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Chlorophenol and nitrophenol metabolism by *Sphingomonas* sp UG30

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***Sphingomonas* sp UG30 is a pentachlorophenol (PCP)-degrading bacterial strain capable of degrading several nitrophenolic compounds, including *p*-nitrophenol (PNP), 2,4-dinitrophenol (2,4-DNP), *p*-nitrocatechol and 4,6-dinitro-*o*-cresol (DNOC). The ability to degrade both chlorophenolic and nitrophenolic compounds is probably not restricted to UG30, but may also be possessed by other pentachlorophenol-degrading *Sphingomonas* spp. The interesting question arises as to whether there is any point of convergence between the initial pathways of PCP and nitrophenol degradation in these microorganisms. There is some experimental evidence that PCP-4-monooxygenase is involved in metabolism of both *p*-nitrophenol and 2,4-dinitrophenol. Further studies are needed to confirm this and to examine the role(s) of other PCP-degrading enzymes in nitrophenol metabolism by this microorganism. In this paper, we review some of the taxonomic, biochemical, physiological and ecological properties of *Sphingomonas* sp UG30 with respect to biodegradation of PCP and nitrophenolic compounds.**

Keywords: dechlorination; degradation; nitrophenols; pentachlorophenol (PCP); *Sphinogomonas*; xenobiotics

Introduction

Chlorophenols and nitroaromatic compounds are of environmental concern because they comprise two major classes of chemicals used as herbicides, explosives, solvents and industrial chemicals or precursors. Widespread use of these compounds has led to contamination of soils, waste streams and surface waters. Many chlorophenolic and nitroaromatic compounds and their metabolites pose a health hazard due to their toxicity to numerous organisms. It is both relevant and important to reduce the input of toxic chemicals into the environment and to study methods for their removal from contaminated sites. Understanding the microbial metabolism of these compounds will assist in management methods to minimize their persistence in the environment.

Members of the genus *Sphingomonas* can degrade numerous naturally occurring compounds and environmentally important xenobiotics, including chloro- and nitrophenols. Some *Sphingomonas* spp have multiple mechanisms for dehalogenation, transformation of nitroaromatics and subsequent aromatic ring cleavage of these compounds [40]. Their ability to survive in diverse environments suggests that sphingomonads may be ubiquitous. In the past, they have sometimes been identified as pseudomonads or *Flavobacterium* spp or listed as unidentified isolates.

For the past 5 years, we have been studying pentachlorophenol (PCP) biodegradation by *Sphingomonas* sp UG30. Recently, we found that *Sphingomonas* sp UG30 and *Sphingomonas* spp strains RA2 and ATCC 39723 can metabolize *p*-nitrophenol (PNP) with the release of nitrite, and mineralize it to CO_2 [23]. We have also observed that *Sphingomonas* sp UG30 can mineralize 2,4-dinitrophenol (2,4-DNP) to CO_2, along with nitrite release [42]. In addition, it is able to transform *p*-nitrocatechol and 4,6-dinitrocreosol [42].

Degradation of PCP has been observed in *Sphingomonas* spp isolated from different geographic areas. In addition, related chlorophenols and other xenobiotics can be degraded by *Sphingomonas* spp. For example, *Sphingomonas* sp NB6 degrades napthalenesulfonate and produces an extradiol dioxygenase that can also catalyze 1,6 cleavage of 3-chlorocatechol to 3-chloro-2-hydroxymuconic semialdehye [34]. In this review, we summarize the current state of knowledge on chlorophenol and nitrophenol biodegradation by *Sphingomonas* sp UG30.

