Demonstration of chemotaxis of *Ralstonia* sp. SJ98 towards *p*-nitrophenol in soil

**Introduction**

Bacterial chemotaxis is the movement of bacteria under the influence of a chemical gradient, either toward or away from the gradient. This phenomenon helps bacteria to find optimal conditions for growth and survival. Chemotaxis-mediated biodegradation of several pollutants such as naphthalene, nitroaromatic compounds and chloroaromatic compounds have been reported (Parales and Harwood, 2002; Pandey and Jain, 2002; Bhushan et al., 2004). It has been therefore argued that this phenomenon might have a role in bioremediation applications (Pandey and Jain, 2002). Some assays have been developed to show bacterial chemotaxis in semi-solid medium, e.g. swarm assay, drop assay, agarose-plug assay, etc (Bhushan et al., 2000; Samanta et al., 2000; Pandey et al., 2002; Bhushan et al., 2004). Recently, Olson et al. (2004) reported bacterial chemotaxis toward trichloroethylene (TCE) in porous medium using magnetic resonance imaging and have predicted that this phenomenon may be potentially exploited to improve rates of *in situ* biodegradation in the subsurface environment, particularly when the pollutant is dissolved in water trapped in low-permeability formations or lenses. However, no attempts have been made to demonstrate bacterial chemotaxis in soil or other heterogenous medium and this continues to a fertile area of research.

Several methods have been previously described to monitor the survivability of the introduced microorganism in soil during laboratory and field experiments such as plate counting or by molecular techniques such as polymerase chain reactions (PCR). The application of selective plate counting techniques are limited due to the difficulties in culturability of certain microbes (Oliver et al., 1995; Heidelberg et al., 1997; Kozdroj and van Elsas, 2001) and PCR techniques, although sensitive, lack specificity (Thiem et al., 1994; Jjemba et al., 2006). Therefore, newer and recent techniques have been employed to study the spread and survivability of organisms in soil using fluorescent stains such as 4,6-diamino-2-phenyllindole (DAPI), 5-(and-6)-carboxytetramethylrhodamine (TAMRA), fluorescamine, etc (Fleminger and Shabtai, 1995; Fuller et al., 2000). New fluorescent compounds that allow cells to be stained
without the loss of activity and viability, and are stable over a long time span have been
discovered for staining cells or cellular components. Fuller et al. (2000) critically
evaluated the labeling of bacteria with numerous fluorescent stains and developed an
easy and effective high-throughput detection procedure for quantifying fluorescent-
labeled cells during in situ transport experiments. Similar techniques are now being
employed by researchers for monitoring and quantifying environmentally released
microbes (Hansen et al., 2001; Klauth et al., 2004).

For the first time, qualitative and quantitative assays for bacterial chemotaxis in
soil microcosm and its role in pollution abatement has been demonstrated in this work.
Using techniques such as plate counting, vital staining followed by fluorescence
activated cell sorting (FACS) and slot blot hybridization experiments the movement of
chemotactic cells though soil has been quantified over a given time frame.

**Bacterial chemotaxis**

Motile bacteria are able to elicit tactic responses to stimulants in their
surrounding environment (Lengeler et al., 1999; Parales and Harwood, 2002). Stimulants
include chemicals (chemotaxis), light (phototaxis), oxygen (aerotaxis), cellular energy
levels (energy taxis), pH (pH taxis), osmolarity (osmotaxis), and temperature
(thermotaxis). Responding to stimulants allows a bacterium to move toward an
environment that is most suitable for its survival and growth (Taylor et al., 1999; Pandey
and Jain, 2002). The tactic motion of bacteria could have macroscopic impacts such as
pathogenesis and symbiosis (Bashan, 1999; De Weert et al., 2002; McGee et al., 2005;
Terry et al., 2005), the formation and structure of bacterial communities, oceanic nutrient
cycling (Blackburn et al., 1998; Barbara and Mitchell, 2003 a,b), and pollutant
biodegradation (Table 1). Examination of the nucleotide sequences of bacterial genomes
reveals that a bacterium can possess many receptors for taxis (Alexandre et al., 2004).
Therefore, motile bacteria probably have the ability to integrate multiple stimuli to find
an optimal environment. The optimal environment for a bacterium is one in which
energy generation is maximum, such as when there is a balance between the amount of
electron donor and electron acceptor available (Zhulin et al., 1997; Taylor et al., 1999).
Since the chemoeffectors to which bacteria are attracted (chemoattractants) are often
electron donors bacteria consume, metabolism of the chemoattractant in the cell produces
a gradient of electron acceptors to which the bacteria can also respond.
Flagella is indispensable for bacterial chemotaxis. The bacterial flagellar motor is a rotary molecular machine which rotates the helical filaments to propel various species of swimming bacteria. The rotor is a set of rings up to 45 nm in diameter in the cytoplasmic membrane; the stator contains about ten torque-generating units anchored to the cell wall at the perimeter of the rotor. The chemotactic swimming is a result of rotation of flagella at speeds of app. 18,000 rpm and it is powered by the proton motive force (DeRosier, 1998). Flagellar motors are reversible in nature that help to change bacterial tumbling into a directional swimming by reversing the flagellar rotation from clockwise to counterclockwise direction (DeRosier, 1998; Parkinson, 1993). An environmental stimulus, e.g., light, oxygen, chemical, etc., is sensed by a receptor and signal(s) in the form of two-component regulatory systems is transmitted to the flagellar motors, which then move in the required direction (Parkinson, 1993; Bren and Eisenbach, 2000) thereby propelling bacteria toward or away from it.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Pollutant(s)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agrobacterium radiobacter</em> J14a</td>
<td>Atrazine</td>
<td>Park et al., 2003</td>
</tr>
<tr>
<td><em>Clostridium</em> sp. EDB2</td>
<td>RDX, CL-20, HMX</td>
<td>Bhushan et al., 2004</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. ADP</td>
<td>Atrazine</td>
<td>Park et al., 2003</td>
</tr>
<tr>
<td><em>P. putida</em> PRS2000</td>
<td>Aminobenzoate, nitrobenzoate, 3-4-chlorobenzoate</td>
<td>Parales, 2004</td>
</tr>
<tr>
<td><em>P. putida</em> F1</td>
<td>Benzene, toluene, trichloroethylene, ethylbenzene, isopropylbenzene, perchloroethylene, dichloroethylene, trifluorene, naphthalene</td>
<td>Parales et al., 2000</td>
</tr>
<tr>
<td><em>P. putida</em> G7</td>
<td>Naphthalene</td>
<td>Grimm and Harwood, 1997</td>
</tr>
<tr>
<td><em>Ralstonia</em> sp. SJ98</td>
<td><em>p</em>-Nitrophenol, 4-nitrocatechol, o-nitrobenzoate, <em>p</em>-nitrobenzoate, 3-methyl-4-nitrophenol, 1, 2,4-dinitrophenol, 2,5-dinitrophenol, 2,6-dinitrophenol, 3,5-dinitrobenzoate</td>
<td>Pandey et al., 2002</td>
</tr>
<tr>
<td><em>R. eutropha</em> JMP134</td>
<td>2,4-Dichlorophenoxyacetate</td>
<td>Hawkins and Harwood, 2002</td>
</tr>
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Table 1. Pollutants that are known to act as chemoattractants to hydrocarbon utilizing bacteria
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Bacterial chemotaxis toward pollutants and its role in bioremediation

