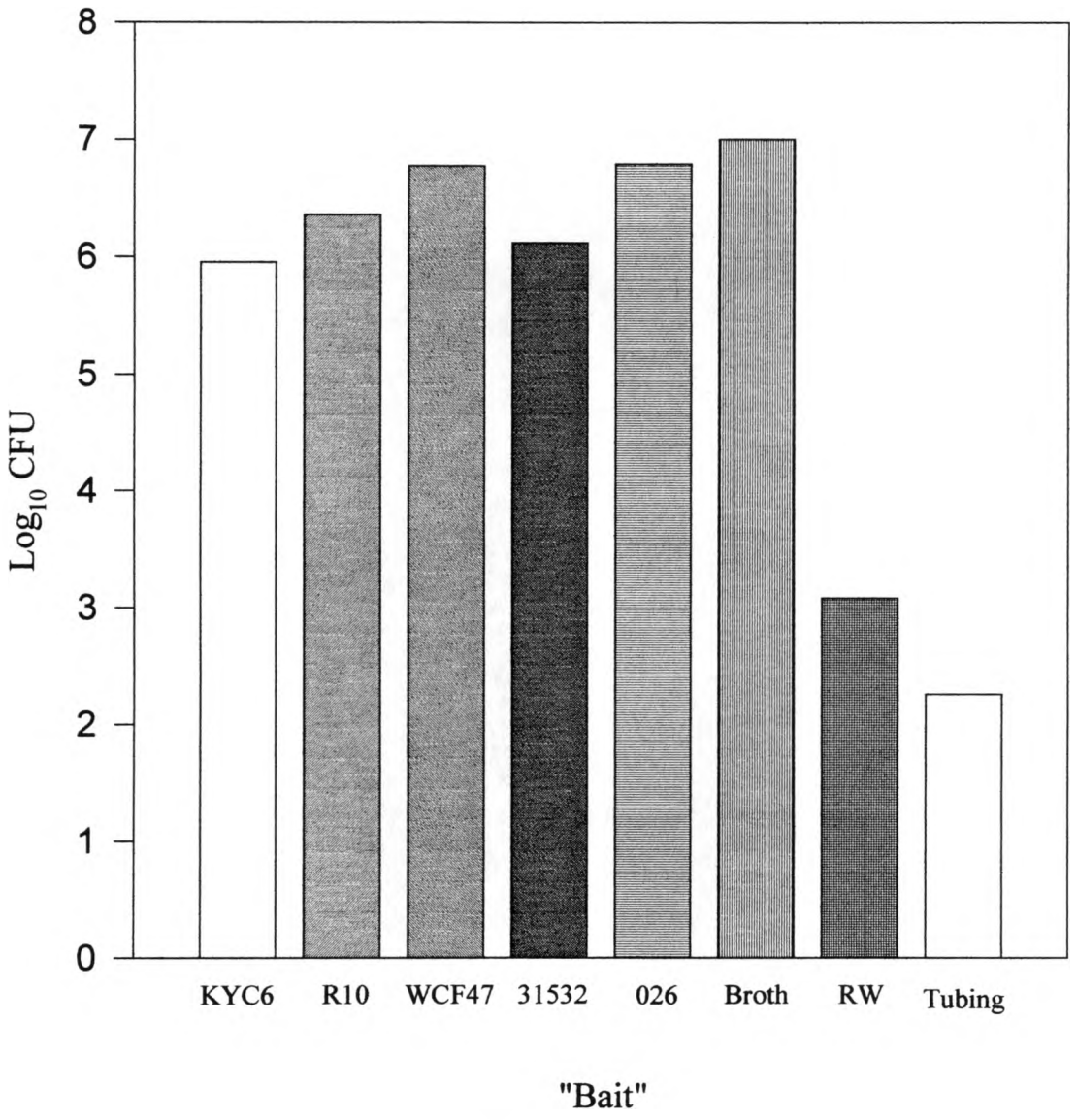

Figure 6. Graph showing the effects of acyl HSLs on bacterial attachment in the San Marcos River after 24 hours. Supernatant was an autoclaved inoculum.

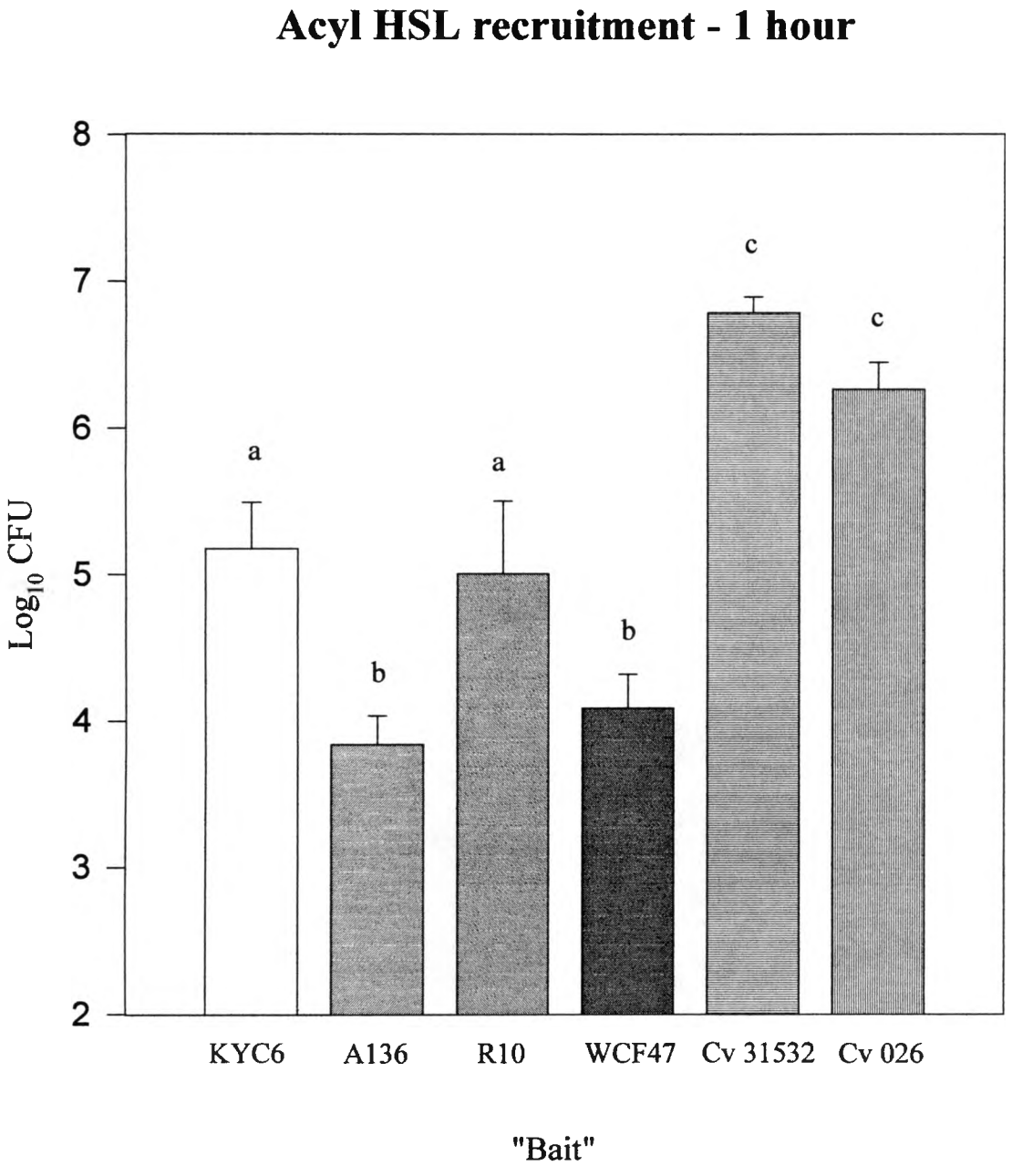

Figure 7. Graph showing effects of acyl HSLs on bacterial attachment to dialysis tubing in the San Marcos River after 1 hour. Error bars represent standard deviation. Values with the same letter do not differ significantly at P=0.05. Supernatant was not autoclaved.