Isolation and characterization of *Sphingomonas* sp UG30

Early studies in our laboratory included attempts to isolate a psychrotrophic PCP degrader but we were not able to do so. We turned our efforts to finding a PCP degrader that could mineralize PCP, particularly in soil, and might be suitable for genetic manipulation, eg, insertion of a genetic marker. Three soils contaminated with PCP were sampled from different locations in Canada. *Sphingomonas* sp UG30 was isolated from one of these soil samples and characterized in our laboratory at the University of Guelph [22]. Eighty-nine bacterial isolates from PCP-contaminated soil samples were tested for PCP dechlorination activity and hybridization to *pcpB* (encoding PCP-4-monooxygenase) and *pcpC* (encoding tetrachlorohydroquinone dehalogenase) gene probes synthesized by PCR from *Sphingomonas chlorophenolica* sp ATCC 39723 genomic DNA. Seven isolates dechlorinated PCP, hybridized to both *pcpB* and *pcpC* DNA probes, and mineralized sodium pentachlorophenate (NaPCP) in a liquid medium. Two of the

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isolates, designated UG25 and UG30 (Figure 1), exhibited excellent mineralization rates and a minimal growth lag in the presence of PCP, and were selected for further research. They were initially classified as *Pseudomonas* spp based on biochemical characteristics. Both isolates were Gram-negative, motile and neither isolate contained detectable plasmids. Both isolates mineralized PCP and exhibited stoichiometric release of Cl^- ions as PCP was degraded [22]. The release of Cl^- began concomitantly with PCP disappearance from the growth medium. Both strain UG25 and strain UG30 degraded NaPCP at concentrations up to 250 μg ml^{-1} in a minimal salts medium. Supplementation of the medium with glutamate (MMG) increased the NaPCP degradation threshold of strain UG25 to 300 μg ml^{-1}, but did not affect that of strain UG30. ^{31}P-NMR spectra of strains UG25 and UG30 cell suspensions exposed to PCP showed decreased intracellular ATP levels and a more acidic cytoplasmic pH relative to untreated cells. These results are not surprising since PCP uncouples oxidative phosphorylation. This de-energization may explain the lack of growth in the presence of high PCP concentrations.

Subsequent growth and mineralization studies suggested that strain UG30 was the most promising isolate for further experimentation. It exhibited similarities to other PCP-degrading bacteria, but there were also some differences. Saber and Crawford [35] isolated a yellow-pigmented bacterium originally classified as *Flavobacterium* sp ATCC 39723 from a wood-treatment site in Minnesota, USA. It used PCP as a sole source of carbon and energy and mineralized PCP when PCP was present at concentrations up to 200 μg ml^{-1} in liquid media. These researchers reported that the bacterium was non-motile, had a nonfunctional flagellum, and contained an 80–100 kb plasmid. Radehaus and Schmidt [32] reported isolation of a motile, yellow-pigmented *Pseudomonas* sp RA2 from a wood treatment site in Colorado, which mineralized up to 160 μg ml^{-1} PCP in liquid media. Resnick and Chapman [33] isolated a PCP-degrading bacterium from a wood treatment facility in Florida. Based on biochemical tests, substrate utilization, a mole percent GC content of 64.2 and its fatty acid profile, it was assigned to the genus *Pseudomonas* and designated as strain SR3. Strain SR3 cells were motile, rod-shaped and grew slowly, producing opaque, off-white colonies. The organism was capable of mineralizing PCP up to a concentration of 200 μg ml^{-1}, although maximal rates of PCP removal were observed from 75 to 150 μg ml^{-1}, decreasing considerably below 50 μg ml^{-1} and above 150 μg ml^{-1}. Edgehill and Finn [10] isolated a bacterium from soil which was rod-shaped, nonmotile, and formed dark yellow colonies. Removal of up to 300 μg ml^{-1} of PCP was observed using pure cultures of this bacterium in an aqueous system. The strain was a member of the coryneform group of bacteria and it was classified as *Arthrobacter* sp ATCC 33790. The above four PCP-mineralizing bacteria were all recently reclassified as *Sphingomonas chlorophenolica* strains on the basis of 16S rRNA gene sequence comparisons, REP (repetitive extragenic palindromic) and ERIC (enterobacterial repetitive intragenic consensus)-PCR finger-printing and serological typing [19] as well as chemotaxonomic data, biochemical and physiological properties and whole cell protein profiles [28]. Recent data from our research, such as the presence of sphingolipids, similarity to other PCP-degrading *Sphingomonas* spp [23], and partial 16S rDNA sequence comparison, convinced us to reclassify our *Pseudomonas* sp UG30 as *Sphingomonas* sp UG30. PC/Gene (Release 6.8; IntelliGenetics, Inc, Mountain View, CA, USA) was used to align approximately 935 bases of partial 16S rRNA sequences of *Arthrobacter* ATCC 33790 (bases 266–1201), *Flavobacterium* ATCC 39723 (bases 1–936), and strains RA2 (bases 41–967), SR3 (bases 1–935) and UG30 (bases 260–1187). Results demonstrated that the strain UG30 sequence is 95–98% similar to the 16S rRNA sequences of the other four bacteria in this sequence region. A dendrogram generated by Clustal analysis (v 1.20; IntelliGenetics, Inc) (Figure 2) shows that strain UG30 clusters separately from the other PCP-mineralizing *Sphingomonas* strains. However, it does align more closely with strains