The first step in bioremediation is the bioavailability of a compound to the bacterial cells which may be facilitated by chemotaxis. Bioavailability of organic contaminants has been identified as a major limitation to efficient bioremediation of contaminated sites (Head, 1998; Stelmack et al., 1999). Contaminated soils contain a separate non-aqueous-phase liquid that may be present as droplets or films on soil surfaces. Biodegradation takes place more readily when the target contaminants are dissolved in an aqueous medium (Stelmack et al., 1999, Pandey and Jain, 2002; Law and Aitken, 2003). Many pollutants, especially those that are hydrophobic, are virtually insoluble in water and remain adsorbed in the nonaqueous-phase liquid (Head, 1998; Stelmack et al., 1999; Parales and Haddock, 2004). In order for biodegradation to occur, bacteria must have access to the target compounds either by dissolution of the target compounds in the aqueous phase or by adhesion of the bacteria directly to the non-aqueous phase liquid water interface. In order to gain access to such adsorbed pollutants, degradative bacteria need to find and attach to surfaces possibly through biofilm formation. Chemotaxis has been shown to play an important role in biofilm formation in several microorganisms (O'Toole and Kolter, 1998; Pratt and Kolter, 1998; Prigent-Combaret et al., 1999; Watnick and Kolter, 1999) that may guide a bacterium to swim toward nutrients (hydrophobic pollutants) adsorbed to a surface, followed by attachment using its flagella. It has been shown that flagella are required for attachment to abiotic surfaces, thus facilitating the initiation of biofilm formation (Pratt and Kolter, 1998; Stelmack et al., 1999). In addition, chemotaxis and/or motility might be required for the bacteria within a developing biofilm to move along the surface thereby facilitating growth and spread of the biofilm (Fenchel, 2002).

In past few years several microbes have been reported to be chemotactic towards different environmental pollutants (Table 1). Some aromatic hydrocarbons, components of petroleum and petroleum products behave as chemoattractants for several bacteria. It is relatively easy to isolate aerobic bacteria that degrade simple aromatic hydrocarbons such as benzene, toluene or naphthalene. Toluene is a good chemoattractant for three toluene-degrading bacterial strains (P. putida F1, Ralstonia pickettii PKO1 and Burkholderia cepacia G4) each with a different toluene degradation pathway (Parales et al., 2000). In each strain chemotaxis to toluene was induced during growth in the presence of toluene. P. putida F1 was also shown to respond to the growth substrates
benzene and ethylbenzene, as well as to aromatic hydrocarbons, such as isopropylbenzene and naphthalene, that do not serve as its growth substrates. An analysis of a series of catabolic mutants of P. putida F1 that are blocked at various steps in the toluene degradation pathway demonstrated that metabolites of toluene degradation do not behave as chemoattractants. In addition, regulatory mutants of P. putida F1 that lack the two-component regulatory system required for induction of the toluene degradation genes were not chemotactic to toluene.