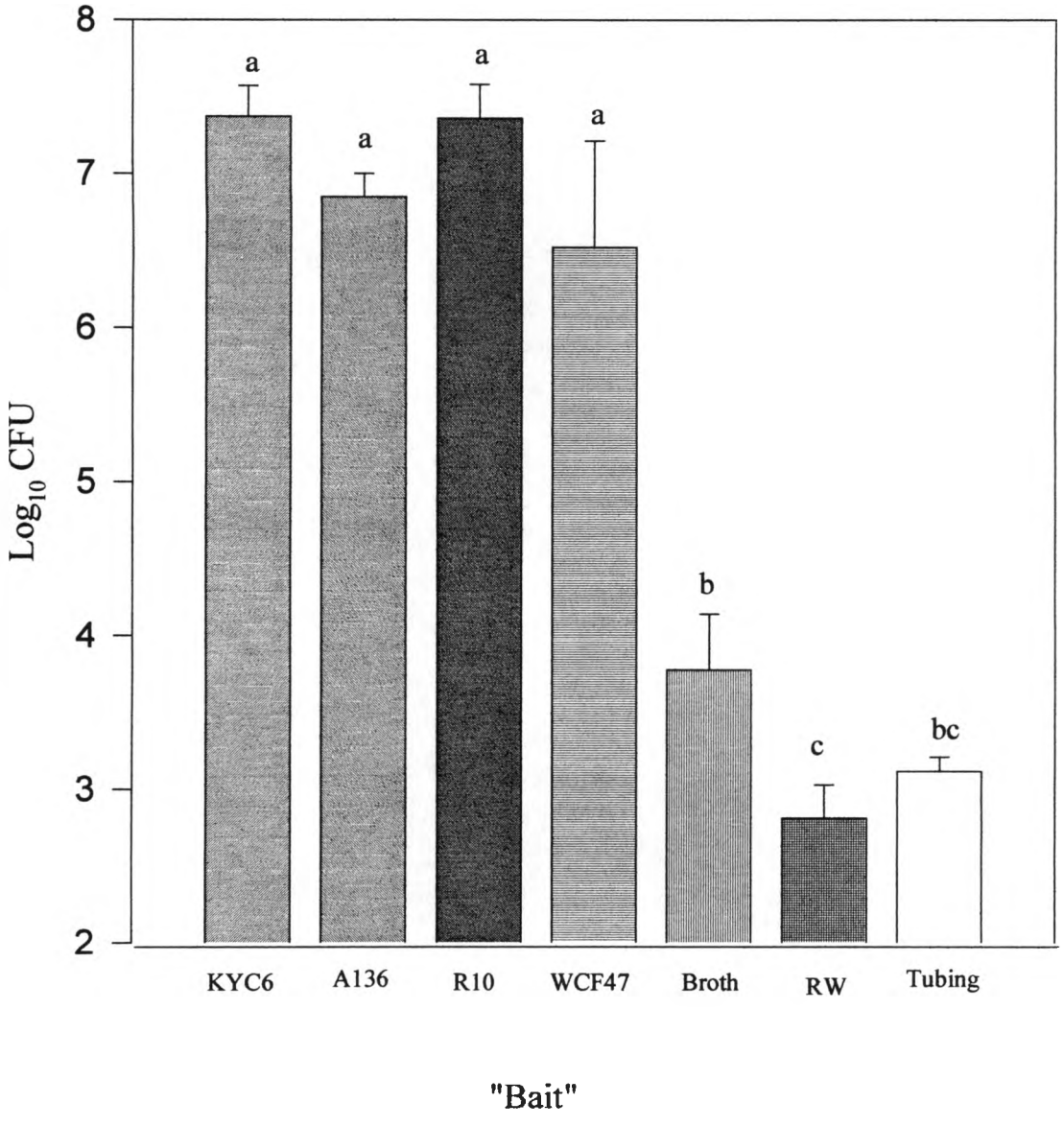

Figure 8. Graph showing effects of 3-oxo-C8-HSLs on bacterial attachment to dialysis tubing in the San Marcos River after 5 hours. Error bars represent standard deviation. Values with the same letters do not differ significantly at P=0.05. Supernatant was not autoclaved.

