Geobacter detection and enrichment of MtBE-degrading organisms from gasoline-contaminated soil and ground water

and

Chemotaxis of para-hydroxybenzoate degrading organisms

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Abstract

Two projects were undertaken to gain experience using a variety of culturing, molecular and other techniques. The aim of the first project was to obtain isolates from gasoline-contaminated groundwater and subsurface soil that could degrade methyl *tert*-butyl ether (MtBE) under aerobic or iron-reducing conditions, respectively. It was hypothesized that *Geobacter* species would be responsible for MtBE transformation under iron reducing conditions, were it to occur. This work was therefore complemented with DNA isolation from the soil samples and PCR using the *Geobacter*-specific Geo825R primer (and general bacterial 8F primer) and fluorescent *in situ* hybridization (FISH) using a FITC-conjugated probe of the same sequence. In the aerobic groundwater enrichments, growth in the presence of MtBE was only observed in the most contaminated sample, and gro in one MtBE transfer tube was finally observed on the last day of the course. Since the soil samples arrived with less than a week remaining in the course, the enrichments did not have a chance to become established, but *Geobacter* small subunit rDNA was amplified from DNA isolated from 6 and 10 ft depth sediment samples. FISH results were difficult to interpret, but the samples with the most fluorescence corresponded to sediment depths whose enrichments showed visible signs of iron reduction.

Three chemotaxis assays were used to test a variety of compounds on four marine p-hydroxybenzoate degraders. All four isolates gave identical results. Using the swim plate method, they all chemotaxed toward p-hydroxybenzoate, dextrose, glutamate, and succinate. The capillary and agarose plug assays were attempted to see if the isolates would chemotax toward compounds that they could not use as a sole growth substrate, however the results were ambiguous since the bacteria tended to lose their motility over the course of the assay and stick to the plug or move around by Brownian motion.

Geobacter detection and enrichment of MtBE-degrading organisms from gasoline-contaminated soil and ground water

Introduction

Geobacter species are gram-negative bacteria that are capable of coupling the complete oxidation of organic compounds with the reduction of metals. A number of electron acceptors can be used by these microorganisms, but the most relevant in the groundwater environment is probably the reduction of ferric iron. Although several species in different genera can use ferric iron as a terminal electron acceptor, the substrate range of *Geobacter* species under these conditions is broader than most. They may therefore be important in the natural attenuation of pollution at contaminated sites (Nealson and Saffarini, 1994).

MtBE is a fuel oxygenate that is highly water soluble and quite recalcitrant to biodegradation and so tends to persist in groundwater. There are a few published reports of degradation of MtBE under aerobic conditions (Bradley et al., 1999; Mo et al., 1997; Salanitro et al., 1994), but none under anaerobic conditions. This combination of water solubility and recalcitrance to degradation has lead to concern over contamination of drinking water supplies and the environment. For this reason, the use of MtBE as a fuel oxygenate has been banned in some states.

The balanced equation for the complete mineralization of MtBE under ferric iron reducing conditions is:

$$CH_3OC(CH_3)_3 + 9\ H_2O + 30\ Fe^{3+} \Rightarrow 5\ CO_2 + 30\ H^+ + 30\ Fe^{2+} \qquad (1)$$

The equations shows that acid will be produced (although hydroxide will also be released as Fe(III) is oxidized) in the reaction and that 30 ferric iron molecules are required to oxidize one MtBE molecule. The free energy of reaction is difficult to determine for this reaction since the form of iron and presence of other ligands will affect the result.

Methods and Materials

Samples

The groundwater samples were obtained from LaGrange, ME. The Bedrock sample had an initial MtBE concentration at last sampling of 35 ppb. MW was taken from just below a layer of free product, and may have been saturated with respect to various gasoline components. The well sediment core was taken from the MW site and arrived in plastic core sheaths of 2 ft lengths.