Naphthalene is another aromatic hydrocarbon that serves as chemoattractant for some strains, e.g. Pseudomonas putida G7, Pseudomonas sp. strain NCIB 98164 and P. putida RKJ1 (Marx and Aitken, 1999; Samanta and Jain, 2000; Parales and Harwood, 2002; Dennis and Zylstra, 2004). The response in all three strains is dependent on the presence of the resident naphthalene catabolic plasmid; cured strains were not attracted to naphthalene (Grimm and Harwood, 1997). The chemoreceptor in P. putida G7, NahY, is a MCP that is encoded downstream of the naphthalene catabolic genes on the NAH7 plasmid (Grimm and Harwood, 1999). NahY is part of an operon that contains genes for salicylate degradation (the naphthalene lower pathway). As such it is coordinately regulated with the naphthalene and salicylate degradation genes. Marx and Aitken (2000) have also shown that naphthalene is degraded more rapidly by a wild-type bacterial strain than by its non-motile or non-chemotactic mutant in a heterogenous aqueous system.

Various nitroaromatic compounds used as pesticides, herbicides, dyes, explosives and as precursors in polymer production are also chemoattractants. Samanta et al. (2000) used a chemotactic enrichment technique to isolate Ralstonia sp. SJ98 from pesticide-contaminated agricultural soil. The strain was initially grown on p-nitrophenol (PNP) and was found to be chemotactic toward 4-nitrocatechol, 3-methyl-4-nitrophenol and o- and p-nitrobenzoate. It was also found to be chemotactic to other nitroaromatics that were transformed by it in the presence of an alternate carbon source using qualitative and quantitative assays (Pandey et al., 2002). Recently, Parales reported chemotaxis of P. putida PRS2000 toward nitrobenzoates and aminobenzoates, and Bhushan et al. (2004) demonstrated bacterial chemotaxis toward cyclic nitramine explosives, CL20, HMX and RDX. On the basis of these reports it may be argued that during the course of evolution chemotaxis might have been adapted by bacteria as it provided them the selective advantage to sense or locate environmental pollutants so that they could move toward or away from these compounds. Ortego-Calvo et al. (2003) agreed with previous
reports (on chemotaxis toward naphthalene and toluene) in suggesting that the genes involved in chemotaxis might share a common pathway of regulation. The close proximity of chemotaxis and biodegradation genes and their coordinate expression implies a natural link between chemotaxis and biodegradation, thereby indicating the applicability of this phenomenon for biodegradation purposes.

**Vital staining for detection of bacteria in soil during bioremediation**

There is increasing interest in using degradative microbes to bioremediate soil and groundwater contaminated with recalcitrant pollutants, a process known as bioaugmentation. Bioaugmentation has been used successfully to remediate groundwater contaminated with chlorinated solvents (Dybas et al., 1998; Steffan et al., 1999; Piskonen et al., 2005) and is expected to be useful for other compounds as well. Bioaugmentation requires that effective concentrations of microorganisms be predictably transported to and through contaminated areas of the subsurface. Current tracking technologies are of limited use due to the effects of labeling compounds on cell viability or other properties, high detection limits or interferences from indigenous organisms, or regulatory concerns about the release of genetically modified or antibiotic-resistant microbial strains. Therefore, new methods for tracking viable bacterial cells under both laboratory and field conditions are being developed as part of a major research project examining the physical, chemical, and biological controls on bacterial transport under way under the auspices of the Acceleration Element of the Natural and Accelerated Bioremediation Research Program at the U. S. Department of Energy (DOE) South Oyster site (Oyster, Va.). In order to examine the processes in detail, the movement and distribution of introduced bacteria must be monitored. Several methods have previously been developed to monitor microbial transport through, and microbial interactions with, porous media during laboratory and field experiments. Plating methods can have detection limits around 100 to 1,000 culturable cells per ml of sample, providing that there is no overgrowth of indigenous bacteria on the plates (Fuller et al., 2000). Selective plating may allow lower detection limits but requires extensive a priori screening of every potential degradative organism for antibiotic sensitivity and carbon source utilization profile. A major disadvantage of this method is that it does not detect "viable but nonculturable" cells (Oliver et al., 1995; van Overbeek et al., 1995; Pommepuy et al., 1996; Heidelberg et al., 1997), which still may be metabolically active but unable to form colonies on solid media. Stable isotopes are also increasingly being used for field
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experiments. Bacteria grown on $^{13}$C-enriched glucose prior to injection into the superficial, uncontaminated aquifer at the North Oyster, Va., site were detected in down gradient monitoring wells by converting collected particulate organic matter to carbon dioxide and measuring the $^{13}$CO$_2$ by Carlo-Erba isotope ratio mass spectrometry (DeFlaun et al., 1997). However, additional methods were required to unequivocally establish that the $^{13}$C represented intact cells of the bacteria injected rather than protozoans or other indigenous microbes which may have incorporated labeled cellular material via predation of live target cells or consumption of dead target cells (W. Holben, University of Montana, personal communication, 1999). One of the molecular methods available, PCR, has been used to count very low numbers of bacteria in a variety of quantification. However, this technique can usually be performed only in the laboratory, and is costly and may give rise to spurious bands leading to unauthentic results. Genetically engineered microorganisms carrying the *genes coding for the green fluorescent protein have been developed (Errampalli et al., 1998; Normander et al., 1999; Errampalli et al., 1999), which has allowed enumeration of these organisms by measuring fluorescence. However, the use of these genetically engineered microorganisms has been restricted to laboratory, greenhouse, and lysimeter experiments because of regulatory concerns.

