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Publisher's version / Version de l'éditeur:
https://doi.org/10.1139/w99-129
Canadian Journal of Microbiology, 46, 2, pp. 133-142, 2000-02

Molecular analysis and development of 16S rRNA oligonucleotide probes to characterize a diclofop-methyl-degrading biofilm consortium

Louise Laramée, John R. Lawrence, and Charles W. Greer

Abstract: Genomic DNA from nine individual bacteria, isolated from a diclofop-methyl-degrading biofilm consortium, was extracted for genetic characterization. The degradation of diclofop-methyl produces metabolites that are known intermediates or substrates for bacteria that degrade a variety of chlorinated aromatic compounds. Accordingly, oligonucleotide primers were designed from specific catabolic genes for chlorinated organic degradation pathways, and tested by PCR to determine if these genes are involved in diclofop-methyl degradation. DNA homology between the PCR products and the known catabolic genes investigated by Southern hybridization analysis and by sequencing, suggested that novel catabolic genes are functioning in the isolates. Specific fluorescent oligonucleotides were designed for two of the isolates, following 16S rDNA sequencing and identification of each of the isolates. These probes were successfully used for fluorescent in situ hybridization (FISH) studies of the two isolates in the biofilm consortium.

Key words: consortium, catabolic gene, diclofop-methyl, 16S rDNA, FISH, SCLM.

Résumé : L'ADN génomique de neuf bactéries, isolées d'un consortium d'un biofilm dégradant le méthyldiclofop, a été extrait pour fin de caractérisation génétique. Les métabolites produits par le consortium lors de la dégradation du méthyldiclofop sont des intermédiaires ou des substrats de bactéries dégradant des composés chlorés. Conséquemment, des amorces oligonucléotidiques, spécifiques pour des gènes cataboliques impliqués dans la dégradation de composés chlorés, ont été testées par PCR pour déterminer l'implication de ces gènes dans la dégradation de méthyldiclofop. L'homologie existant entre les produits de PCR et les gènes étudiés, évaluée par séquençage et transfert de Southern, suggère l'existence de nouveaux gènes cataboliques au sein du consortium. Suite au séquençage de l'ADNr16S et à l'identification des isolats, deux oligonucléotides fluorescents, spécifiques pour deux isolats, ont été utilisées avec succès dans des études d'hybridation in situ en fluorescence (FISH) de ces deux isolats du consortium dans le biofilm.

Mots clés : consortium, gène du catabolisme, méthyldiclofop, ADNr16S, FISH, SCLM.

Introduction

Biofilms, chains, mats, and microcolonies are the predominant forms of bacteria in nature (Costerton et al. 1987; Shapiro 1995). Multicellularity brings the same advantages to bacteria as it does to eukaryotic organisms: strength in numbers and the potential for specialization and cellular division of labor (Shapiro 1995). A spatial organization of metabolically interacting populations, and protection against antibacterial agents and phagocytic eukaryotic cells, are some of the benefits of bacterial multicellularity.

Received September 1, 1999. Revision received October 26, 1999. Accepted October 27, 1999.

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One of the most complex microbial multicellular organizations is a biofilm. Biofilms will form on almost any immersed surface and consequently can be found in a variety of locations, including water and waste water conduits, bioreactors, submarine periscopes, teeth, intestines, rumen, medical biomaterials, streams, and sewage effluents (Costerton et al. 1987; Characklis and Marshall 1990). In streams and rivers, degradative consortia that form attached films are mainly responsible for the transformation and the degradation of natural and xenobiotic organic compounds (Peyton and Characklis 1995).

Growth in biofilms facilitates metabolic cooperativity which is functional in the catabolism of organic compounds, particularly in the case of biodegradation of industrial and agricultural chemicals. Although research has concentrated on interdependence within anaerobic consortia, there is increasing evidence of the importance of microbial interactions in aerobic processes as well (Massol-deya et al. 1995; Wolfaardt et al. 1994*c*; De Souza et al. 1998). A bacterial consortium of at least nine individual microorganisms, able to degrade the herbicide diclofop-methyl, was isolated from soil by continuous culture enrichment (Wolfaardt et al. 1994*b*; Wolfaardt et al. 1994*c*). The members of this consortium formed a specific and highly structured biofilm when diclofop-methyl was used as sole carbon source. It was

suggested, as in other studies of bioreactor biofilms, that the nature of this organization represented an optimization of relationships between members of the consortium.

Diclofop-methyl, the methyl ester of 2-(4-(2,4-dichlorophenoxy)phenoxy)methylpropanoic acid, is used as a post-emergence herbicide for the control of annual grasses such as wild oats (*Avena fatua*) in a variety of crops, particularly in western Canadian cereals (Smith 1977; Smith et al. 1986; O'Sullivan 1990). Although it was classified as a non-persistent chemical, diclofop-methyl has been detected in river waters, air samples, irrigation waters, and groundwater samples in western Canada (Williamson 1984; Muir and Grift 1987; McNaughton and Crowe 1995).