Media

Media Stock Solutions (composition used by J. Leadbetter):

100 X FW media base

Component	Amount
Water	1 L
NaCl	100g
$MgCl_2.6H_2O$	40 g
$CaCl_2.2H_2O$	10 g
KH_2PO_4	10 g
KCl	50 g

1 M MOPS buffer, pH 7.2, filtered

1 M sodium sulphide stored in the dark under N_2 at 4 °C

1 M Sodium Bicarbonate, autoclaved under 100 % CO_2 in serum bottles

100 X ammonium chloride

Component	Amount
Water	1 L
NH_4Cl	25g

1000 X EDTA-Chelated Trace Elements

Component	Amount
Water	987 mL
EDTA	5200 mg
Adjust pH to 6.0 with NaOH	
$FeSO_4.7H_2O$	2100 mg
H_3BO_3	30 mg
$MnCl_2.4H_2O$	100 g
$CoCl_2.6H_2O$	190 mg
$NiCl_2.6H_2O$	24 g
$CuCl_2.2H_2O$	2 mg
$ZnSO_4.7H_2O$	144 mg
$Na_2MoO_4.2H_2O$	36 mg
Sodium vanadate	25 mg
$Na_2SeO_3.5H_2O$	6 mg
$Na_2WO_4.2H_2O$	8 mg
Filter to sterilize	

1000 X Vitamin B_{12} solution

Component	Amount
Water	100 mL
Cyanocobalamin	100mg
Titrate with HCl until dissolved, filter sterilize and freeze in 10 mL aliquots	

1000 X 12-Vitamin Solution

Component	Amount
Phosphate buffer, 10 mM, pH 7.2	100 mL
Riboflavin	10 mg
Thiamine-HCl	100 mg
L-Ascorbic acid	100 mg
D-Ca-pantothenate	100 mg
Folic acid	100 mg
Niacinamide	100 mg
Nicotinic acid	100 mg
4-Aminobenzoic acid	100 mg
Pyridoxine-HCl	100 mg
Lipoic acid	100 mg
NAD	100 mg
Thiamine pyrophosphate	100 mg
Titrate with NaOH until dissolved, filter sterilize and freeze in 10 mL aliquots	

For 1 L media

Constituent	Groundwater (aerobic)	Sediment (anaerobic)
100X FW base	10 mL	10 mL
Ammonium chloride stock (100 X)	10 mL	10 mL
Sodium sulphate stock (1M)	0.5 mL	
Autoclave (in Widdel flask for anaerobic media) and cool		
$NaHCO_3$ (1M)		30 mL
MOPS (pH 7.2, 1 M)	10 mL	5 mL
12-vitamin solution	1 mL	0.5 mL
Trace elements	1 mL	0.5 mL
Sodium sulphide (1M)		1 mL
Amorphous ferric hydroxide (about 800 mM)		0.625 mL/5 mL
MtBE (150 mM, added at time of inoculation)	50 µL/5 mL	50 µL/5 mL
Toluene (added neat at time of inoculation)	1 µL/5 mL	

When it was first described, *Geobacter* was enriched for and cultured on a slightly different medium (Snoeyenbos-West et al., 2000). All of the major constituents are

present in the media used here, but a few more trace elements and vitamins were added, and yeast extract was excluded.

After autoclaving, the anaerobic media was cooled under a stream of anoxic gas. The remaining media constituents were added through the ports at the top of the Widdel flask and then the ports were closed and the media dispensed. Electron acceptors and C sources were added separately later. Homoacetogen enrichments, which were set up to see if MtBE would be converted to tert-butyl alcohol by this group of organisms, did not receive any Fe(III) in addition to the HCO_3^- in the media.

Ferric iron hydroxide was made by titrating $FeCl_3$ with NaOH to pH 7.0. This is amorphous, so it should be reasonably bioavailable. It was washed 3X with DI water to remove the NaCl that is formed and the pH was then checked to make sure it was still at about 7. Another source of Fe that can be used is $FePO_4$, which is more bioavailable and less tightly bound. The iron used in this experiment was not autoclaved to avoid physical changes brought about by the heating and pressure. The approximate concentration of the iron slurry was 800 mM, so 0.625 mL was added to 5 mL media in tubes.

Five mL media, the C source and 0.625 mL Fe(III) slurry, when used, were added to Balch tubes, and the headspace was flushed with N_2:CO_2 (90:10) prior to being stoppered with butyl rubber stoppers. About 1 g soil was added to each serum bottle. The iron reducing enrichments were to be observed for iron reduction and turbidity, and the media pH monitored. DNA extracted from subsamples was also intended to be monitored by PCR for *Geobacter* enrichment. The enrichments without iron (homoacetogens) were to be monitored for production of TBA, acetate and loss of HCO_3^- when they showed signs of activity. The late arrival of the samples did not allow completion of this part of the project.