Labeling cells with fluorescent stains has been employed to examine bacterial attachment to surfaces (Fleminger and Shabtai, 1995; Johnson et al., 2001), to count the numbers of total and active cells in a variety of environmental samples (Schaule et al., 1993; Yu et al., 1995; Vogt et al., 2005), and for in situ injection of groundwater bacteria (Harvey et al., 1993; Bales et al., 1995; Zilles et al., 2002; Caruso et al., 2002). One of the stains used, 4,6-diamino-2-phenylindole (DAPI), specifically binds to nucleic acids (RNA and DNA), which enables it to universally label cells in an organism-independent manner (Button and Robertson, 2001). The binding mechanism adversely affects normal cell function (Parolin et al., 1990; Bernander et al., 1998), resulting in a loss of viability. The potential effects of this compound on the transport properties of an organism have been shown to be minimal for short-term experiments (Kucukcolak et al., 1998). Another stain, 4-phenyl spirofuran-2-(3H), 1-phenylalan-3,39-dione (Fluram, fluorescamine), forms covalent bonds with free amino groups. Fluram was shown not to effect the adhesion of a *Rhodococcus* strain to titanium-rich particles (Fleminger and Shabtai, 1995), but its effects on other species have not been examined. For fluorescent stains to be useful for studying bacterial transport and monitoring bioaugmentation, they must
have minimal effects on bacterial adhesion, viability, and metabolic activity, while at the same time they must be retained in the cells for at least several weeks. New fluorescent compounds which may allow cells to be stained without a loss of activity or viability or changes in adhesive properties have been and continue to be developed. Many of these dyes have been developed specifically for eukaryotic cell staining, but the principles underlying their use makes them applicable to prokaryote staining as well. Some of the newer dyes specifically stain cell membranes, whereas, while others cross the membrane and covalently bond to intracellular proteins. In either case some of these dyes have been shown to be retained in cells for at least 3 to 4 weeks without a loss of cell viability or alterations in cell function or adhesion (Haugland, 1996).

Materials and Methods

Bacterial strain

Ralstonia sp. SJ98 was isolated from a pesticide contaminated soil, using “chemotactic enrichment technique” (Samanta et al., 2000). Burkholderia cepacia RKJ200, a non-chemotactic p-nitrophenol (PNP) degrading strain, was also isolated from an agricultural field sprayed with parathion/methyl parathion (Chauhan et al., 2000).

Growth medium

In order to prepare a cell suspension of the bacterial strains, nutrient broth medium was inoculated with overnight grown seed cultures of strains SJ98 and RKJ200 and incubated at 30°C under shaking conditions (200 rpm). The cells were induced with 0.4 mM PNP, harvested at mid log phase, washed and finally resuspended in minimal medium (Prakash et al., 1996). PNP is a yellow compound and disappearance of this color in the culture medium indicates mineralization of PNP as a carbon and energy source by the bacteria.

Bacterial chemotaxis in semi-solid medium

The chemotactic behavior of strains SJ98 and RKJ200 was studied in semi-solid agar medium by two different methods, viz. drop assay and swarm plate assay as described earlier (Bhushan et al., 2000; Samanta et al., 2000). For drop assay (Pandey et al., 2002) cells were grown in nutrient broth and induced at early log phase (OD600 0.3) with 0.3 mM PNP. They were grown further for 3-5 h, washed and resuspended in drop
assay medium (MM containing 0.3% bactoagar) and poured in petriplates. Crystals of desired nitroaromatic compound were placed in the center of the petriplate and chemotactic response of *Ralstonia* sp. SJ98 was observed within 3-4 h of incubation at room temperature (25°C) (Fig. 1). For swarm plate assay *p*-nitrophenol (final concentration 0.3 mM) was added to the swarm plate medium (MM containing 0.16% bacto-agar) before pouring the plates. About 75-100 μl of induced and washed cell suspension (OD$_{600}$ 2.0) in minimal medium was gently poured in a petri dish and incubated at room temperature (25°C). 1 mM Glucose was added to the cell suspension for providing energy to the cells. Formation of rings was observed after 12-16 h of incubation (Fig. 1).

**Figure 1.** A diagrammatic representation of the drop assay and swarm plate assay for demonstrating bacterial chemotaxis in semi solid agar medium. For drop assay the inoculum is added to the medium; whereas, in swarm plate assay cells are added as a thick suspension to the center of the plate.

**Bacterial chemotaxis in soil**

Chemotaxis of strain SJ98 toward PNP was demonstrated in the laboratory using different assays in semi-solid medium; for demonstration in soil microcosm two different assays were designed, i.e. the qualitative plate assay in a smaller scale and a quantitative tray assay on a larger scale. For these experiments cell suspensions of both strains were prepared from 250 ml cultures as indicated above and finally resuspended in 2 ml of phosphate buffer saline (PBS) in order to achieve a required cell density of $\sim 10^{10}$ cells/ml.
(i) Plate assay: 10 g soil was spiked with 70 ppm PNP dissolved in 30 ml MM and allowed to dry. In a petri dish concentric zones of soil and bacto agar were made such that the organism could travel radially from the center of the plate traversing the alternate soil and agar zones (Fig. 2). Change in yellow color of PNP to colorless indicated the depletion of PNP and hence the end point of the experiment. One of the controls was inoculated with strain RKJ200 and the other was uninoculated. Moisture content of the soil was maintained at 40-50% of its water holding capacity by sprinkling water whenever necessary.