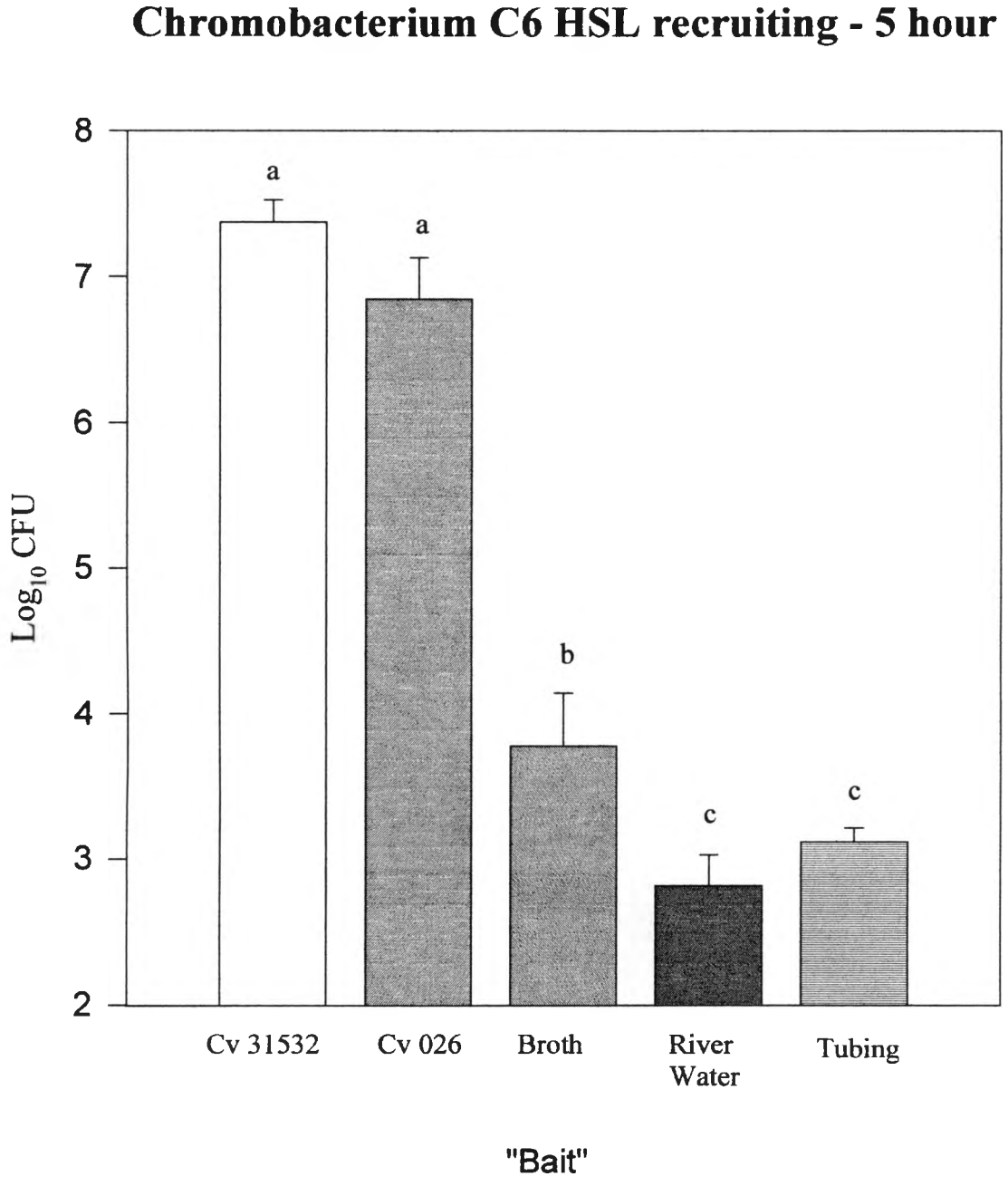

Figure 9. Graph showing effects of C6-HSLs on bacterial attachment to dialysis tubing in the San Marcos River after 5 hours. Error bars represent standard deviation. Values with the same letter do not differ significantly at P=0.05. Supernatant was not autoclaved.

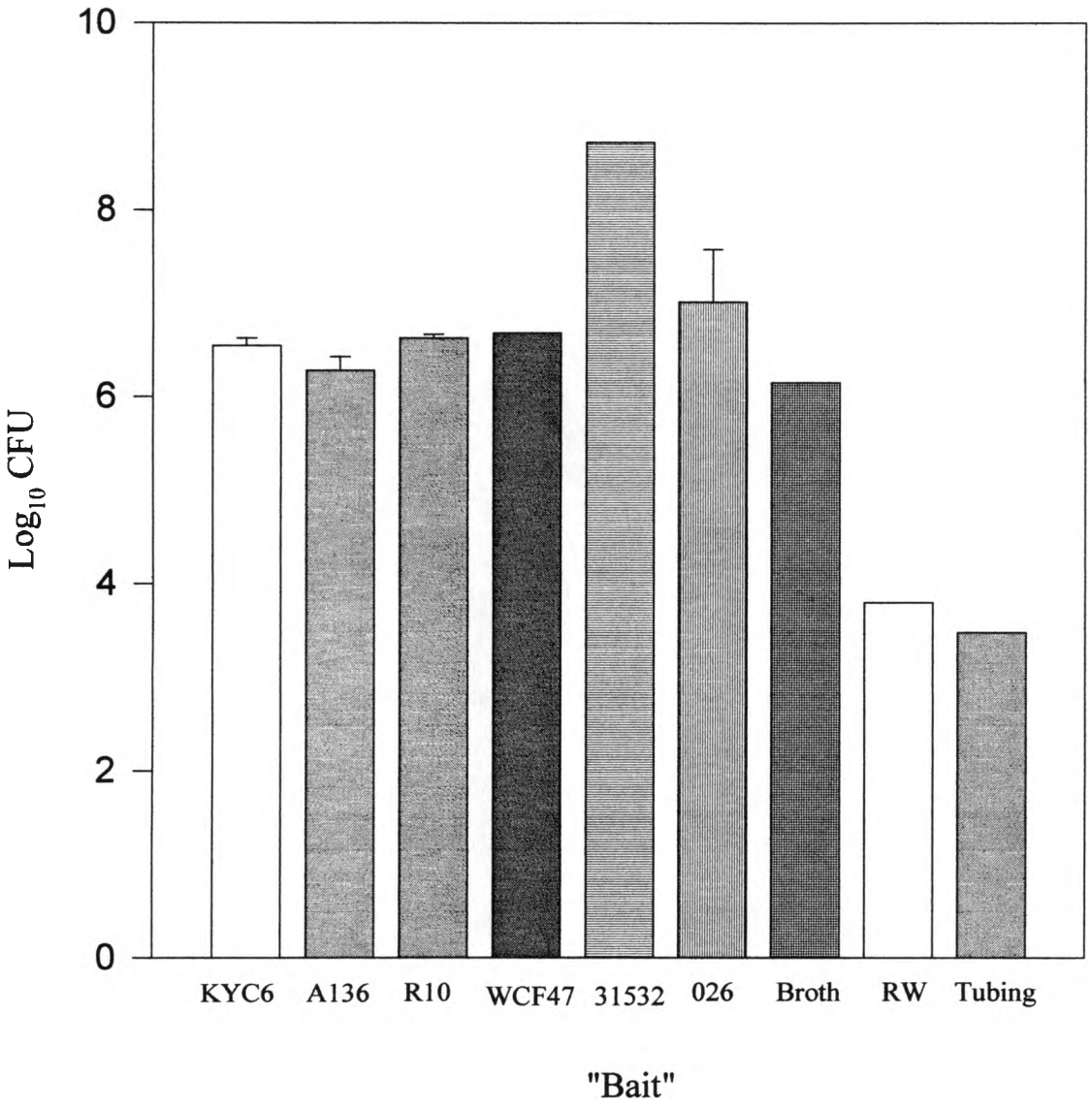

Figure 10. Graph showing effects of acyl HSLs on bacterial attachment to dialysis tubing after 18 hours. Supernatant was not autoclaved.