A hypothetical degradation pathway for diclofop-methyl in soil was postulated by Smith (1977) and adopted by Martens (1978). Besides the liberation of carbon dioxide, the identified metabolites were 4-(2,4-dichlorophenoxy)phenol; 4-(2,4-dichlorophenoxy)phenetole; and trace amounts of other unidentified metabolites. In sterile soils, no metabolites were detected, suggesting a biodegradation process for diclofop-methyl. More recently, a study performed by Wolfaardt et al. (1994*a*) with the degradative microbial consortium, confirmed the involvement of microorganisms in the degradation of the herbicide, and identified other metabolites such as 2,4-dichlorophenol; 2-chlorophenol; 1,3-dichlorobenzene; 4-(2,4-dichlorophenoxy)-dehydrophenetole; and 4-phenoxyphenol. Several metabolites from the degradation of diclofop-methyl such as 2,4-dichlorophenol; 2-chlorophenol; and 1,3-dichlorobenzene, suggest the possible involvement of genes similar to those from aerobic bacterial pathways that degrade compounds like 2,4-dichlorophenoxyacetic acid (2,4-D) or 1,3-dichlorobenzene.

The specialized organization of the biofilm consortium, as well as the chemical structure of diclofop-methyl, make this diclofop-methyl-degrading bacterial biofilm a good model to study the relationships between organization and degradation capabilities, and the ecology of a complex microbial system in general. Information on the genetic characteristics could be used to develop molecular tools, to facilitate investigations of the microbial ecology of the degradative consortium, and to define the roles of individual members in complex organizations. The purposes of this study were: (*i*) to perform the genetic characterization of the diclofop-methyl-degrading bacterial consortium isolated previously by Wolfaardt et al. (1994*b*); (*ii*) to use available molecular information to investigate if known catabolic genes for the degradation of compounds such as 2,4-D, PCBs and dichlorobenzene, are involved in diclofop-methyl degradation; (*iii*) to identify the isolates by 16S rDNA sequence analysis; (*iv*) to generate 16S rDNA oligonucleotides to localize specific isolates within the biofilm community.

Materials and methods

Bacterial strains and culture media

The bacterial isolates from the diclofop-methyl consortium were previously isolated by Wolfaardt et al. (1994*b*). Isolated bacterial colonies from a nutrient agar plate were used to inoculate sterile 10% tryptic soy agar (TSA) plates (10% tryptic soy broth supplemented with 1.5% of agar, USB, Becton Dickinson, Cockeysville, U.S.A.) and sterile liquid 10% tryptic soy broth (TSB) (USB, Becton Dickinson, Cockeysville, U.S.A.) for genomic DNA preparation. The bacterial isolates were stored at –80°C in 10% TSB supplemented with 15% glycerol.

Total DNA extraction

The genomic DNA of each isolate was extracted using an adapted protocol (Fortin et al. 1998) from Ausubel et al. (1990), except that a second DNA precipitation step was added after the isopropanol precipitation. The isopropanol-precipitated DNA was resuspended in 400 µL of 3 M sodium acetate and transferred to Eppendorf tubes. One millilitre of 100% ethanol was added to each tube. The DNA was reprecipitated 15 min at –80°C, and centrifuged 15 min (15 000 × *g*) at 4°C. The DNA pellet was washed with ice cold 70% ethanol, and then dried. Each DNA pellet was resuspended in 100 µL 1× TE pH 8.0 and stored at –20°C.

DNA quality was first verified by agarose gel electrophoresis with 5 µL of each DNA preparation on a 0.7% Tris-acetate-EDTA (TAE) agarose gel (ICN Biomedicals). The DNA concentration was estimated using a spectrophotometer (Spectronic 2000, Bausch & Lomb) by diluting it in sterile distilled water (1:100), and reading the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}). The concentration of DNA was estimated by the A_{260}, considering that 1 absorbance unit equals 50 µg/mL of double stranded DNA. The quality was also evaluated by the A_{260}/A_{280} ratio. PCR reactions were performed using each DNA preparation, and 23S eubacterial universal primers (Thiem et al. 1994) to confirm the absence of any PCR inhibitory compounds in the DNA preparations.

DNA fingerprinting

Genomic DNA preparations were analyzed by arbitrary primed PCR (AP-PCR) to generate a DNA fingerprint (Welsh and McClelland 1990; Williams et al. 1990; Yu and Pauls 1992; Brousseau et al. 1993; Martin-Kearley et al. 1994) to confirm the unique character of each isolate. Three different primers were used independently to generate a DNA fingerprint of each bacterial isolate: primer 0940–10 (5′-AGA AGG CCG-3′), primer 0940–10.1 (5′-AGA AGG CCG A-3′), and primer 0940–10.3 (5′-AGA AGG CCG C-3′). All the primers were prepared with a DNA synthesizer (Applied Biosystems model 380A). Primer 0940–10 originated from a primer collection of arbitrary nucleotide sequences (Brousseau et al. 1993). The latter two primers were derived from 0940–10, and differed by the addition of one nucleotide. The average G+C content was 65.6%. The PCR amplification protocol was adapted from Martin-Kearley et al. (1994). Each PCR was prepared using a 10× *Taq* DNA polymerase buffer (100 mM Tris-HCl pH 9; 500 mM KCl; 15 mM $MgCl_2$) (Pharmacia Biotech). Each PCR tube contained the following ingredients: 50 to 100 ng of bacterial genomic DNA, 2.5 units of *Taq* DNA polymerase (Pharmacia Biotech), 5 µL of 10× *Taq* DNA polymerase buffer, 60 to 80 pmol of primer, 8 µL of 1.25 mM deoxyribonucleoside triphosphates (200 µM each: dATP, dGTP, dCTP, and dTTP), 1 µL of 100 mM $MgCl_2$, and sterile distilled water to bring the final volume to 50 µL. Each sample was covered with 75 µL of light mineral oil. The negative controls contained the same mixture as described above except that the DNA was replaced with sterile water. One negative control reaction was performed for each PCR primer. Prior to the addition of the *Taq* DNA polymerase/10× buffer mixture, the samples were heated for 2 min at 100°C in the thermal cycler, and then transferred on ice to add the enzyme mixture. The amplification conditions were: denaturation through a 1 min ramp time from 72 to 96°C, followed by a 1 s hold at 96°C, annealing with a 3 min ramp time from 96 to 35°C, followed by a 1 min hold at 35°C and elongation with a 75 s ramp time from 35 to 72°C, followed by a 30 s hold at 72°C. A total of 30 cycles were performed after which the sample block was kept at 4°C until agarose gel electrophoresis was performed. Five microlitres of each PCR