Figure 2. A diagrammatic representation of the plate assay for demonstrating chemotaxis of bacteria in soil

(ii) Tray assay: A glass tray was fabricated such that it contained three parallel lanes marked at an interval of 1 cm (Fig. 3). Two glass stoppers were fixed to prevent mixing of soil and agar before its solidification. Zones 1 and 2 contained 0.5 mM p-nitrophenol suspended in 0.4% bacto agar and soil mixed with 70 ppm PNP was added to zone 3 of each lane. The I<sup>st</sup> lane was inoculated with *Ralstonia* sp. SJ98 (~10<sup>10</sup> cells), II<sup>nd</sup> lane with *B. cepacia* RKJ200 (~10<sup>10</sup> cells) and the III<sup>rd</sup> lane was kept uninoculated. Disappearance of yellow color of PNP in zone 1 indicated that bacteria reached soil after diffusing through the bacto-agar; this time was considered as the starting time of the experiment; the experiment was terminated when zone 2 in the experimental lane was decolorized.

Figure 3. A diagrammatic representation of one of the channels in the tray assay. Zones 1 and 2 contain 0.4% agarose containing 0.5 mM PNP and zone 3 contains soil mixed with 70 ppm PNP. The inoculum is added in zone 1 as shown by the colorless area such that it travels toward zone 3, enters zone 2 thereafter depleting the yellow color of PNP in that region.
CFU count and estimation of residual PNP concentration in soil

CFUs were calculated by plating suitable dilutions of bacteria from app. 1 g soil collected at various time intervals from all the three lanes (Fig. 3). To determine the residual PNP concentration samples were collected at the start and end of the experiment at a distance of about 1, 3, 6 and 8 cm from zone 1; PNP was extracted and analyzed by high-performance liquid chromatography (HPLC) as reported earlier (Labana et al., 2005). The residual PNP content in the soil samples was estimated using a standard curve constructed on the basis of known concentrations of PNP. The standards were extracted from soil as in case of unknown samples.

Staining bacterial cells for flow cytometry

Cells were grown in nutrient broth to early stationary phase, harvested, washed and resuspended in 5 ml PBS to a concentration of ~10^9 cells/ml. 5-(and 6-)-Carboxyfluorescein diacetate (CFDA) was added to the cell suspension at a final concentration of 200 μM, incubated with stirring at 37°C for 3 h and the stained cells were harvested, washed and finally resuspended in 2ml PBS. The efficiency of staining was visualized using Fluorescent Microscopy (Nikon Eclipse 600). One ml of unlabelled cells of each strain served as negative control.

Drop assay was performed to confirm the viability and chemotactic ability of ‘stained cells’ as described earlier (Samanta et al., 2000). The tray was set as described previously and inoculated with about ~10^10 fluorescent labeled cells (SJ98 and RKJ200). Soil samples were taken at 12, 24, 32 and 36 h intervals from each of the lanes, fixed (please see below) and stored at 4°C for flow cytometry (FCM) analysis.

Fixation of cells and FCM analysis

2.5% Glutaraldehyde and 2% paraformaldehyde was used for fixation. To 1 gm of soil sample 1 ml PBS was added, vortexed, centrifuged briefly to allow the settling of soil particles and the supernatant containing cells was transferred to a fresh tube. Equal amount of glutaraldehyde solution was added to the supernatant and incubated at 4°C for 1 hr. The cells were collected by centrifugation, washed, resuspended in 300 μl of PBS and finally stored for FCM analysis.

The FCM data was acquired using FACScalibur™ flow cytometer (BD Biosciences, San Jose, CA) equipped with an argon laser (488nm) and CELLQuest™
software (Becton Dickinson). The FACScalibur was calibrated on regular basis by using CaliBRITE beads (BD Biosciences, CA, USA). FACSFlow solution (Becton Dickinson) was used as the sheath fluid during data acquisition. Samples were analyzed in triplicate for every condition and the instrument was decontaminated by running sodium hypochlorite solution followed by distilled water. For fluorescence measurements by detectors FL1, FL2 and FL3, the photomultiplier tube (PMT) voltages and threshold levels were adjusted. PMT voltages were adjusted to place the unstained population of diluted bacteria in the lower left quadrant of two parameter plots ($\leq 10^1$ log scale). Voltages were set at 518 V for side scatter (SSC), 579 V for detector FL1, 600 V for detectors FL2 and 670 V for FL3 (fluorescence detectors). The bacterial population was positioned so that it was entirely on scale on an FSC (forward scatter) versus SSC plot.

Positive samples consisted of soil extract containing stained SJ98 cells. These samples were analyzed in order to set the threshold just above the majority of the background fluorescent particles (beyond $10^1$ log scale on X-axis) using fluorescence detector FL2 ((585/42 band pass)). A polygonal gate (R1) was defined around the population of positive control bacteria on a bivariate dot plot of log SSC versus log FSC in order to locate and gate the cells. Samples containing soil extract without fluorescent cells were analyzed as negative control to determine the level of background fluorescence occurring within the gated region. Sample filtrates (300 µl) were added to 1 ml of sheath fluid in Falcon tubes (BD Biosciences, San Jose, Calif.) and vortexed prior to FCM analysis. Samples were analyzed on medium flow rate such that 50,000 cells are analyzed by the detector.