The anaerobic incubation tubes were kept upside down to prevent oxygen from entering and loss of the substrates through the butyl rubber stoppers. In addition, controls were made with 1) no C source, 2) no soil. The latter control is intended to show the abiotic loss of the substrates. The former is to control for growth due to other factors (OC in the soil, etc.). Samples were incubated from groundwater sediment taken from depths of 2, 4, 6, 8 and 10 feet.

Groundwater (aerobic) enrichments

Since the groundwater samples were oxic, and *Geobacter* species are obligate anaerobes, aerobic enrichments were made for MtBE degraders from these samples. Transfers and filters were made into the media described above. The original tubes were made with undiluted groundwater and the added media components. The following enrichments were prepared:

1, 2	Bedrock groundwater +/- MtBE
3, 4	MW groundwater +/- MtBE
5	Bedrock filter + MtBE
6	MW filter + MtBE
7, 8	Bedrock groundwater +/- toluene
9, 10	MW groundwater +/- toluene
11, 12	Bedrock or MW groundwater, no C source added

13, 14 MtBE or toluene, no groundwater added

Samples were agitated at 28 °C and checked daily for turbidity and by microscopy.

Sediment (anaerobic) Enrichments

The following enrichments were prepared:

Iron reducers:

1, 2	2 ft depth sediment +/- MtBE
3, 4	4 ft depth sediment +/- MtBE
5, 6	6 ft depth sediment +/- MtBE
7, 8	8 ft depth sediment +/- MtBE
9, 10	10 ft depth sediment +/- MtBE
11	MtBE, no sediment

Homoacetogens:

12, 13	4 ft depth sediment +/- MtBE, no Fe(III)
14, 15	6 ft depth sediment +/- MtBE, no Fe(III)
16, 17	8 ft depth sediment +/- MtBE, no Fe(III)
18	MtBE, no Fe(III), no sediment

The samples were agitated at 28 °C.

DNA extraction/PCR

Total DNA was extracted from groundwater sediment at 4, 6, 8 and 10 ft depths using the Mo Bio Ultra Clean Soil DNA kit or the Mega Prep kit from the same company according to the manufacturere's instructions. The resultant DNA was amplified by PCR using the Geo825R primer (Snoeyenbos-West et al., 2000) and the bacterial 8F primer (SD Bact 0008F20). General bacterial DNA was also amplified by replacing the Geo825 primer with a universal primer (U1392R). The temperature regime used was 94 °C/5 min followed by 25 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C and 1 minute at 72 °C. A final elongation step (72 °C for 7 minutes) was followed by holding at 4 °C. The amplified DNA was run on 1.25% agarose gels and the bands were stained with GelStar.

Fluorescent in situ hybridization (FISH)

Hybridization Buffer:

Stock	Amount	Final Conc.
ddH_2O	295 mL	
3 M NaCl	150 mL	0.9 M
1 M Tris, pH 8	50 mL	100 mM Tris
10 % SDS	5 mL	0.1 % SDS

Wash Buffer:

Stock	Amount	Final Conc.
ddH_2O	357 mL	
3 M NaCl	83 mL	0.9 M
1 M Tris, pH 8	50 mL	100 mM
0.5 M EDTA	5 mL	5 mM
10 % SDS	5 mL	0.1 %

1 g of sediment from depths 2, 4, 6, 8, and 10 feet were added to tubes containing 0.1 M pyrophosphate solution and glass beads. These were vortexed at maximum speed for 1 minute. 100 µL of this was transferred to tubes containing 3% formaldehyde in particle-free (0.2 micron filtered) water. From these fixed preparations, 25 µL were mixed with 10 mL particle-free water and filtered through 0.2 µm polycarbonate filters placed on top of a GF/C filter on the housing. The filters were then treated with 50% ethanol (in the

filter housing with no vacuum applied) to improve the permeability of the cells. The filters were dried and cut in four.

The hybridization buffer was mixed with probe (Geo825-FITC, 50 ng/μL) at 24:1 and a 25 μL drop was added to each slice. These were placed in a parafilm-sealed pipette tip box with a few drops of water to maintain the humidity in the chamber and incubated at 37 °C for 6 hours. The filter slices were then flushed with wash buffer (holding the filter slice on the slide using forceps, then the slide was flooded with pre-warmed wash buffer and placed at the wash temperature for 10 minutes. The wash was repeated, then the filter slices were flushed with water and dried. Two wash temperatures were used: 47 °C and 52 °C. The third chosen wash temperature (57 °C was not done because of the low signal found using the less stringent wash conditions). The wash temperatures were chosen as the T_d and plus or minus 5 °C. The T_d was calculated from the following formula (Ramsing et al., 1993):

$$T_d = 81.5 + 16.6 \log_{10}M + 0.41[\%(G+C)] - 820/n - 0.7(\%f)$$

M = molar cation concentration (0.5 M)
% (G+C) = GC content of the probe (55.9% for Geo825R)
n = length of probe (17 bases)
%f = formamide concentration (0)

The fourth slice was stained with acridine orange (DAPI binds non-specifically to particles, so it is not ideal for sediments, but AO can't be used as a counterstain on the same filter since it fluoresces at the same wavelength as FITC)) to see the total number of cells present.