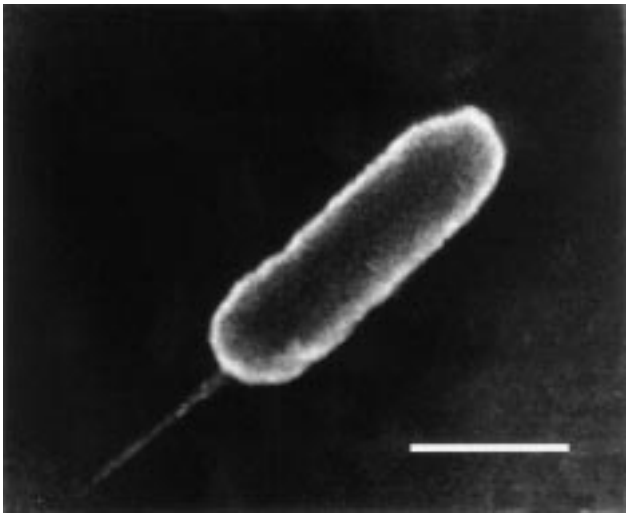

Figure 1 Scanning electron micrograph of *Sphingomonas* sp UG30. (Bar = 0.5 μm).

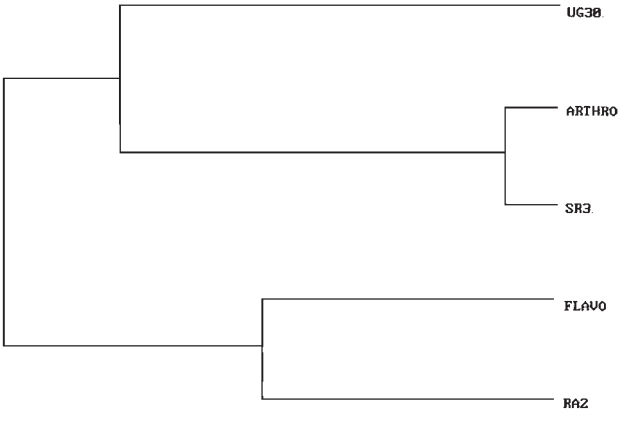

Figure 2 Dendrogram of alignment of partial 16S rRNA gene sequences from PCP-degrading bacteria using Clustal analysis (v 1.20) from PCGene software program. SR3 (*Sphingomonas chlorophenolica* strain SR3, GenBank accession No. U60174); Arthro (*Sphingomonas chlorophenolica* strain ATCC 33790, GenBank accession No. X87161); Flavo (*Sphingomonas flava* strain ATCC 37923, GenBank accession No. U60172); RA2 (*Sphingomonas flava* strain RA2, GenBank accession No. U60173); UG30 (*Sphingomonas* strain UG30, GenBank accession No. AF170090).

ATCC 33790 and SR3 than with strains ATCC 39723 or RA2. It is possible that there is even more similarity as not all bases have been determined for each sequence. Perhaps elucidation of the complete 16S rRNA sequences will shed further light on the phylogenetic relationships of these bacteria.