**Slot blot hybridization**

In order to confirm the migration of cells throughout soil slot blot hybridization was carried out. The total soil DNA was extracted from samples collected at different time intervals using a FastPrep DNA isolation kit according to the manufacturer's instructions (Qbiogene/BIO101). The DNA was further purified by gel extraction using a Qiaquick gel extraction kit (QIAGEN, Germany). In order to quantify strain SJ98 in soil using hybridization techniques a specific probe was designed and radio-labeled that hybridized specifically to genomic DNA of strain SJ98. This probe, a ~540 bp segment of the benzenetriol dioxygenase gene involved in the degradation of several nitroaromatic compounds, was amplified (AY866518). The reaction mixture consisted of 50 ng of genomic DNA of SJ98, 1 U of Taq DNA Polymerase (New England Biolabs,
Mass.), 1X buffer (10mM Tris-HCl [pH 9.0], 1.5 mM MgCl₂, 500 mM KCl, 10 mM deoxynucleoside triphosphate, and 100 ng of each primer (BtD-F: 5' Agg AgT TCA TCC TgC TSW g 3'; BtD- R: 5' CgC ACS CCg AAC ACW gCg TC 3'). Initial DNA denaturation and enzyme activation steps were performed at 95°C for 30s, annealing at 50°C for 1 min and extension at 72°C for 30 s, and a final extension for 2 min at 72°C in a Thermal Cycler (Eppendorf, Germany).

DNA isolated from the soil was transferred onto a Hybond N⁺ membrane using PR648 Slot blot filtration manifolds (Amersham Pharmacia Biotech. Inc., USA) and hybridized with the 540 bp fragment of a gene encoding ‘benzenetriol dioxygenase’ involved in nitroaromatic degradation. The membrane was then washed two times at room temperature in 2X SSC (Ambion, Gmbh, Germany) [1X SSC is 0.18M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA (pH 7.7)] followed by two washes at 50°C in 0.1X SSC and 0.1X SDS. Absence of positive signals from the slots containing DNA from control plots reconfirmed the specificity of the probe. The use of a standard curve based on known concentrations of DNA makes it theoretically possible to quantify DNA from any source (Okano et al., 2004). In this study a standard curve was constructed by plotting counts/unit area generated by known amounts of DNA using Molecular Imager® FX system (BioRad, Hercules, CA) and the Quantity One Software (BioRad, Hercules, CA). The DNA concentration of strain SJ98 from soil samples was determined from the standard curve.

Chemicals

*p-Nitrophenol, CFDA, glutaraldehyde and para formaldehyde were obtained from Sigma Chemical Co. USA. All other chemicals were of highest purity grade available locally.

Results and Discussion

*Ralstonia* sp. strain SJ98 was previously shown to be chemotactic toward *p*-nitrophenol, 4-nitrocatechol, *o*-nitrobenzoate, *p*-nitrobenzoate and 3-methyl-4-nitrophenol. These compounds also serve as sole source of carbon and energy to the organism (Bhushan et al., 2000; Samanta et al., 2000). Complete catabolic pathways for mineralization of these compounds were not elucidated; however, 1,2,4-benzenetriol was reported as a pathway intermediate based on TLC GC and GC-MS studies.
Since chemotaxis has been hypothesized to enhance bioremediation of organic pollutants (Bhushan et al., 2004) and insoluble metals (Childers et al., 2002), *Ralstonia* sp. SJ98 was selected for the demonstration of bacterial chemotaxis in a heterogeneous medium i.e. soil. Chemotaxis of strain RKJ200 towards PNP was also tested using drop assay, swarm plate assay and capillary assay. The formation of concentric rings was not observed in drop assay and swarm plate assay indicating that strain RKJ200 is non-chemotactic towards PNP (Fig. 4 a, b). Furthermore, the results were re-confirmed by the capillary assay, where the chemotaxis index obtained for strain RKJ200 (1±0.3) was found to be similar to that obtained in case of the negative control where PNP was not added (1±0.2). Therefore, it was concluded that strain RKJ200 is non-chemotactic towards PNP and, consequently, was used as a negative control for the present study.

![Chemotaxis response](image)

**Figure 4.** Chemotaxis response *Ralstonia* sp. strain SJ98 in (a) drop assay and (b) swarm plate assay toward different nitroaromatic compounds: (A) p-nitrophenol and (B) 4-nitrocatechol. (C) Aspartate was used as positive control. (D) Strain RKJ200 was used as negative control as it is non-chemotactic toward these compounds.

*p*-Nitrophenol was selected as the chemoattractant not only because strain SJ98 is chemotactic toward it but also because its complete degradation is evident by the decolorization of its bright yellow color. Firstly, the ‘plate assay’ was developed to demonstrate chemotaxis in soil qualitatively and, thereafter, a quantitative ‘tray assay’ was developed to monitor the movement of cells (Figs. 2, 3). For ‘plate assay’ cell suspension of both strains was added separately to the center of the agar zone (Fig. 5 a). It was observed that strain SJ98 not only degraded PNP in the central agar zone, but also
Chapter 5
degraded PNP from the peripheral zone in 48 h, whereas, strain RKJ200 (a non-chemotactic PNP degrading strain) could only degrade PNP from the central agar zone. In the uninoculated plate PNP was not degraded as indicated by the yellow color. This experiment indicated that unlike strain RKJ200, strain SJ98 had the capability to degrade PNP from a zone distant to the point of inoculation.

Figure 5 (a). Plate assay showing presence of PNP as indicated by yellow color in the inner and outer agarose zone in the uninoculated plate. The second plate inoculated with strain RKJ200 shows depletion of PNP in the inner zone. The third plate inoculated with strain SJ98 shows complete depletion of PNP in both layers.