Results and Discussion:

Groundwater (aerobic) enrichments

Toluene enrichments set up at the same time as the MtBE degradation enrichments showed growth in all tubes, although samples from the MW site came up much more quickly than the Bedrock samples. Microscopic examination of the samples showed that there were much higher numbers of organisms in the MW water. In the tubes containing MtBE, the only sample to show any signs of growth was the 0.2 μm filtered MW groundwater tube. This was transferred to a new tube of media, but the transfer did not grow, indicating that most of the growth was probably at the expense of other material on the filter. A later transfer from the initial filter tube didn't show any signs of growth until one week later, when some motile cells were observed microscopically. This observation was made on the last day, however, so no further characterization or transfers were made.

It is likely that nothing came up in the first transfer because the bulk of the cells accounting for the turbidity in the culture were using other substrates. Once these substrates were exhausted, however, the enrichment for MtBE degraders could begin. The cells in the second transfer were highly motile short, fat rods. These will continue to be monitored and additional transfers made from the original inoculum to see if an MtBE-degrading isolate or consortium can be maintained.

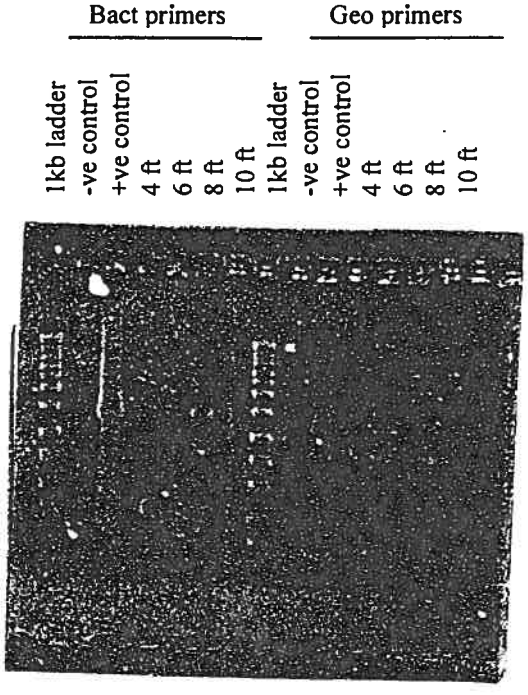
Sediment (anaerobic) Enrichments

The enrichments were not going for long enough to determine if iron reduction could be coupled to MtBE degradation (or if homoacetogens in groundwater sediment could demethylate MtBE). From the colour of the Fe(III)-reducing media, however, it was clear that Fe(III) reduction was occurring (sediment changes from bright orange to brown). The order of activity was as follows: 6 ft> 8 ft> 2, 4 ft> 10 ft depth. The reduction of iron occured whether or not MtBE had been added to the medium, so the reduction could not be attributed to growth at the expense of MtBE (at least until MtBE measurements in the media are made). These enrichments will also continue to be studied.

DNA extraction/PCR

The initial small-scale DNA purification step did not yield DNA that was amplified using the Geo825R primer except for a faint band of the appropriate size in the 6 ft sample. There are a couple of possible explanations: there were no *Geobacters* in the other samples, the bacterial population was low and the concentration of target DNA in the final extract was too low, or the contaminants in the groundwater inhibited either the PCR reaction or efficient extraction of the DNA. A larger scale DNA extraction was done using the Mega Prep kit and PCR products of the right size were obtained in the 6 and 10 ft depth samples. General bacterial primers used with these same samples, however, only amplified the right sized band in the 8 ft sample (fig. 1). Since *Geobacter* species rDNA should also be amplified using the latter primer set, it seems likely that the DNA needs to be further purified and that the PCR conditions must be optimized. Given the clear evidence of microbial activity from all the 2-8 ft enrichments, bands should have been obtained from all of those samples using the general bacterial primers. This DNA will be further purified and the PCR repeated. A more reliable DNA extraction/purification protocol is required to use this as a method of monitoring the enrichments for the presence of *Geobacters.*