sample was mixed with 1 µL of loading buffer (30% (v/v) glycerol, 0.15% (w/v) bromophenol blue, 0.15% (w/v) xylene cyanol), and loaded onto a 1.4% (w/v) TAE agarose gel together with the 100 and 1000 bp DNA ladders (Gibco BRL). The PCR bands were stained in ethidium bromide and observed by using a UV transilluminator at a wavelength of 254 nm. The gels were photographed using type 57 Polaroid film.

Detection of catabolic genes

Genes encoding the large subunits of the enzymes biphenyl dioxygenase; 2,4-dichlorophenol hydroxylase; catechol 1,2-dioxygenase; chlorocatechol 1,2-dioxygenase; catechol 2,3-dioxygenase; and the polypeptide P3 (one of the components of the multicomponent phenol hydrogenase) were selected as targets for PCR amplification. These genes encode enzymes involved in the hydroxylation or the ring-cleavage of (halo) aromatic compounds. For each enzyme investigated, a search in GenBank was performed, and the corresponding sequences were retrieved for comparative DNA and protein alignments using the software GENEWORKS (Intelligenetics Inc., Campbell, Calif.). Genes sharing homologous DNA sequences long enough to allow design of PCR primers (forward and reverse primers for each catabolic gene) were selected. PCR primers were then designed based on one of these homologous DNA sequences. The PCR primer sequences as well as related information are presented in Table 1. Analysis of the designed primers was performed using the FASTA (Pearson and Lipman 1988) and BLASTN (Altschul et al. 1990) search programs, and showed good specificity for the target sequences. All the primers were made with a DNA synthesizer (Applied Biosystems model 380A). Positive controls contained DNA from strains known to possess one of the reference catabolic genes. The negative control was prepared using sterile distilled water instead of DNA to reveal possible non-specific DNA contamination. Both controls were performed for each set of PCR. Typically, one PCR contained 50–100 ng of genomic DNA, 30–40 pmol of each primer (forward and reverse), 2.5 units of *Taq* DNA polymerase, 5 µL of 10× *Taq* polymerase buffer, 8 µL of 1.25 mM dNTP, 0.5 µL of 100 mM $MgCl_2$, and sterile distilled water to make up the volume to 50 µL. Each sample was covered with light mineral oil. Prior to the addition of the *Taq* DNA polymerase/10× buffer mixture, the samples were heated 2 min at 100°C in the thermal cycler, then transferred to ice to add the enzyme mixture. The standard amplification conditions for 30 cycles were: 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. The last cycle was followed by a final extension for 3 min at 72°C, and the reactions were kept at 4°C until the electrophoresis was performed. Several annealing temperatures were tested for each set of primers. The first annealing temperature used was one of medium stringency (50°C) and the others varied between 37 and 68°C, depending on the number of bands in the preceding reaction. The test was stopped when no bands or too many bands were observed. Ten microlitres of the PCR reaction were mixed with 2 µL of loading buffer, and loaded onto a 1 to 1.4% (w/v) TAE agarose gel, depending on the size of the expected PCR bands. The 100 and 1000 bp ladders were also loaded onto each gel as molecular weight standards. The PCR products were visualized with ethidium bromide and UV transillumination at 254 nm. Putative positive PCR bands were analyzed to verify the specificity of the amplification either by cloning and sequencing, or by Southern hybridization of PCR fragments using the gene probe from the reference strain.

PCR amplification and sequencing of 16S rDNA

The genomic DNA preparation was used for the amplification by PCR of the 16S rDNA gene using 16S universal primers. The PCR amplified 16S rDNA fragment was then sequenced directly or after cloning, and the sequences obtained were analyzed to identify the isolate. With the exception of primer R14b (5′-CCA TGA GGA CTT GAC GTC AT-3′), all the 16S universal primers used were previously published by Dorsch and Stackebrandt (1992). The forward primer F1 and the reverse primer R13 were used to amplify by PCR the 16S rDNA of each isolate. The desired PCR band was about 1.5 kbp. The other primers as well as the primers F1 and R13 were used to sequence both strands of the amplified 16S rDNA.

The PCR mixture used in this section is similar to the one for the amplification of catabolic genes. The volume of each ingredient used was doubled, except for the primers, to give a final volume of 100 µL, which was covered with mineral oil. Sixty picomoles of each primer were used per reaction. The positive controls were performed with DNA known to yield a band of 1.5 kp, and the negative control was prepared using sterile distilled water instead of template DNA to reveal possible non specific DNA contamination. Both controls were performed for each set of PCR. A denaturation step of 2 min at 100°C in the thermal cycler was performed prior to the addition of the *Taq* DNA polymerase/buffer solution and amplification was carried out for 30 cycles. The annealing temperature required for 16S rDNA amplification for each isolate was evaluated by preliminary tests. Each cycle had the following conditions: 1 min at 94°C; 1 min at 65°C (isolates 2 to 6, and 9), 50°C (isolate 8), 42°C (isolate 1), or 37°C (isolate 7); and 1 min at 72°C. The last cycle was followed by a final extension at 72°C for 3 min. The samples were kept at 4°C until the electrophoresis was performed. Each PCR sample (100 µL of oil free) was mixed with 10 µL of loading buffer, and loaded onto 1 or 2 well(s) of a 0.7% TAE agarose gel. The PCR products were visualized after staining with ethidium bromide using a UV transilluminator at 254 nm. The 1.5 kbp PCR fragments were purified with the QIAEX II Gel extraction kit (Qiagen Inc., Mississauga, Ont.) as described by the manufacturer. The DNA concentration was estimated by running 5 µL of the purified material on a 0.7% TAE agarose gel.