Figure 5 (b). Tray assay showing depletion of PNP in zones 1 and 2 of the channel inoculated with strain SJ98 and only in zone 1 in the case of strain RKJ200; in the uninoculated lane PNP remains undegraded.

To investigate further, another assay i.e. the ‘tray assay’ was set up in sterile conditions such that the population of cells could be calculated at various time points at different zones of soil (Fig. 5 b) and also to quantify PNP depletion. As in case of ‘plate assay’ it was observed that after 3 days PNP was depleted from zone 2 in the case of strain SJ98, whereas, strain RKJ200 did not degrade it even after 5 days. Similarly, in the uninoculated control there was no change in zone 1 and 2. CFUs of soil samples collected at different time intervals from all the three lanes were determined (Table 2). These results show a significant increase in the number of SJ98 cells with time in
samples collected at a distance of 8 cm from zone 1, with a simultaneous decrease in CFUs at distance of 1 cm from zone 1, indicating the migration of cells from zone 1 to zone 2 through soil (Fig. 3 and 5b). In the case of strain RKJ200 the CFUs increased at a distance of 3 cm from zone 1, however at 8 cm from zone 1 the viable count was zero. This observation indicated that non-chemotactic RKJ200 cells diffused in soil along with the water absorbed by soil from the agar zone.

<table>
<thead>
<tr>
<th>Time</th>
<th>Dist. from zone 1</th>
<th>CFU (cells/g soil)</th>
<th>Residual PNP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SJ98 (10^9)</td>
<td>RKJ200 (10^9)</td>
</tr>
<tr>
<td>12 h</td>
<td>1 cm</td>
<td>~10^8</td>
<td>~10^8</td>
</tr>
<tr>
<td></td>
<td>3 cm</td>
<td>0</td>
<td>~10^7</td>
</tr>
<tr>
<td></td>
<td>8 cm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24 h</td>
<td>1 cm</td>
<td>~4X10^8</td>
<td>~10^9</td>
</tr>
<tr>
<td></td>
<td>3 cm</td>
<td>~10^8</td>
<td>~10^7</td>
</tr>
<tr>
<td></td>
<td>8 cm</td>
<td>~6X10^6</td>
<td>0</td>
</tr>
<tr>
<td>32 h</td>
<td>1 cm</td>
<td>~7X10^6</td>
<td>10^8</td>
</tr>
<tr>
<td></td>
<td>3 cm</td>
<td>~10^8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8 cm</td>
<td>~10^8</td>
<td>0</td>
</tr>
<tr>
<td>36 h</td>
<td>1 cm</td>
<td>~10^6</td>
<td>10^4</td>
</tr>
<tr>
<td></td>
<td>3 cm</td>
<td>~10^7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8 cm</td>
<td>~10^7</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time</th>
<th>Dist. from zone 1</th>
<th>CFU (cells/g soil)</th>
<th>Residual PNP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SJ98 (10^9)</td>
<td>RKJ200 (10^9)</td>
</tr>
</tbody>
</table>

Table 2. Viable plate counts obtained during tray assay from soil sample collected at various time intervals from different distances from zone 1 of channels inoculated with strains SJ98 and RKJ200, and the residual PNP concentration in soil. The CFUs are the mean of experiments performed in triplicate. In the control lane the residual PNP concentration was 100% (70 ppm) at all time intervals.

In addition, in order to quantify the PNP concentration at the end of the experiment (3 days), PNP was extracted from different zones of soil and analyzed by HPLC. There was a significant decrease in the amount of PNP present in soil near zone 2 in case of strain SJ98 as compared to that of strain RKJ200 (Table 2). In the uninoculated control set-up there was no decrease in the level of PNP even after 72 h indicating that PNP
persisted in soil over this time period. This observation indicated that strain SJ98 depleted PNP from zone 1 and also from zone 3, thereby creating a gradient of PNP. The chemotactic SJ98 cells sensed this gradient and as a result they migrated through soil toward zone 2.

For bioremediation purposes it is important to monitor the introduced organism in soil and therefore various methods are being employed including tagging cells with green fluorescent protein (GFP), staining, etc (Rosochacki and Matejczyk, 2002; Abbey et al., 2003; Backman et al., 2004). Suitability of techniques may enhance or adversely affect the success of tracking because many such methods are of limited use due to the effects of labeling compounds on cell viability/other properties or interferences from indigenous organisms (Fuller et al., 2000). Therefore, cells were labeled with CFDA and flow cytometry was used to track the CFDA-stained (fluorescent dye-labeled) bacterial cells in soil over a time period. For this stained bacterial cells were introduced in zone 1 (Figs. 3, 5b) and soil samples were collected at various time intervals from different distances from zone 1.

![Figure 6](image-url)

**Figure 6.** Histogram showing the movement of strain SJ98 through various zones of the tray at different time intervals as determined by flow cytometry. The graph compares the percentage of gated cells present in the soil samples.
Figure 7. (a) Flow cytometric data of stained cells collected at various time points from different zones of soil (in the track assay) represented in a graphical form. The region M2 contains the stained cells and M1 mainly represents background fluorescence. The Y axis shows the number of cells and X axis shows the fluorescence on log scale. Initial pt. refers to soil samples collected adjacent to Zone 1; End pt. refers to those collected adjacent to zone 2. FL2-H is fluorescence recorded by FL2 (height of fluorescence).