Fig 1. Agarose gel with PCR reactions



Fluorescent in situ hybridization (FISH)

Since this was the first attempt to use the *Geobacter* probe for FISH, the conditions were chosen empirically and there were no appropriate controls. It is therefore not all that surprising that the results were ambiguous. All of the samples had some fluorescence associated with the filters. Much of what was seen was fairly randomly shaped, however, and might have simply been debris. The patterns were similar whether the filters were washed at low or medium stringency. Very little was seen at 10 ft depth in the sediment column. At 2 feet there might have been a few cells, but mainly the fluorescent material was randomly shaped debris. At 4 feet there was more of what looked like cells and more still at 6 ft, but these particles were still outnumbered by the debris. The number of cell-shaped (and total) particles fell again at 8 ft depth. The peak at 6 ft corresponds to the peak in iron reduction activity in the enrichments, however the results were so difficult to interpret (and even more difficult to photograph!) that this relationship remains speculative.

Unfortunately time didn't permit the continuation of this work, though the presence of *Geobacter* looked promising. More time was needed to determine if they could degrade MtBE. As in the aerobic enrichments, if MtBE degradation was occurring, it would most likely begin slowly, after the other contaminants in the original sample were exhausted.

Chemotaxis of para-hydroxybenzoate degrading organisms

Introduction

These marine isolates were obtained from Eel Pond during the first part of the course. PCR of p-OHB2 rDNA and partial sequencing of the fragment showed that it clustered fairly closely with *Marinomonas* and *Oceanospirillum* species. *Oceanospirillum* species are rigid, helical, gram negative bacteria, which are motile by bipolar tufts of flagella (Pot et al., 1999-). The *Marinomonas* species were recently separated from *Alteromonas* species and some members of these groups have been isolated from the guts of marine animals as well as associated with submerged materials, seawater and sediment (Gauthier and Brettmayer, 1999).

Methods and Materials:

The same media components were used as described in the previous section, except that a saltwater media base was used (also J. Leadbetter recipe) and a 1M sodium sulphate solution was used as the S source stock.

1 X SW media base

Component	Amount
Water	20 L
NaCl	400g
$MgCl_2.6H_2O$	60 g
$CaCl_2.2H_2O$	3 g
KH_2PO_4	4 g
KCl	10 g

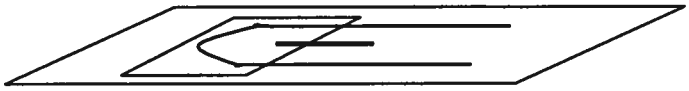
Constituent	Liquid media	Plates	Swim plates
Sea water base (1X)	1 L	1 L	1 L
MOPS (1M, pH 7.2)	10 mL	10 mL	10 mL
Sodium Sulphate (1M)	0.5 mL	0.5 mL	0.5 mL
Ammonium Chloride (1M)	10 mL	10 mL	10 mL
Washed Agar		15 g	3 g
Autoclave – cool to 60 °C			
EDTA-trace elements	1 mL	1 mL	1 mL
12 Vitamin mix	1 mL	1 mL	1 mL
Vitamin B12	1 mL	1 mL	1 mL
0.5 M 4-OH Benzoate	3 mL	3 mL	1 mL

The swim were poured and allowed to stand agar side down since they're too loose to turn over. When other C sources were used, they were applied at the same concentration as p-hydroxybenzoate. Solvents were added to liquid media (in screw cap tubes) neat at 5 µL/5 mL C-free media.

Chemotaxis Assays

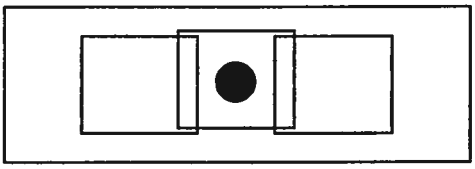
Swim plate assay: Low-agar plates were inoculated with liquid culture by stabbing the centre of the plate with a pipette tip prior to adding 25 μL of the culture. For inoculations from colonies, a sterile toothpick was used to transfer a small amount of material to the centre of the plate (again, stabbed so that the cells move through, rather than on top of the agar. Pictures were taken of plates illuminated from below with fluorescent light (bucket of light) using a Kodak DC290 digital camera and the EDAS200 system.