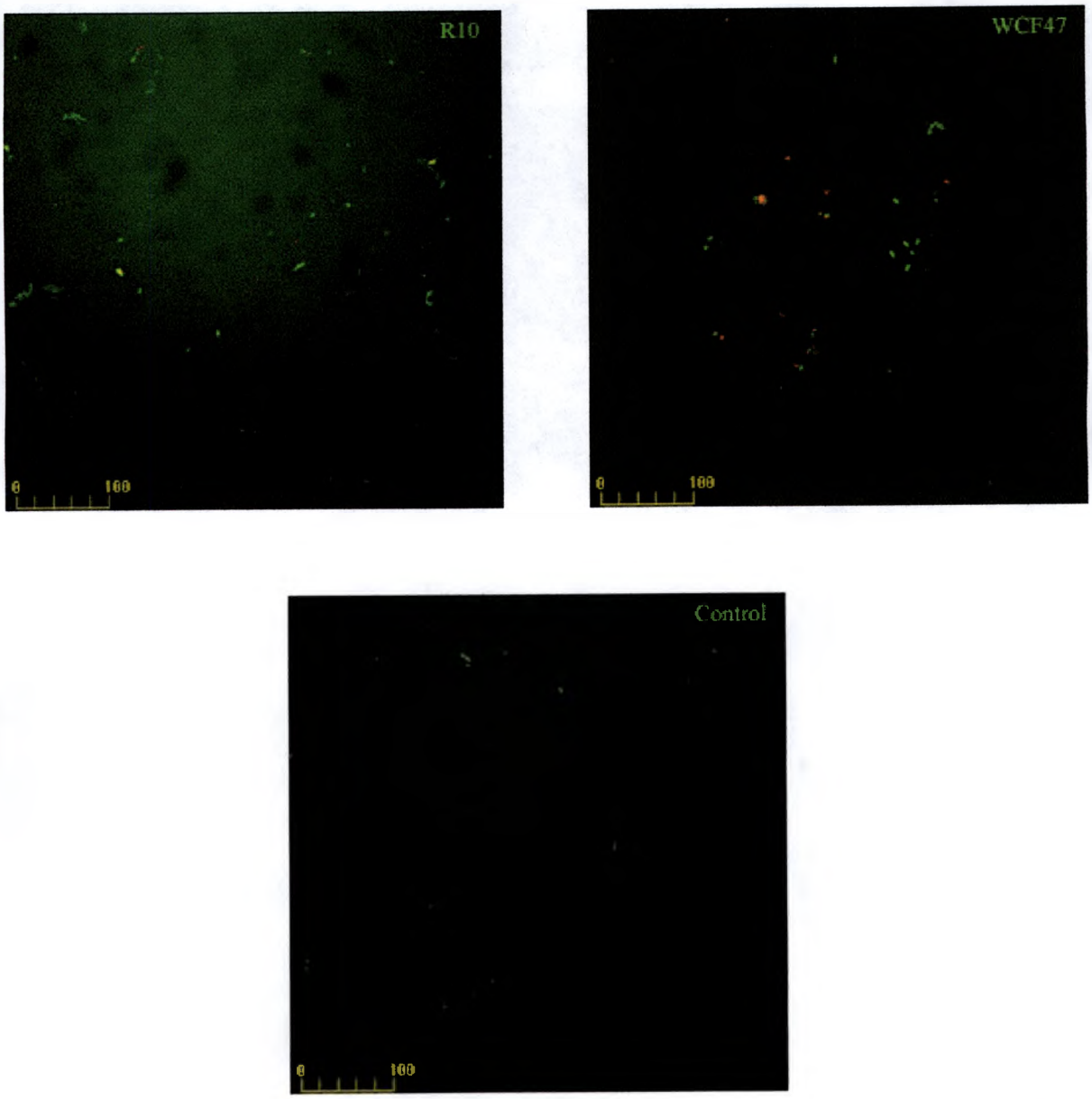

Figure 11. SCLM images showing colonization of dialysis tubing after 2.5 hours.

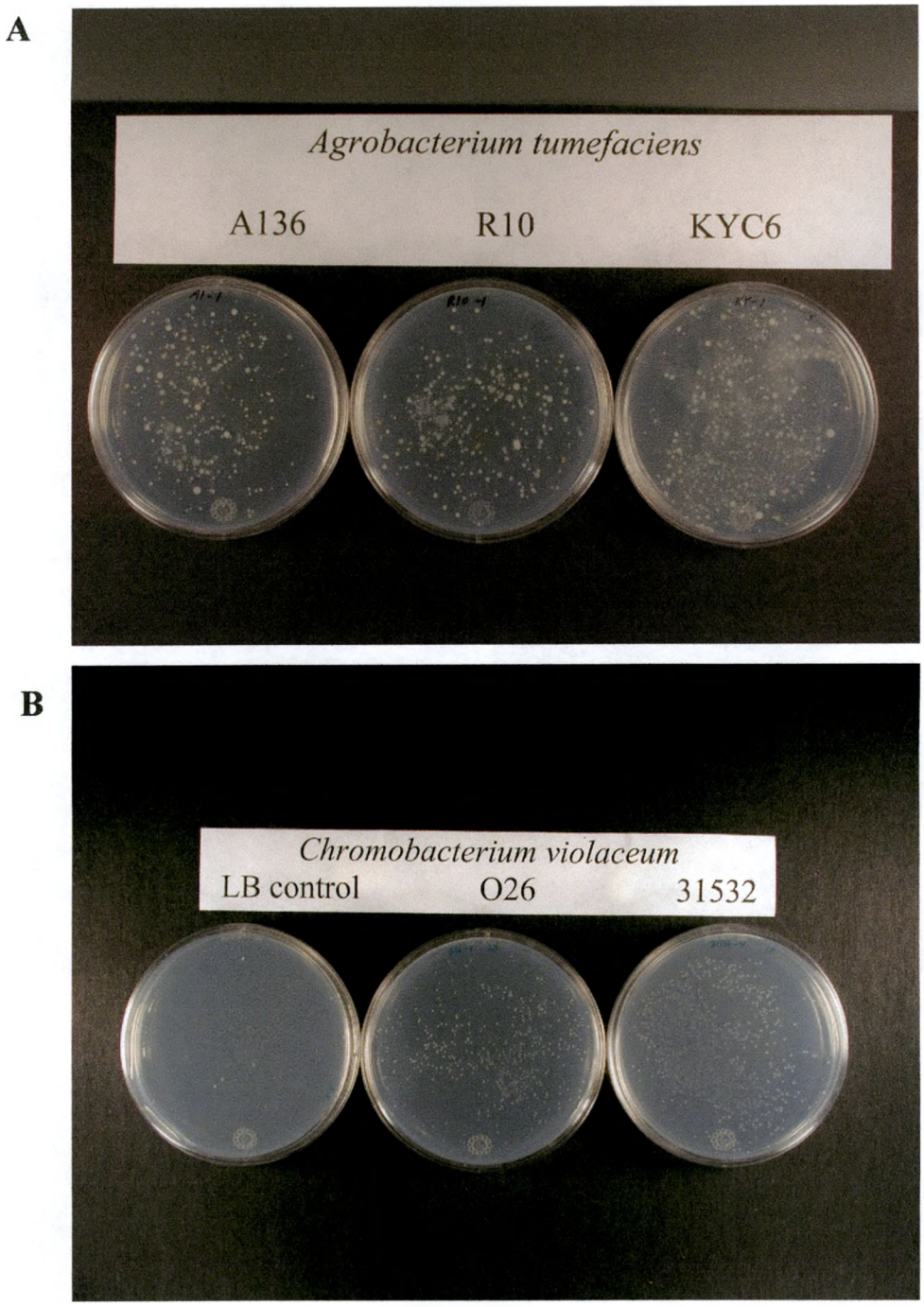

Figure 12. Dilution plates from tubing with acyl HSL producing strains show more growth than non producing strains and nonbacterial controls. (A) Dilution plates (10^{-1}) for *A. tumefaciens* baiting experiment. (B) Dilution plates (10^{-4}) for *C. violaceum* baiting experiment. All plates are from 5 hour experiment.