The purified amplified 16S rDNA fragments of isolates 1 and 9 were cloned into PTZ/PC. The cloning vector PTZ/PC is a modified PTZ19R *E. coli* plasmid (Tessier 1997) with a single T residue overhang on its 3′ ends, following cleavage with the appropriate restriction endonuclease. This last characteristic significantly enhances the efficiency of cloning PCR fragments, since *Taq* DNA polymerase will catalyze the preferential addition of a single dATP residue on the 3′ ends of blunt-ended double-stranded DNAs.

Sequencing reactions were prepared with ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin Elmer), and were performed using the PTZ19R universal or reverse primers (Pharmacia Biotech) and the 16S universal primers. The sequencing reactions were performed as recommended by the manufacturer. The sequencing reactions were purified using G-50 Sephadex quick spin columns (Boehringer Mannheim, Indianapolis, Ind.) to remove traces of unincorporated dye terminators. The columns were used as recommended by the manufacturer. The DNA was resuspended in 4 µL of a deionized formamide–8 mM EDTA pH 8.0 solution. Nucleotide sequences were obtained with an automated sequencing apparatus (ABI 373) and the corresponding analysis was performed using SEQED v. 1.0.3 from Applied Biosystems (Perkin Elmer). The sequencing primers were used to insure that the entire sequence was sequenced two to three times. The software GENEWORKS (Intelligenetics Inc, Calif.) was used to perform DNA alignments. The sequenced 16S rDNA of each isolate was compared to the GenBank non-redundant nucleotide sequence database using both FASTA (Pearson and Lipman 1988) and BLASTN (Altschul et al. 1990) search programs.

Design of 16S rDNA oligonucleotide probes

The sequences of 16S rDNA from each isolate were also analyzed to design specific oligonucleotide probes for selected isolates

Table 1. List of the designed catabolic PCR primers used in this study.

Primer	Sequence (5′- to -3′)	Nucleotides	Expected PCR band size	Based on the following catabolic genes (GenBank accession No.)
Biphenyl dioxygenase				
bphA-F	TCA CCT GCA GCT ATC ACG GCT GG	23	812 to 836 bp	*bphA1*, *Rhodococcus* sp. (D32142)
bphA-R	GGA TCT CCA CCC AGT TCT CGC CAT CGT CCT G	31		*bphA**, *Pseudomonas pseudoalcaligenes* KF707 (M83673)
				bphA, *Pseudomonas* sp. (M86348)
				bphA1, *Rhodococcus globerulus* (X80041)
Phenol hydroxylase (one component)				
tfdB-F	CCG GTT CCA TCA ACT TGC TGT TCG AGG C	28	376 bp	*tfdB**, *Alcaligenes eutrophus* JMP134 (M35097)
tfdB-R	GCC GAG TCC GTT GGT CGG CGG ATG CC	26		
Phenol hydroxylase (multicomponent)				
phem-F	TTC ATG TCC GGT GCC GCC TAC AAC GGC GAT ATG GC	35	499 bp	*pheA4*, *Pseudomonas putida* BH (D28864)
phem-R	GGG CGG TAG TAT TTG TCG AAG GTG TCC GGG TAT TT	35		*dmpN**, *Pseudomonas* sp. CF600 (M60276)
				phhN, *Pseudomonas putida* P35X (X79063)
				phlD, *Pseudomonas putida* H (X80765)
				orf4, *Acinetobacter calcoaceticus* (Z36909)
Catechol 1,2-dioxygenase				
C12O-F	GAA GGA CCG CTA TAT GTT GCA GGT GC	26	361 to 367 bp	*pheB*, *Pseudomonas putida* m (M57500)
C12O-R	TAG TGA ATA TGC GCA GGG CG	20		*catA*, *Acinetobacter calcoaceticus* (M76991)
				orf7, *Acinetobacter calcoaceticus* (Z36909)
Chlorocatechol 1,2-dioxygenase				
chloro12-F	GTA CAG CGG TAT CCA TGA CCA CAT CCC	27	242 bp	*tfdC**, *Alcaligenes eutrophus* JMP134 (M35097)
chloro12-R	CCT TCG AAG TAG TAC TGC GTG GTC A	25		*tfdC*, *Pseudomonas putida* (M36279)
				tcbC, *Pseudomonas* sp. P51 (M57629)
Catechol 2,3-dioxygenase				
C23O-F	AGG TGC TCG GTT TCT ACC TGG CCG A	25	405 to 408 bp	*nahH*, *Pseudomonas putida* pNAH7 (M17159)
C23O-R	ACG GTC ATG AAT CGT TCG TTG AG	23		*dmpB*, *Pseudomonas puitda* pVII50 (M33263)
				*xylE**, *Pseudomonas putida* ATCC 33015 (M64747)
				xylE, pDK1 (M65205) C23O, *Alcaligenes* sp. KF711 (S77084)
				bphE, *Pseudomonas* sp. IC (U01825)
				phlB, *Pseudomonas putida* (X77856)
				phlH, *Pseudomonas putid* (X80765)