Capillary assay: motile liquid cultures were centrifuged gently (5000g) and resuspended in artificial sea water to an OD of approximately 0.2 for the assay. A U shaped, closed-ended capillary was placed on top of a glass microscope slide and covered with a coverslip. A washed cell suspension was pipetted into the space until the coverslip was held in place by surface tension. The cell suspension was made by gently centrifuging (5000g) the cells and resuspending in SW media base. A small capillary was heat sealed at one end, then passed through a flame and into the stock solution of the test compound to draw up some of the liquid as the capillary cooled. The capillary was then placed in the cell suspension and the behaviour of the cells was observed using the 10 X objective and either pseudo-dark field illumination or phase contrast.



The test compounds were p-hydroxy benzoate, phenol, succinic acid, glucose, benzene, m-cresol, xylene, and ammonium chloride. These were used at 0.5 M or neat, for the solvents).

Agar plug assay: This assay is well described by Yu and Alam (1997). Plugs were made of 2% low melting point agarose in SW media base. Test compounds were added at about 5 mM and the mixture was coloured by dipping a dry toothpick into Coomassie brilliant blue crystals and stirring the agarose to mix. The melted agarose-attractant mixtures were kept in a 60 °C water bath throughout, and the slides were kept on a hotplate at the lowest setting. (The alternative is to keep the agarose slightly hotter and just work very fast.) A 10 μL drop of the warm agarose mixture was placed on a warmed slide between two coverslips (strips of plastic can also be used). A third coverslip was quickly placed on top of the agarose drop and the slide was removed to the counter to allow the agarose to harden.



The space between the middle coverslip and the slide was flooded with washed cell suspensions in SW media base (.01% glycerol can be added if the cells lose their motility

before the end of the assay without any fuel). Pictures were taken each minute for 15 minutes, and the appearance of white bands of cells around the plug was sought. This was confirmed by observing under the microscope (10X objective).

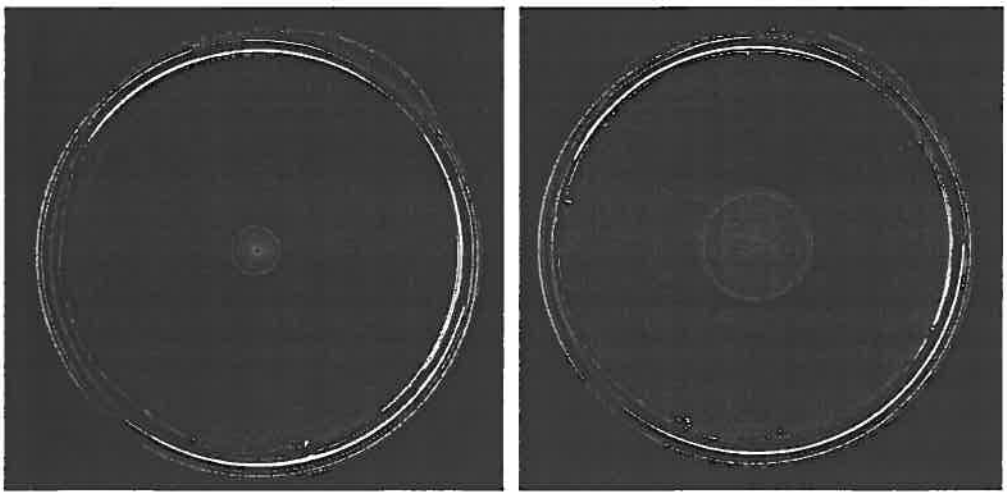
Results and Discussion:

The isolates that were used were all obtained from Eel pond. The ones designated pOHB1, pOHB3 and pOHB4 were similar: short, motile rods that form small creamy white colonies on SW-pOHB agar plates, which spread at the edges with age. POHB4 was slightly more difficult to get started in liquid culture but they tended to be slightly faster and to remain motile for longer. POHB2 was a spirillum which also has a smaller coccoid form that dominates as the culture ages. This change in cell morphology was the source of some confusion at first, however it is typical of *Oceanospirillum* strains (although the coccoid form predominates for most *Oceanospirillum* species only once the culture has aged for 1-2 weeks, as opposed to 2 days observed here (Pot et al., 1999)). None of the isolates grew as dense liquid cultures on pOHB or any of the other growth substrates that were offered and they tended to lose their motility and begin to lyse after two or three days in liquid culture.