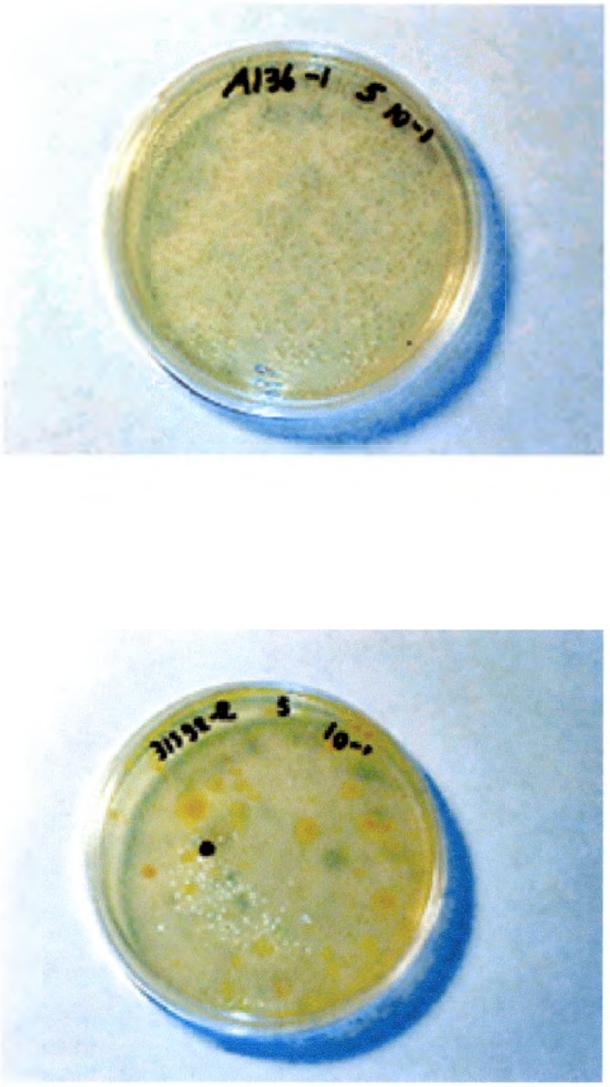

Figure 13. R_2A dilution plates showing bacterial diversity. On top: *A. tumefaciens* A136. On bottom: *C. violaceum* 31532.

Table 2. Identification of bacteria using Biolog or Fatty acid analysis.

Organisms identified with Biolog	**Supernatant attracted to**
Flavobacterium ferrugineum	*A. tumefaciens* A136
Flavobacterium ferrugineum	*C. violaceum* 026
Ralstonia picketti	River water
Sphingobacterium mizutaii	*C. violaceum* 026
Flavobacterium johnsoniae	*A. tumefaciens* KYC6
Micrococcus lylae	*C. violaceum* 026
Flavobacterium johnsoniae	Control

Organisms identified by fatty acid analysis	**Supernatant attracted to**
Pseudoaltermonas haloplanktis	*A. tumefaciens* A136
Hydrogenophaga pseudoflava	*A. tumefaciens* WCF47

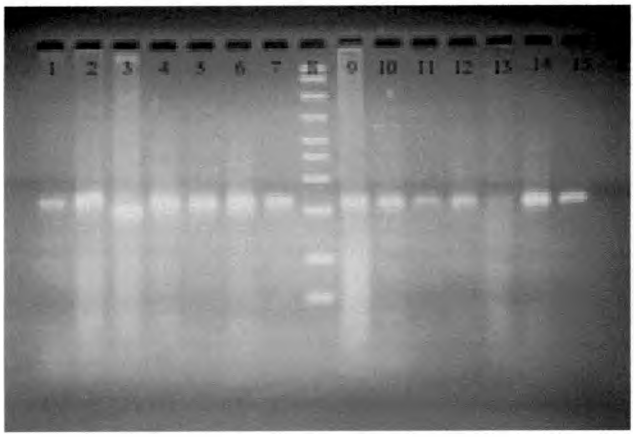

Figure 14. 4% agarose gel of Qiagen purified PCR products for sequencing. DNA from pure cultures was extracted by freeze-thaw method. A (lane 1), B (lane 2), C (lane 3), D (lanes and 5), E (lanes 6 and 7), Amplisize standard (lane 8), F (lane 9), G (lane 10), G (lane 11), H (lane 12), I (lane 13), J (lane 14 and 15).

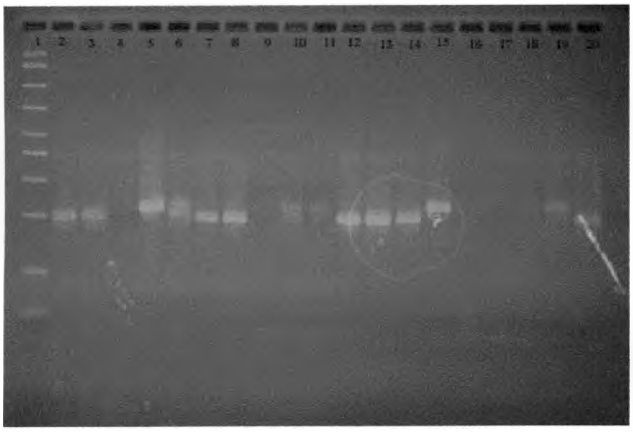

Figure 15. 4% agarose gel of PCR products for DGGE. DNA of mixed cultures was extracted by freeze-thaw method. Amplisize standard (lane 1), KYC6 (lane 2), A136 (lane 3), 31532 (lane 5), Cv 026 (lane 6), R10 (lane 7), WCF47 (lane 8), RW (lane 9), broth (lane 10), Tubing (lane 11): lanes 2-11 are supernatants. R10 (lane 12), KYC6 (lane 13), A136 (lane 14), 31532 (lane 15), tubing (lane 16), RW (lane 17), broth (lane 18), Cv 026 (lane 19), WCF47 (lane 20): lanes 12-20 are sonicates.

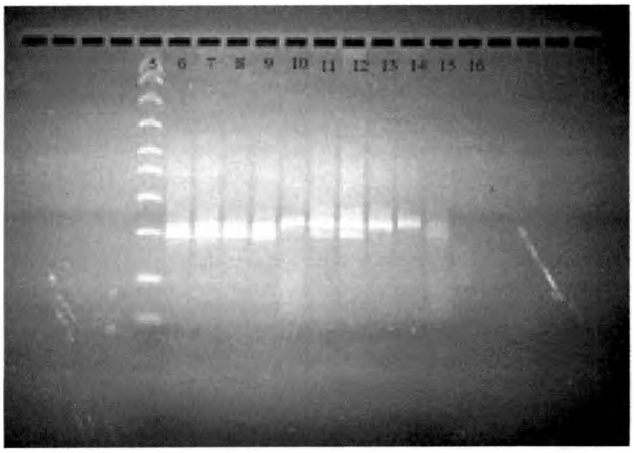

Figure 16. 4% agarose gel of PCR products for DGGE. DNA extracted with Bactozol™ kit from mixed cultures. Amplisize standard (lane 5), KYC6 (lane 6), A136 (lane 7), R10 (lane 8), WCF47 (lane 9), 31532 (lane 10), Cv 026 (lane 11), broth (lane 12), RW (lane 13), tubing (lane 14), sterile water (lane 15): lanes 6-14 are taken from 10^{-1} dilution plates.

Table 3. Identification of sequencing samples based upon BLAST search results.