Note: *, Used as positive control for PCR.

in order to study their localization in the diclofop-methyl degrading biofilm (during or after biofilm formation), and as a function of the carbon source supplied in the biofilm system. Isolates 4 and 6 were selected for these studies because these two isolates were reported to mineralize diclofop-methyl as sole carbon source when supplied with a second carbon source (1% TSB) (Wolfaardt et al. 1994*b*). The selected region for the design of specific oligonucleotide probes for isolates 4 and 6 was chosen based on the 16S rDNA alignment of the nine isolates. Specificity analysis of the designed probes against the 16S rDNA sequences of the nine isolates, was performed using the Amplify software (Bill Engels, copyright). The oligonucleotide probes specific for isolates 4 (4A–R) and 6 (6B–R) were labeled with the FAM NHS dye label (a succinimidyl ester of carboxyfluorescein) and the TAMRA NHS dye label (a tetramethylrhodamine carboxylic acid), respectively (CORE DNA SERVICES, University of Calgary, Alta.). The oligonucleotide probe Eub338 (Amann et al. 1990), which is complementary to a conserved region of the 16S rRNA in the domain *Bacteria*, was labeled with TAMRA NHS, or FAMS NHS, and used as a positive control to hybridize to all bacteria.

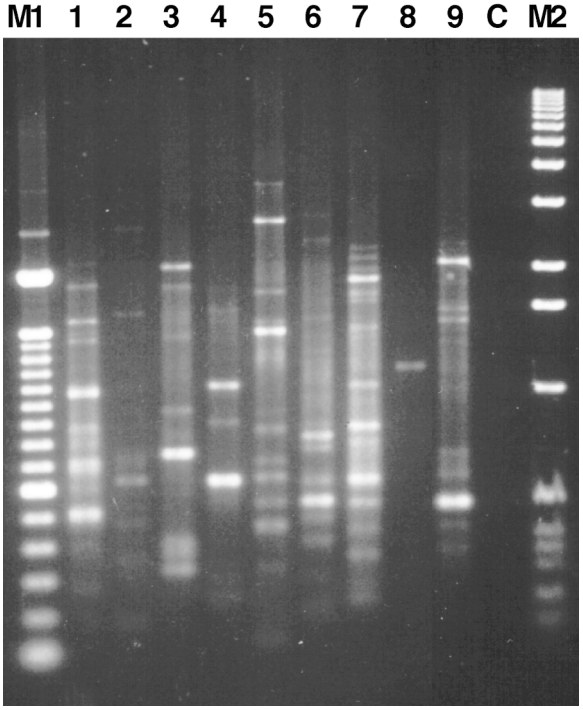
Fluorescent in situ hybridization (FISH)

Specificity of the designed probes (4A–R and 6B–R) against their target DNA sequences was verified using the whole cell hybridization technique. The protocol was adapted from Manz et al. (1992). Hybridization experiments with each probe were performed on isolates 1 (negative control), 4, and 6. Two millilitres of TSB medium were inoculated with a loopful of cells for each isolate, and incubated at 26°C with agitation to an OD_{550} of 0.6. One millilitre of culture was harvested in an Eppendorf tube, and centrifuged for 1 min in a microcentrifuge (15 800 × *g*) at room temperature. The cells were washed with 500 µL of PBS (130 mM NaCl, 10 mM $NaPO_4$ pH 7.2) and recentrifuged for 1 min. The cells were fixed for 3 h (twice for 15 min, once for 2.5 h) in 500 µL of 3% formaldehyde-PBS (v/v). Between each fixation step, the cells were centrifuged and resuspended in fresh formaldehyde solution. After the last fixation step, the cells were washed in PBS, and resuspended in 100 µL PBS/100% ethanol (1:1) and stored at –20°C. Ten microlitres of cell preparation and of a 1:10 dilution were spotted on precleaned microscopic multitest slides (12 wells, ICN Biomedicals) and left to air-dry. The cells were then completely dehydrated by successive 3 min washes with 50% ethanol, 70% ethanol, and 100% ethanol and left to air-dry. Samples of consortium biofilm, attached to glass microscope slides, were fixed in 4% formaldehyde for 30 min and then dehydrated as described above. Ten microlitres of a hybridization solution (20% (v/v) formamide, 0.9 M NaCl, 20 mM Tris/HCl pH 7.2, 0.01% SDS, and 25 ng of isolate 4 or 6 labeled probes) were applied to each well of the microscope slide, and incubated for 1.5 h at 46°C in a humidified atmosphere. The probes were removed from the slides with 2 mL of washing solution (20 mM Tris, 0.01% SDS, 180 mM NaCl, 5 mM EDTA). The microscope slides were immediately immersed in 50 mL of washing solution at 48°C for 20 min. Slides were rinsed briefly with distilled water and air-dried.

Microscopy and confocal laser scanning microscopy

Microscope slides containing the pure cultures were observed using a fluorescence microscope ARISTOPLAN (Leitz). Pictures were digitized using a MICROMAX (Princeton) camera and the software NORTHERN ECLIPSE (EMPIX Imaging, Inc.). A BioRad MRC 1000 CLSM (Hemel Hempstead, England), equipped with a krypton-argon laser and mounted on a Nikon Microphot SA microscope was used to obtain images of the hybridized biofilm samples. The CLSM system was operated using the Pentium host computer, and software provided by Bio-Rad. Observations were made with 60× and 100×, 1.4 numerical aperture, oil immersion lenses (Nikon, Tokyo, Japan).