Some of the physiological properties of PCP degradation by strain UG30 have been characterized [36]. Maximum rates of PCP dechlorination and degradation increased linearly with increasing initial cell densities up to 1×10^9 CFU ml^{-1} and then plateaued. Maximum rates of PCP dechlorination and degradation decreased as the initial PCP concentration increased. Degradation of 400 $\mu g\ ml^{-1}$ of PCP was incomplete and stopped when 120 $\mu g\ ml^{-1}$ Cl^- was released into the medium. Cl^- ions inhibited PCP degradation at a concentration of 100 $\mu g\ ml^{-1}$ and higher. It is possible that Cl^- ions decrease the pH of the medium which increases PCP toxicity. This was observed with PCP-degrading bacteria when pH was adjusted using HCl and NaOH [39]. Enzymes involved in PCP dechlorination and degradation by strain UG30 cells were inducible, and were not repressed by glucose. Strain UG30 cultures degraded PCP at 10°C and degradation rates increased linearly with increasing temperature up to 30°C. The apparent activation energies for PCP dechlorination and degradation were 7.72 and 9.13 kcal mol^{-1}, respectively.

It appeared that we had found a degrader that may have potential for soil bioremediation, but further studies were necessary to determine mineralization abilities.

Mineralization of PCP by *Sphingomonas* sp UG30 in broth

Other studies in our lab had demonstrated metabolic effectiveness of bacterial cells encapsulated in biopolymer matrices. We investigated mineralization by both free and encapsulated cells. We determined the rate and extent of PCP mineralization by both free and κ-carrageenan encapsulated cells in a minimal salts medium amended with a glutamate and incubated at 22°C [3]. Cells of strain UG30 at a density of 1×10^8 CFU ml^{-1} mineralized (55% of $^{14}CO_2$ recovered) 100 $\mu g\ ml^{-1}$ PCP in MMG broth within 2 days, and 200 $\mu g\ ml^{-1}$ PCP within 5 days, but no mineralization was observed with 300 $\mu g\ ml^{-1}$ PCP after 21 days incubation. However, strain UG30 cells at the same density and encapsulated in κ-carrageenan beads mineralized (60% of $^{14}CO_2$ recovered) as high as 600 $\mu g\ ml^{-1}$ PCP within 21 days. The differences in mineralization activity between free and encapsulated cells at the 100 and 500 $\mu g\ ml^{-1}$ levels are depicted in Figure 3.

Others studied the use of encapsulated or immobilized cells to enhance PCP mineralization. Lin and Wang [26] observed PCP mineralization in broth using *Sphingomonas chlorophenolica* (formerly *Arthrobacter* sp) ATCC 33790 as free cells, encapsulated in alginate or co-immobilized in alginate containing activated carbon. Encapsulated cells mineralized ($^{14}CO_2$ recovered) 86% of the PCP compared with 77% mineralization ($^{14}CO_2$ recovered) by free cells in a liquid medium containing 32 $\mu g\ ml^{-1}$ of PCP over 135 h. Co-immobilized cells removed 90% of the PCP within 2 h compared with 50% PCP removal over 22 h with free cells,