The swim plate assays were the easiest ones to use and interpret. This method is limited to substrates that the bacteria can grow on, however. Volatile substrates are also problematic since volatilization and dissolution into the plates would prevent the formation of a good gradient. Slow growth would also make it difficult to use this particular assay. These results are, however, less ambiguous than for the other assays since it was less prone to disruption due to vibrations or brownian motion.

In all assays, plates with pOHB1, 3 and 4 looked similar. With parahydroxybenzoate in the medium, pOHB1,3 and 4 produced large circles while the spreading of pOHB2 was more limited. The pictures in figure 2 show a comparison of pOHB2 and pOHB4 swim plates with para-hydroxybenzoate as the chemoattractant. The plates were inoculated at the same time.

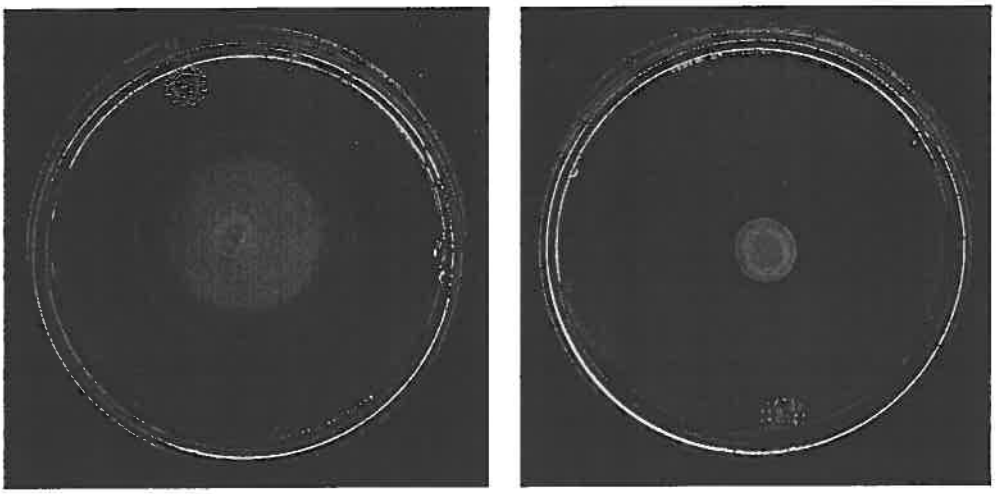
Figure 2: p-hydroxybenzoate swim plates



pOHB2 POHB4

When other compounds were offered as chemoattractants, pOHB1,3 and 4 spread to a lesser extent than they did with para-hydroxybenzoate, and the results were comparable to those obtained with pOHB2. Figure 3 shows pOHB4 on swim plates containing pOHB and dextrose as chemoattractants. Spreading in the dextrose plate is much more limited than in the pOHB plate.

Figure 3: pOHB4 chemotaxis on pOHB and dextrose



pOHB Dextrose

For swim plate inoculated with pOHB2, all of the chemoattractants caused spreading to a similar extent. The results of the swim plate assays are summarized in Table 1.

Table 1. Chemotaxis results from swim plates

Compound	pOHB1	pOHB2	pOHB3	pOHB4
pOHB	+	+	+	+
Benzoate	-	-	-	-
Catechol*	-	-	-	-
Xylene*	-	-	-	-
Cresol*	-	-	-	-
Toluene*	-	-	-	-
Benzene*	-	-	-	-
Glutamate	+	+	+	+
TMB	-	-	-	-
Phenol	-	-	-	-
Glucose	+	+	+	+
Succinate	+	+	+	+

POHB 2 seemed to be growing slowly in the phenol and toluene plates, and *perhaps* in the cresol and catechol plates after several days, but there was never any sign of movement away from the inoculation site. No growth of pOHB2 was observed on any of

these substrates when the cells were grown in liquid culture (see Table 2). In general, the cells looked healthier coming from plates than from liquid culture. The liquid cultures also never exceeded an A_{600nm} of about 0.3 or 0.4 and growth was fastest on pOHB. The compounds marked with asterisks were tried despite being volatile, in the case of the solvents, or unstable (catechol), so the negative results were not surprising. What was strange was the lack of growth on benzoate, being so similar to pOHB.