Fig. 1. Agarose gel electrophoresis of the DNA fingerprint of the individual isolates by arbitrarily primed-PCR (AP-PCR) using primer 0940–10.3 (5′-AGAAGGCCGC-3′). Lanes: M1 and M2, 100 bp DNA ladder and 1 kbp DNA ladder, respectively; C, negative control; 1 to 9, isolates 1 to 9.



Results and discussion

The first step in the genetic characterization was to confirm the unique character of each isolate of the bacterial biofilm by DNA fingerprinting. Distinct band profiles were successfully obtained for each of the nine isolates by AP-PCR using three different AP-PCR primers. The band profiles were generally between 200 to 1500 bp, and were reproducible. The nine strains were clearly different based on the banding patterns (Fig. 1). This confirmed that the biofilm consortium consisted of at least the nine isolates reported by Wolfaardt et al. (1994*c*). Further, the presence of a relatively high number of community members indicated the potential for complex catabolic and ecological interactions.

Based on the chemical structure of diclofop-methyl and the known metabolites, it was hypothesized that degradation pathways, such as those involved in the degradation of 2,4-D; 1,3-dichlorobenzene; or biphenyl might be functional in the diclofop-methyl-degrading consortium. This was examined using PCR in combination with specific primers designed from genes encoding the enzymes biphenyl dioxygenase, catechol dioxygenases (1,2- and 2,3-), chlorocatechol dioxygenase, and phenol hydroxylases (single and multicomponent phenol hydroxylases). These genes encode enzymes which could be involved in hydroxylation or in

ring-cleavage of the aromatic rings of diclofop-methyl. With one exception (single component phenol hydroxylase), all the primers were designed using at least three different known genes (Table 1). In the case of the single component phenol hydroxylase, five different DNA sequences were retrieved from the GenBank database (GenBank accession Nos. L04488, M35097, M57500, M98806, and U17960). Some homology at the amino acid level was found, but this homology was not conserved at the DNA level (data not shown). Less than 5% identity at the DNA level was observed between the five sequences. Therefore only the sequence encoding the dichlorophenol hydroxylase enzyme (*tfdB*) was considered for design of the primers. The latter gene is especially interesting since 2,4-dichlorophenol was identified as a diclofop-methyl metabolite.

Most of the PCR for the catabolic genes did not produce any bands for the bacterial isolates, but all the controls gave the expected results. In some cases PCR bands, approximately of the expected size, were generated and were therefore analyzed in more detail. However, no homology was found between the PCR fragments of the bacterial isolates and the respective catabolic genes based on the PCR band sequences and (or) Southern hybridization analysis with the reference catabolic genes. At least one plasmid was detected in all the isolates with the exception of isolate 1 (data not shown); and although the relationship between plasmid(s) and catabolic genes was not established in this study, the occurrence of large plasmids in most of the isolates is probably relevant. The genes encoding many known catabolic enzymes involved in the degradation of aromatic compounds are located on plasmids, and mobile genetic elements, such as plasmids, are very important in the adaptation of catabolic potential in microbial communities (Barkay et al. 1993). Moreover, high population densities, and the long residence time of the cells in a biofilm, favor natural gene transfer (Characklis et al. 1990). However, at this point, there is no evidence that pathways used by aerobic bacteria to degrade compounds like 2,4-dichlorophenoxyacetic acid or 1,3-dichlorobenzene are functioning in the diclofop-methyl-degrading consortium.

Genes encoding for the targeted enzymes could not be detected by the catabolic primers designed in this study. However, given the large diversity of catabolic genes known for specific degradation steps, primer design, or selection can be problematic. For example, sixteen different genes encoding for catechol 2,3-dioxygenase were retrieved from the GenBank database, but only eight of them showed enough homology to allow the design of a PCR primer set. Up to four different classes of *bph* genes exist based on DNA:DNA hybridization experiments with known *bph* genes as probes (Furukawa et al. 1990; Williams and Sayers 1994; Gutierrez et al. 1996). In some instances, this diversity is due to the evolutionary time scale of exposure; microbes were exposed to phenolic compounds originating from the degradation products of plant material long before human industrialization (Powlowski and Shingler 1994). The enterobacteria are also known to produce phenols and methyl-phenols from tyrosine (Kumagai et al. 1970; Spoelstra 1977). Divergence of the phenol-degrading genes may reflect the different strategies developed with time by microorganisms to neutralize these toxic compounds. A great diversity in the phenol hydroxylase flavoproteins (single component phenol hydroxylases) has also been noted (Kukor and Olsen 1992). Designing additional PCR primer sets might be useful to determine the unique character of all steps in the degradation of diclofop-methyl by this nine member consortium, but detection will remain limited to genes with sufficient homology to known catabolic genes to be amplified by the primers. Additionally, if homologous genes are detected, their role in diclofop-methyl degradation would require confirmation by biochemical tests.