Sequencing samples	Plate isolated from	Closest matches with BLAST search	% base pairs matched
A	*A. tumefaciens* A136	Aquatic bacterium RUB R2A 15	100
		Beta proteobacterium A0618	95
B	*A. tumefaciens* A136	*Curacaobacter baltica* OS 140	95
		Uncultured sheep mite bacterium Llangefni 75	94
C	River water	*Sphingomonas* sp. 2MPII	100
		Alpha proteobacterium MBIC1549	100
		Flavobacterium capsulatum	99
D	Tubing	*Plesiomonas shigelloides* (ATCC 14029T)	95
		Erwinia chrysanthemi	92
E	*C. violaceum* 026	Gamma proteobacterium HTB010	97
		Budvicia aquatica (strain DSM 5075)	95
F	*C. violaceum* 026	*Curacaobacter baltica* OS 140	96
		Uncultured sheep mite bacterium Llangefni 75	95
		Pectobacterium caratovorum subsp. Odoriferum	94
G	*A. tumefaciens* WCF47	Aquatic bacterium RUB R2A 15	100
		Beta proteobacterium A0618	95
H	*A. tumefaciens* WCF47	Uncultured epsilon proteobacterium 1063	97
		Unidentified eubacterium	96
		Leptothrix discophora (strain SP-6)	96
I	Broth	Elbe River snow isolate Iso19neu	100
		Aeromonas veronii strain HM 231	100
		Uncultured gamma proteobacterium BioIuz k41	99
		Aquatic bacterium RUB GSP 02	97
J	*C. violaceum* 31532	*Flavobacterium* sp. B17	97
		Soil bacterium is120	95
		Uncultured eubacterium clone MT20	94
K	C. violaceum 31532	Uncultured bacterium WkB35	92
		Elbe River snow isolate Iso 18	92
		Burkholderia cepacia isolate LMG 12614	91

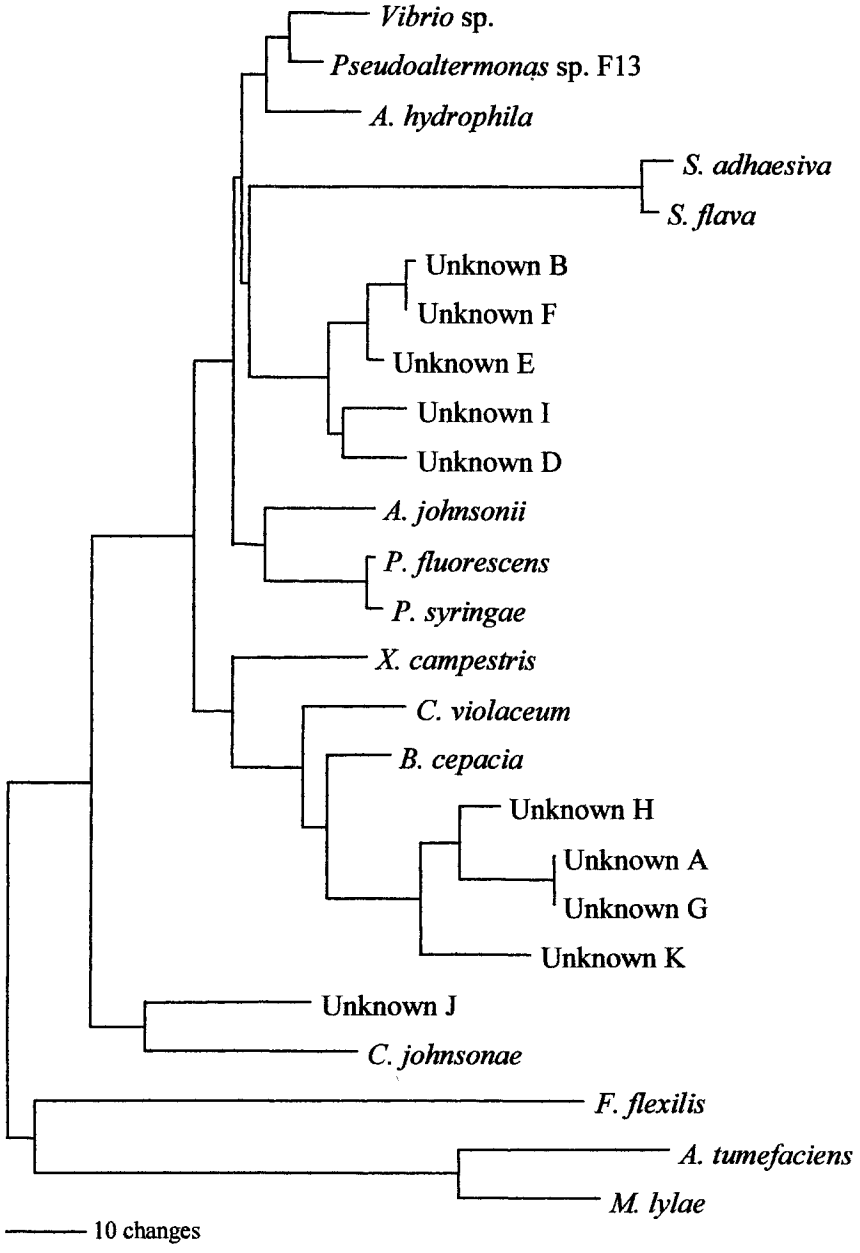

Figure 17. Phylogenetic tree showing the relationship between 16s rRNA sequence fragments of organisms isolated from the San Marcos River (unknowns) and some commonly known aquatic organisms. The tree was generated by the neighbor joining method. *A. tumefaciens* and *Micrococcus lylae* were used as outgroups.