Table 2. Growth in liquid media (5 days at RT):

Compound	1	2	3	4
POHB	+	+	+	+
Glutamate	+	+	+	+
Succinate	+	+	+	+
Dextrose	+	+	+	+
Benzoate	-	-	-	-
TMB	-	-	-	-
Toluene	-	-	-	-
Benzene	-	-	-	-
Xylene	-	-	-	-
Cresol	-	-	-	-
Benzoate	-	-	-	-
Phenol	-	-	-	-

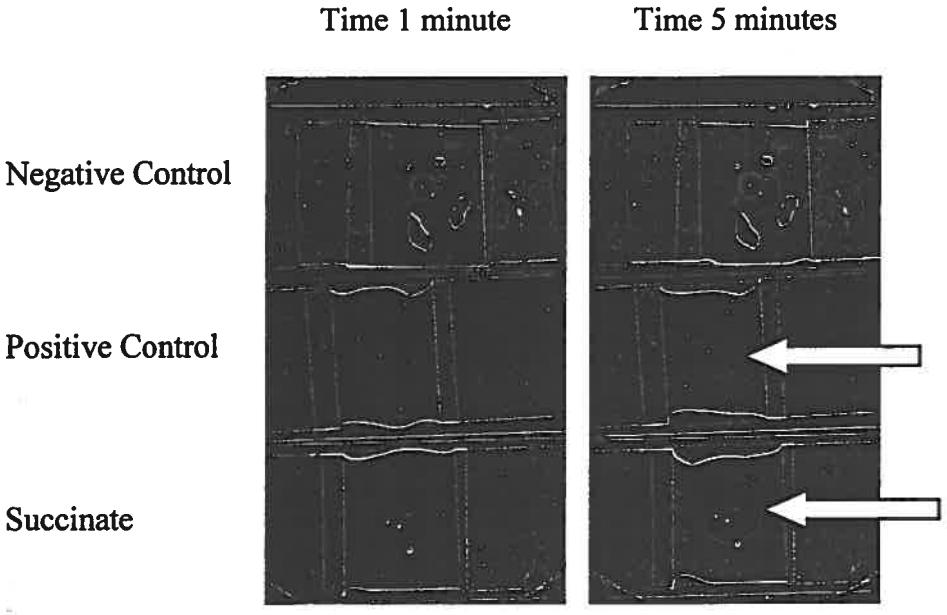
Several attempts were made to use the other two assays to determine chemotaxis to aromatic solvents and other compounds. Despite a number of attempts using cultures at different growth stages, clear results were not obtained for most compounds. The cells were always observed for motility before starting an experiment. They were then carefully washed (low-speed centrifugation) and suspended in SW media base for the assay. Typically, only a single assay could be done before the cells lost their motility. Since the window of vigorous motility in liquid culture was short, it was difficult to do the assays (particularly because the cells took 2 days to grow up and a positive control, pOHB, was required to see that the assay and cells were working). This was a problem for both the capillary tube assay and the agarose plug assay.

The capillary tube assay was tried a number of times. The cells usually responded to pOHB, but vibrations in the lab bench and brownian motion of the cells that were no longer motile made it difficult to separate real motility as a response to the chemoattractant from mass movement of the liquid. For this reason it was very difficult not to bias the results since the response to a number of the compounds was already "known". The assay should also be optimized for each compound so that the concentration that is presented is not so high that it inhibits the bacteria, while being sufficiently high to produce the effect.

The agarose plug assay was done as described in the methods section, and the results were confirmed by checking for rings of cells by microscopy. In many cases this was necessary since no obvious rings were visible on the slides. Only tests using pOHB1 were successful using this approach since the other cultures were not sufficiently motile, and the non-motile cells seemed to get stuck to the agarose plug when they bumped into it by brownian motion, even in the negative controls (no test compound). An example of

the agarose plug assay using pOHB1 is shown in figure 4. No test compound in the plug is the negative control, pOHB is the positive control and succinate is the test compound. The arrows indicate the white ring of cells around the plugs. Several other attempts at this assay were unsuccessful.

Figure 4: Agarose plug assay using pOHB1



It appeared that pOHB1 chemotaxed toward benzoate in addition to succinate, dextrose, pOHB and glutamate. Catechol and TMB gave ambiguous results. It could be that some of the assays were difficult to interpret because the cells didn't have enough fuel to maintain their motility in the absence of a C source, but addition of glycerol to the SW didn't improve the success rate. In future, it might be wise to try scraping cells from a plate and resuspending them in SW for the assays since the cultures seemed to remain robust for longer on solid media, and preparing the cells for each individual assay immediately prior to beginning.

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