PCR amplification and sequencing of the 16S rDNA was performed to identify each isolate. The 16S rDNA sequence of the isolates was compared with all entries in the GenBank non-redundant nucleotide sequence database using both FASTA and BLASTN search programs. The best score for both programs along with the Biolog identifications, previously performed by Wolfaardt et al. (1994*b*), and the GenBank accession numbers for the 16S rDNA sequences of the isolates, are presented in Table 2. Generally, the results obtained by both search programs were very similar, if not identical. Homology scores against known 16S rDNA sequences were between 92 and 99.7%. The bacterial genus designation from the 16S rDNA identification matched well with the Biolog identification (based on substrate oxidation) for three of the isolates. The 16S rDNA based analysis was successful at identifying isolates 3 and 5, which was not possible with the Biolog system (Wolfaardt et al. 1994*b*). Most of the isolates belonged to the Pseudomonaceae and Enterobacteriaceae families. As the identity of isolate 1 was not firmly established either by the Biolog or by the 16S rDNA databases, it may represent a novel bacterial species. In general, use of substrate utilization patterns (Biolog system) is most successful with clinical isolates and has an identification rate for environmental isolates of >70% (Konopka et al. 1998), observations which are consistent with those in the present study.

Phenoxy herbicide degrading bacteria may be frequently found in soils and natural waters, however, studies of diclofop-methyl-degrading microorganisms are extremely limited. In the only other study of degradation of diclofop-methyl by soil isolates Grenier-Smith and Adkins (1996*a*) reported that a *Sphingomonas paucimobilis*, *Acinetobacter baumanni*, *Chrysomonas luteola*, *Pseudomonas aureofaciens*, *Pseudomonas cepacia*, and a *Pseudomonas fluorescens* were capable of growth when diclofop-methyl was supplied as sole source of carbon and energy. However, none of the isolates of Smith-Grenier and Adkins (1996*a*, 1996*b*) were capable of mineralization of diclofop methyl to CO_2. Indeed the majority of the isolates derived carbon and energy primarily from hydrolysis of the ester bond to produce diclofop acid. The authors did not assess the possible influence of cometabolites on diclofop-methyl degradation by their isolates. Cometabolism has been indicated as an important prerequisite for degradation of diclofop-methyl (Wolfaardt et al. 1994*c*). Members of these genera are commonly found to be capable of degradation of phenoxy acid herbicides (Kilpi 1980) and have also been reported as members of mixed cultures degrading mecoprop (Lappin et al. 1985). In general, previous studies have reported the predominance of Gram-negative phenoxy herbicide and

Table 2. Identification of the bacterial isolates based on the Biolog system and the 16S rDNA sequence analysis (FASTA and BLASTN program analysis servers).

Isolate (GenBank Accession No.)	Biolog identification[a]	16S rDNA identification based on FASTA analysis[b] [accession No.] (% identity/ nucleotides or nt)	16S rDNA Identification based on BLASTN analysis[c] [accession No.] (% identity/ nucleotides or nt)
1 (AF181568)	*Sphingobacterium thalopophilium**	*Flexibacter santi* [M62795] (92.3%/1470 nt)	*Flexibacter santi* [M62795] (93%/1257 nt & 92%/166 nt)
2 (AF181569)	*Xanthomonas maltophilia*	*Stenotrophomonas* sp. [AJ002814] (99.4%/1490 nt)	*Stenotrophomonas* sp. [AJ002814] (99%/1490 nt)
3 (AF181570)	no id	*Pseudomonas pseudoalcaligenes* [Z76666] (99.4%/1473 nt)	*Pseudomonas pseudoalcaligenes* [Z76666] (99%/1473 nt)
4 (AF181571)	*Pseudomonas paucimobilis*	*Sphingomonas* sp. [X87165] (99.7%/1438 nt)	*Sphingomonas* sp. [X87165] (99%/1438 nt)
5 (AF181572)	no id	*Sphingomonas* sp. [Z73631] (98.1%/1425 nt)	*Sphingomonas* sp. [Z73631] (98%/1425 nt)
6 (AF181573)	*Bacillus coagulans*	*Bacillus polymyxa* [D16276] (93.4%/1490 nt)	*Paenibacillus popilliae* [AF071860] (93%/1417 nt)
7 (AF181574)	*Enterobacter aerogenes**	*Klebsiella planticola* [X93216] (99.7%/1495 nt)	*Klebsiella planticola* [X93216] (99%/1495 nt)
8 (AF181575)	*Comamonas acidovorans*	*Comamonas* sp. [AJ002803] (99.3%/1483 nt)	*Comamonas* sp. [AJ002803] (99%/1483 nt)
9 (AF181576)	*Pseudomonas fluorescens*	*Pseudomonas monteilii* [AF064458] (99.3%/1518 nt)	*Pseudomonas monsselii* [AF072688] (98%/1514 nt)