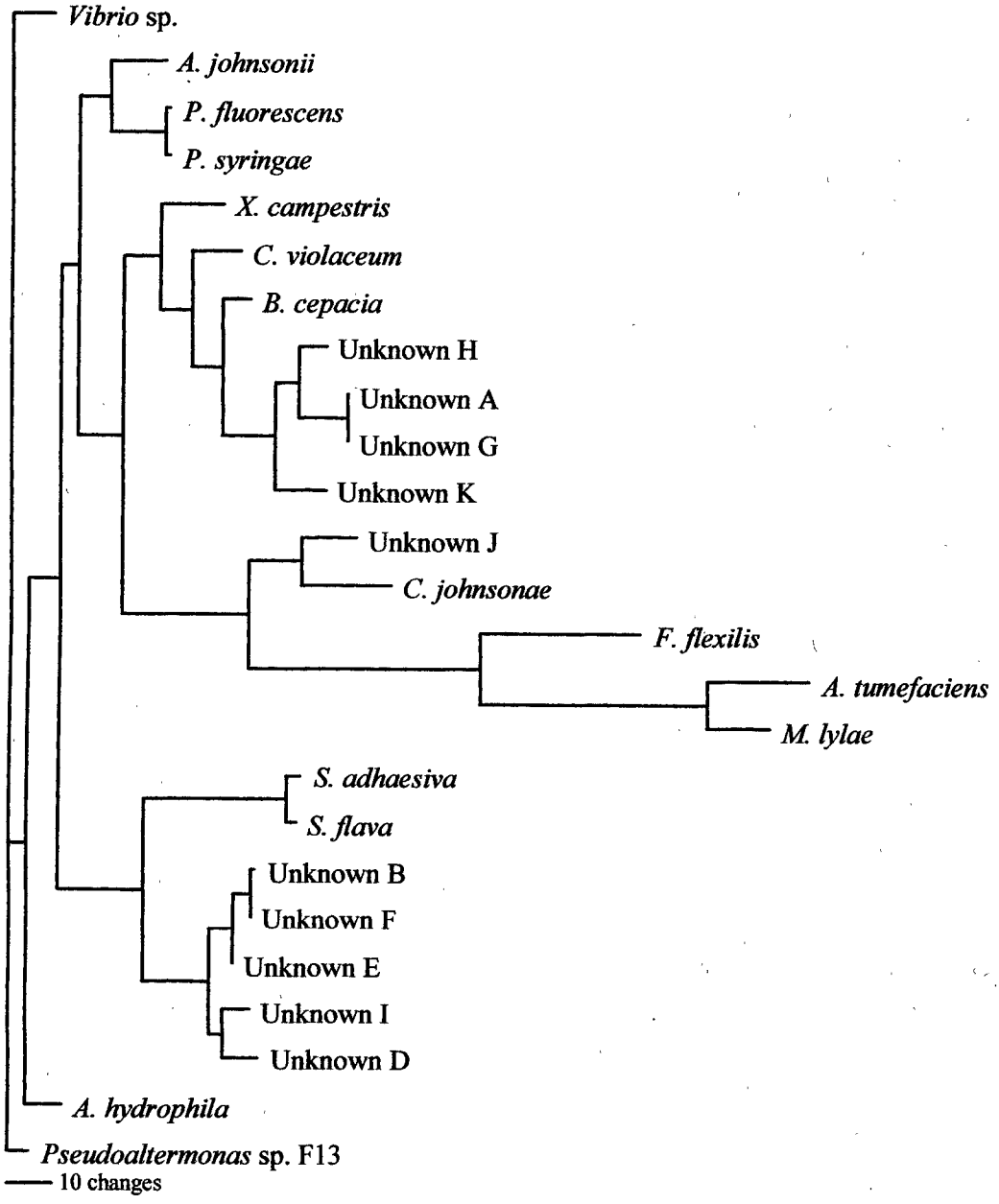

Figure 18. Neighbor joining tree showing the relationship between 16s rRNA sequence fragments of organisms isolated from the San Marcos River (unknowns) and some commonly known aquatic organisms using midpoint rooting.

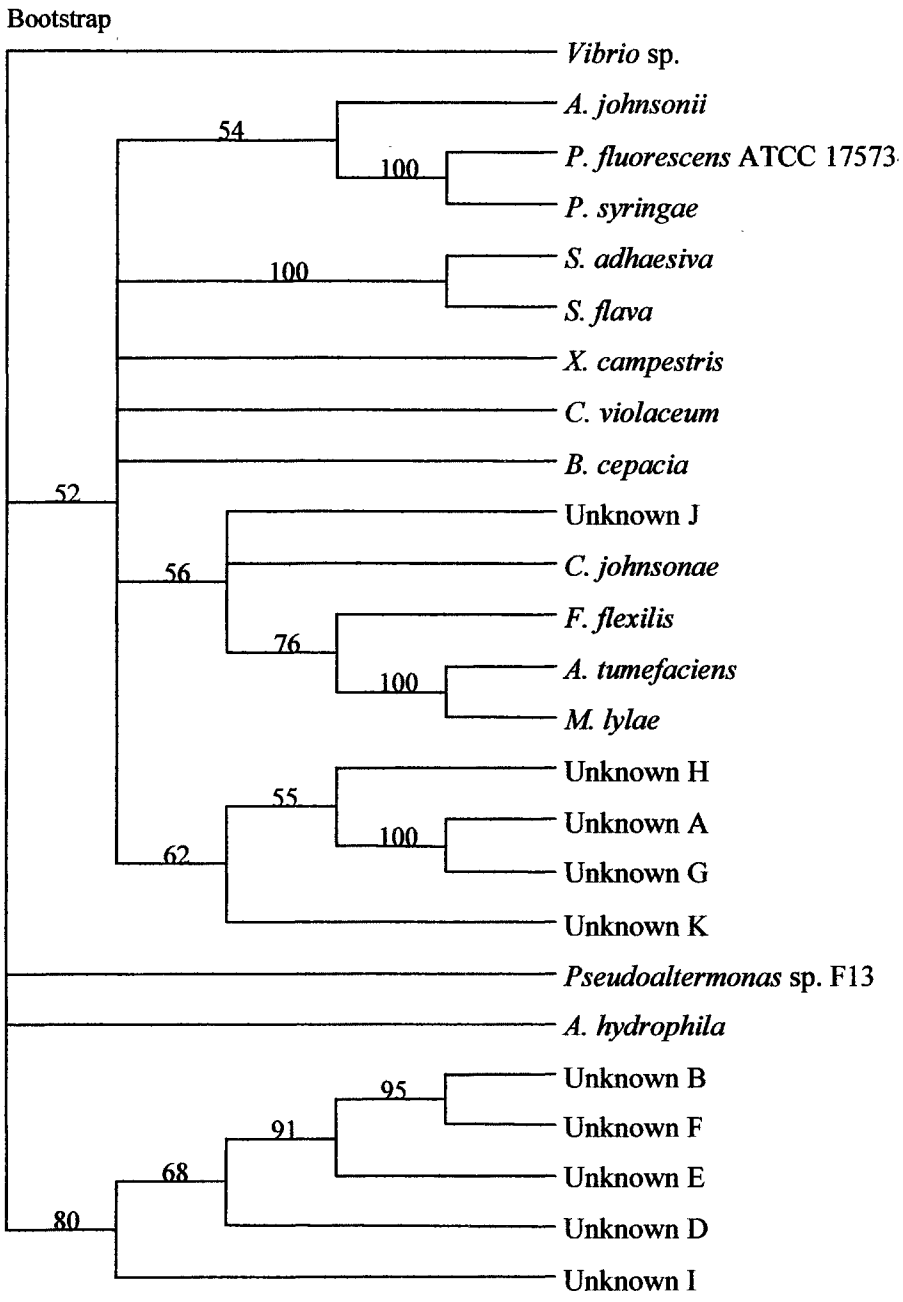

Figure 19. Neighbor joining bootstrap tree showing the relationship between 16s rRNA sequence fragments of organisms isolated from the San Marcos River (unknowns) and some commonly known aquatic organisms. Nodes with support derived from 1000 bootstrap replicates are depicted.