(b) FCM data as shown in dot plot and in the form of graph. Fluorescent cells have been gated by a polygonal gate to differentiate the soil particles from the cells. The absence of signals in the M2 region in the case of uninoculated controls establishes the accuracy of gating indicating that auto fluorescence of soil particles has been eliminated from analysis.
Based on the analysis of FCM data the percentage of cells in various samples collected at different time points have been compared in figure 6. These results clearly showed a gradual increase followed by a decrease in signals (0-29 %, 29-13 %) in M2 region initially; thereafter, an increase in signals (0-21 %) towards the end (at a distance of 8 cm from zone 1) indicating the migration of cells from zone 1 to 2. In strain RKJ200 no fluorescence was detected above the background level at a distance of 8 cm from zone 1. The histograms obtained from FCM data also clearly showed an increase in the number of gated SJ98 cells with increasing distance from the point of inoculation as compared to gated RKJ200 cells (Fig. 7a). There was insignificant fluorescence in the case of the uninoculated control at different time intervals indicating that fluorescence detected was mainly due to stained cells and not soil particles (Fig. 7b). The above results show that being chemotactic SJ98 cells traversed the zone of soil to reach zone 2 with simultaneous PNP depletion; however, RKJ200 cells diffused through soil up to a very short distance. Therefore, the application of chemotactic cells for bioremediation could facilitate detoxification of a larger area as compared to non-chemotactic ones.

![Figure 8](image-url)

**Figure 8.** Slot blot hybridization studies to determine the concentration of DNA extracted from soil samples on the first day (row A) and last day (row B) of the experiment at distances 1 cm (7A, 7B), 3 cm (8A, 8B), 6 cm (9A, 9B) and 8 cm (10A, 10B) from the point of inoculation. Rows 3A-6A and 3B-6B have DNA extracted from samples collected from the uninoculated control lane. DNA of known concentration has been used for construction of a standard curve (last row). The four corners of the blot represent positive control (genomic DNA of strain SJ98).

To further confirm the FCM results slot blot hybridization was performed. Soil samples were collected at a distance of 3, 6 and 8 cm from zone 1 (Fig. 5b) on the first day and after the third day (when PNP in zone 2 was degraded) and DNA was extracted
from these samples. The ~540 bp fragment of benzenetriol dioxygenase gene involved in PNP degradation in strain SJ98 was used as probe. The autoradiogram shows that in slot blot hybridization there are no hybridization signals in the uninoculated slots indicating that the probe did not bind non-specifically to soil organisms other than strain SJ98 (Fig. 8). DNA concentration of each sample was calculated using a standard curve based on counts generated by known concentrations of DNA. Results indicated significant changes in cell populations at different distances from zone 1 (Table 3). DNA concentration was significantly higher in samples collected at a distance of 8 cm from zone 1 as compared to that collected on the 1st day as evident from the quantitation results indicated in table 3. This result can be extrapolated to conclude that the cell population showed considerable changes at different time points at various distances in soil again suggesting the movement of cells through soil.

<table>
<thead>
<tr>
<th>Distance from Zone 1</th>
<th>12 h</th>
<th>72 h</th>
<th>12 h</th>
<th>72 h</th>
<th>12 h</th>
<th>72 h</th>
<th>12 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counts/mm²</td>
<td>36518</td>
<td>31758</td>
<td>18725</td>
<td>28862</td>
<td>0</td>
<td>26846</td>
<td>0</td>
<td>25529</td>
</tr>
<tr>
<td>DNA conc. (ng/μl)</td>
<td>60.0</td>
<td>57.5</td>
<td>0</td>
<td>55.0</td>
<td>0</td>
<td>45.0</td>
<td>0</td>
<td>32.5</td>
</tr>
</tbody>
</table>

Table 3. Slot blot hybridization showing radioactive counts per unit area and corresponding DNA concentration from samples collected at different distances from zone 1 at various time points. Zone 1 refers to the bacto-agar zone of tray assay in which strain SJ98 was inoculated.

Earlier, Pandey and Jain (2002) argued the potential role of chemotaxis in bioremediation of soils contaminated by organic pollutants. However, this phenomenon was never demonstrated in soil. Olson et al., (2004) analyzed both the diffusive and the chemotactic behavior of *Pseudomonas putida* F1 in the presence of the chemical stimulus trichloroethylene (TCE). This study represented the first quantification of bacterial chemotactic parameters within a packed column. They proposed that for conditions under which chemotaxis occurred in porous media, the same may be potentially exploited to improve rates of *in situ* biodegradation in the subsurface environment, particularly for pollutants dissolved in water trapped in low-permeability formations or lenses. In another report, Childers et al. (2002) demonstrated the ecological role of chemotaxis for accessing insoluble Fe(III) oxide by *Geobacter metallireducens* and postulated that such a mechanism helps *Geobacter* species to predominate over other Fe(III) oxide-reducing microorganisms in sedimentary environments. The results of this
study demonstrate the movement of SJ98 cells in soil for the first time. It strengthens the argument that chemotaxis may enhance bioavailability of pollutants, thereby facilitating bioremediation in soil. Labana et al. (2005) successfully performed bioaugmentation of PNP-contaminated sites by exogenously introducing a PNP degradative strain in small-scale field studies. It may be hereby envisaged that introduction of a degradative strain showing chemotaxis towards the pollutant would enhance bioremediation as the same inoculum could be used for detoxification of a larger area. The assays developed for this study may also be used to study chemotactic behavior of other organisms in soil microcosms and the results may be extrapolated for field-scale studies.
References


Chapter 5