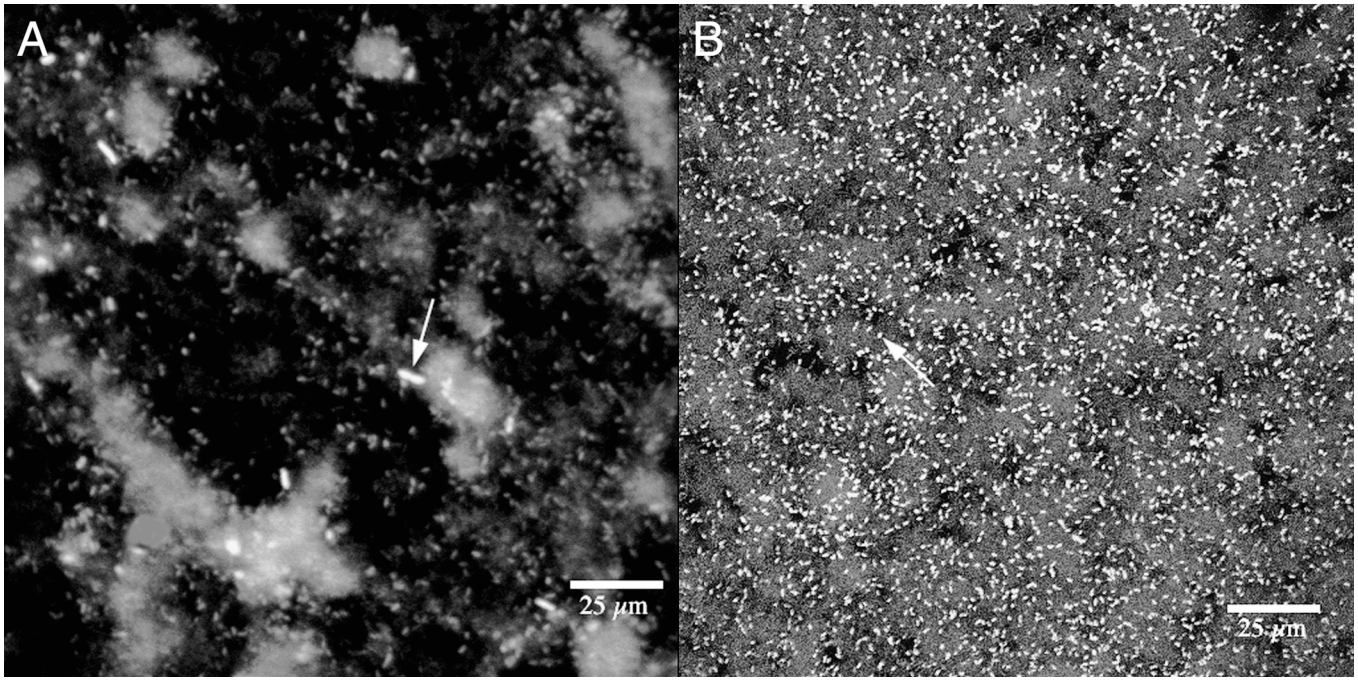
[a]From Wolfaardt et al. 1994*b*.
[b]Pearson and Lipman 1988.
[c]Altschul et al. 1990.
*Indicates doubtful identification.

Fig. 2. Location of the hybridization probes 4A–R and 6B–R in the 16S rDNA sequences of the consortium isolates. The consensus nucleotides in each sequence are shaded.

diclofop-methyl-degraders, but the present study also indicates the potential importance of Gram-positive bacilli in these communities.

Specific oligonucleotide probes were designed for isolates 4 and 6 based on the 16S rDNA alignment of all nine isolates (Fig. 2). The 16S rDNA alignment regions for the design of the probes were from nucleotides 648 to 665 for isolate 4, and from nucleotides 712 to 729 for isolate 6. The designed oligonucleotide probes specific for isolates 4 (4A–R) and 6 (6B–R) had the sequences 5′- ATA CCA GTC CAG TGA GCC -3′, and 5′- GTT ACA GGC CAG AAA GTC-3′, respectively. The specificity analysis of the designed probes, using the AMPLIFY software, was conclusive, i.e., no significant homology between the designed probe sequences and the 16S rDNA sequences of the other isolates was found. Thus, the designed oligonucleotide probes were used to perform in situ fluorescent hybridization (FISH) test trials in biofilm samples.

Fig. 3. In situ fluorescent hybridization (FISH) of the biofilm consortium. Samples were hybridized with either TAMRA NHS-labeled 6B–R and FAMS NHS-labeled Eub338 (A), or FAM NHS-labeled 4A–R and TAMRA NHS-labeled Eub338 (B). The target cells (isolate 6 in A, and isolate 4 in B) are indicated by arrows.



Specificity of the designed probes (4A–R and 6B–R) against their target DNA sequences was verified using the whole cell hybridization technique. A strong fluorescence was detected for isolates 4 and 6 with their labeled probes 4A–R and 6B–R, respectively. Only a weak response was detected for both cross-reactions, as well as for isolate 1 with both probes, corresponding to non-specific hybridization. These results confirmed the specificity of the designed probes (4A–R and 6B–R) against their target DNA sequences.

Application of the two specific probes in biofilms formed by the mixture of the nine isolates also indicated that they were specific and lacked cross-reactivity with other community members. Two typical fields showing the form and arrangement of the biofilm and the distribution of cells hybridized with labeled probes 4A–R and 6B–R are presented in Fig. 3. Although the structure of the mixed culture biofilms differed from those reported for the biofilm community by Wolfaardt et al. (1994*c*), it was possible to demonstrate that isolate number 6 had a diffuse distribution within the biofilm, occurring as individual dividing cells or small microcolonies, and contributing on average less than 1×10^{11} cells $\cdot$ cm^{-2} (Fig. 3A). In contrast, isolate 4, which had been shown to mineralize ^{14}C diclofop in the presence of cosubstrates, was extensively distributed within the biofilm and the predominant biofilm community member with up to 5×10^{11} cells $\cdot$ cm^{-2} (Fig. 3B). Thus application of these species-specific probes will allow detailed examination of the population dynamics within the biofilm community in response to treatment effects such as changes in nutrient source, community composition, presence of protistan grazers, providing a basis for ecological studies of this degradative community.

Conclusions

Molecular characterization of bacterial isolates from a diclofop-methyl-degrading biofilm consortium, with catabolic genes from known degradation pathways for structurally-related compounds, suggested that novel catabolic genes are functioning in the isolates. Specific fluorescent oligonucleotides were designed for two of the isolates following 16S rDNA sequencing and identification of each of the isolates. The distribution and abundance of isolates 4 and 6 in the biofilm consortium were examined by fluorescent in situ hybridization, validating the specificity of the probes, and demonstrating their potential for ecological studies of this degradative consortium.

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