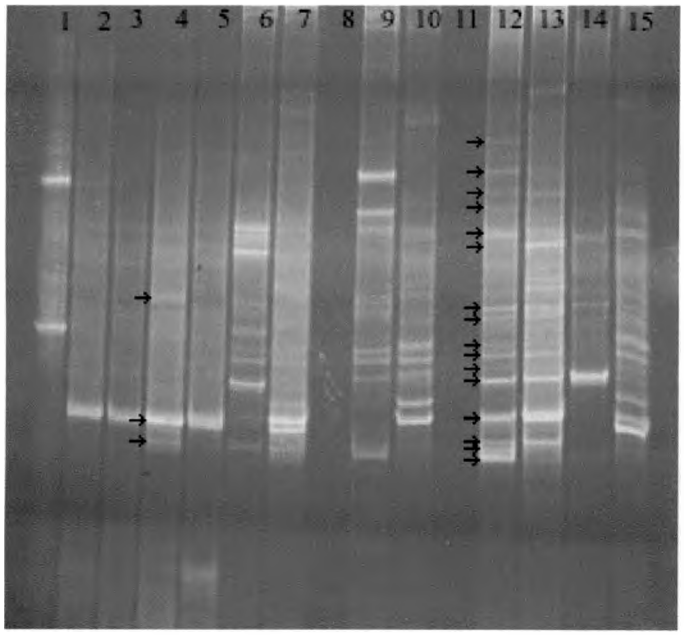

Figure 20. DGGE banding patterns from 16s rRNA PCR amplification of biofilm communities attached to dialysis tubing after 5 hours. *E. coli* DS291 and *P. aeruginosa* 10145 (lane 1), 31532 supernatant (lane 2), 31532 plate (lane 3), 31532 sonicate (lane 4), Cv 026 super (lane 5), Cv 026 plate (lane 6), Cv 026 sonicate (lane 7), RW super (lane 8), RW plate (lane 9), RW sonicate (lane 10), broth super (lane 11), broth plate (lane 12), broth sonicate (lane 13), tubing plate (lane 14), tubing sonicate (lane 15). DNA was extracted from sonicates by freeze-thaw and from plates with Bactozol™ kit. Arrows used to highlight banding patterns.

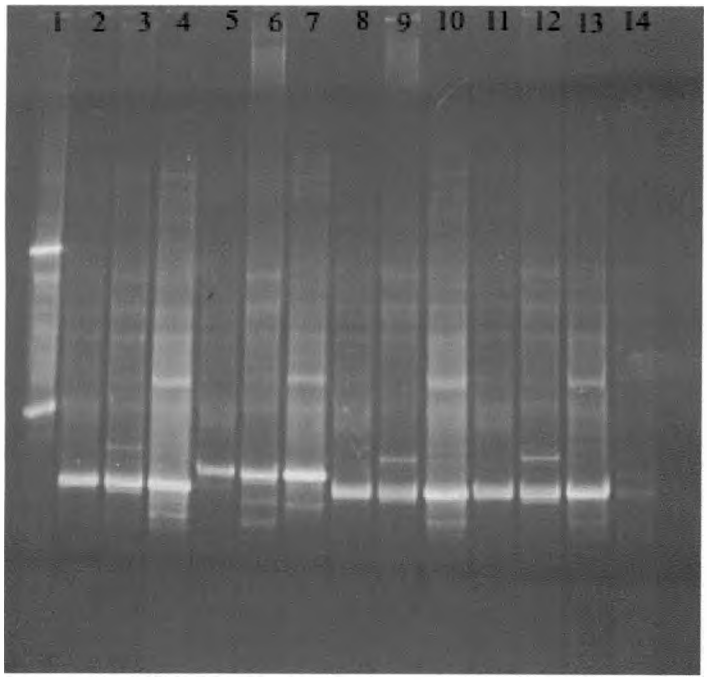

Figure 21. DGGE banding patterns from 16s rRNA PCR amplification of biofilm communities attached to dialysis tubing after 5 hours. *E. coli* DS291 and *P. aeruginosa* 10145 (lane 1), KYC6 supernatant (lane 2), KYC6 plate (lane 3), KYC6 sonicate (lane 4), A136 super (lane 5), A136 plate (lane 6), A136 sonicate (lane 7), R10 super (lane 8), R10 plate (lane 9), R10 sonicate (lane 10), WCF47 super (lane 11), WCF47 plate (lane 12), WCF47 sonicate (lane 13), sterile water (lane 14). DNA was extracted from sonicates by freeze-thaw and from plates with Bactozol™ kit.

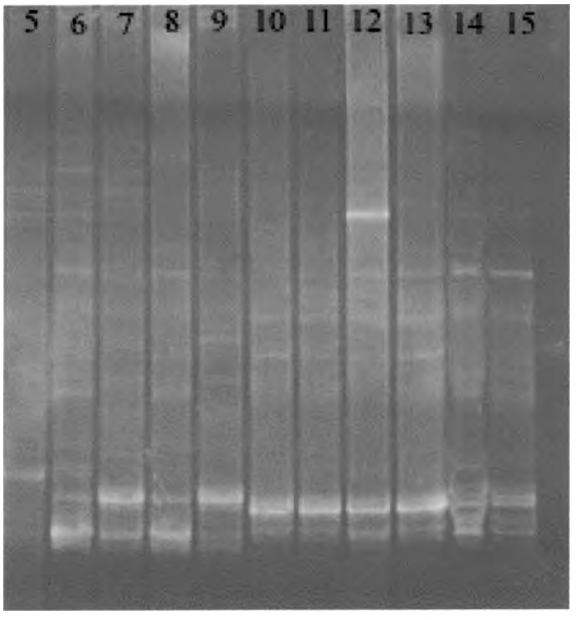

Figure 22. DGGE banding patterns for 16s rRNA PCR amplification of biofilm communities attached to dialysis tubing after 5 hours. A136 plate (lane 5), R10 sonicate (lane 6), R10 plate (lane 7), WCF47 sonicate (lane 8), WCF47 plate (lane 9), 31532 sonicate (lane 10), 31532 plate (lane 11), Cv 026 sonicate (lane 12), Cv 026 plate (lane 13), sterile water (lanes 14 and 15). All DNA extracted with Bactozol™ kit.

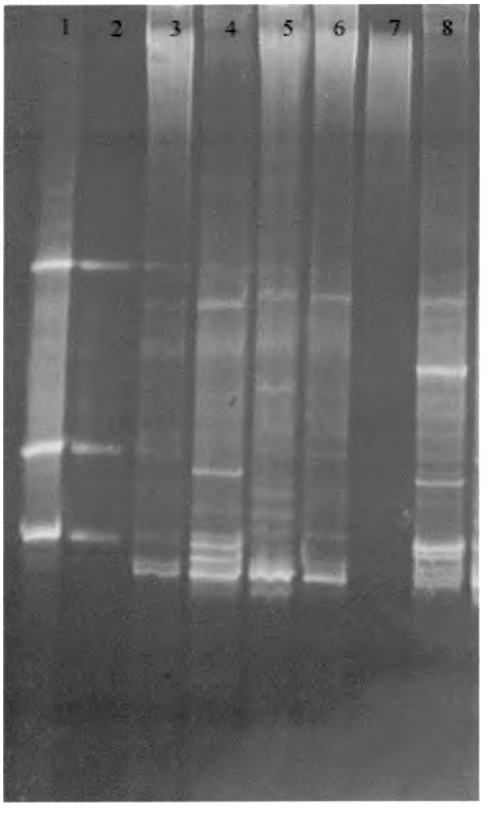

Figure 23. DGGE banding patterns from 16s rRNA PCR amplification of biofilm communities attached to dialysis tubing after 5 hours. *E. coli* DS291, *P. aeruginosa* 10145, *C. violaceum* 31532 (lane 1 and 2), tubing sonicate (lane 3), tubing plate (lane 4), broth sonicate (lane 5), broth plate (lane 6), RW sonicate (lane 7), RW plate (lane 8). All DNA extracted with Bactozol™ kit.