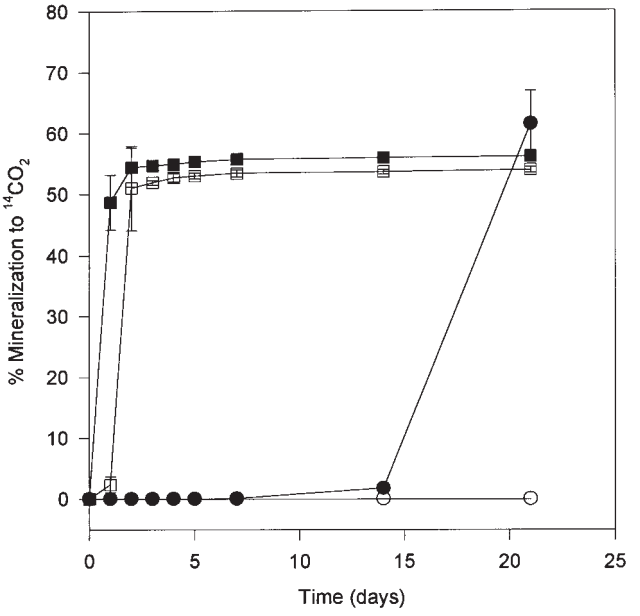

Figure 3 Mineralization of [U-^{14}C]PCP by *Sphingomonas* sp UG30 free and encapsulated cells in minimal medium with glutamate. 100 $\mu g\ ml^{-1}$ PCP (□, free cells; ■, encapsulated cells) and 500 $\mu g\ ml^{-1}$ (○, free cells; ●, encapsulated cells). Values are means ± SD from three independent trials [3].

as determined by HPLC. Co-immobilization resulted in 55% mineralization ($^{14}CO_2$ recovered) over 458 h compared with 86% mineralization ($^{14}CO_2$ recovered) over 135 h for free cells. This difference in removal and mineralization indicated a marked difference in characteristics between PCP removal and PCP mineralization. O'Reilly and Crawford [29] immobilized *S. chlorophenolica* (formerly *Flavobacterium* sp) ATCC 39723 cells in polyurethane foam (PUF) and observed mineralization (70–80% of $^{14}CO_2$ recovered) of up to 300 $\mu g\ ml^{-1}$ PCP in liquid media compared to no mineralization at 200 $\mu g\ ml^{-1}$ PCP and above for the same density of free cells. Hu *et al* [16] demonstrated the ability of PUF-immobilized *S. chlorophenolica* (*Flavobacterium*) cells to mineralize PCP (as $^{14}CO_2$ recovered) at concentrations as high as 700 $\mu g\ ml^{-1}$. These studics illustrated the important beneficial and protective effects of encapsulation in catabolism of toxic compounds by bacteria [2].

Mineralization of PCP in soil by *Sphingomonas* sp UG30

The use of both free and encapsulated cells was investigated for the potential use of strain UG30 in bioremediation of contaminated soil. Mineralization of PCP by free and encapsulated cells and the effect of N, P and K fertilizer amendments were investigated using microcosms containing PCP-contaminated soil [4]. We chose to use a contaminated soil sample from a wood-treatment site in Ontario, Canada containing 350–370 mg PCP kg^{-1} dry soil and 21 000 mg total petroleum hydrocarbons kg^{-1} dry soil in order to observe effects from a soil as close to field conditions as possible. The soil was amended with [^{14}C]-PCP (10 666 bq kg^{-1}), and inoculated with cells of strain UG30

either encapsulated in κ-carrageenan or as free cells. Uninoculated control soil evolved about 19% of the initial radioactivity as $^{14}CO_2$ after 30 weeks of incubation at 22°C. Addition of 1000 ppm phosphate increased PCP mineralization, whereas addition of 1000 ppm nitrogen generally inhibited mineralization in soil. No enhancement of PCP mineralization was observed in soil inoculated with 1×10^8 CFU g dry soil^{-1} of free cells. However, inoculation of the soil with κ-carrageenan-encapsulated cells at the same inoculum density enhanced PCP mineralization to 65% of the initial radioactivity after 26 weeks. Repeated inoculations (six times over 6 weeks) of the soil microcosms with 1×10^8 encapsulated strain UG30 cells g^{-1} dry soil resulted in a more rapid mineralization rate (65% mineralization of radiolabelled PCP within 9 weeks), while addition of sterile beads (beads with no cells) resulted in less than 17% mineralization after 16 weeks.

Different densities of encapsulated cells were used to determine their effects on PCP mineralization. Treatments with cells grown inside beads, and treatments with higher initial cell densities exhibited greater mineralization activity in the first few weeks, but by 20 weeks, PCP mineralization (about 70% recovered as $^{14}CO_2$) was not significantly different among all soil treatments. Our results showed that encapsulation of strain UG30 cells can enhance PCP mineralization in a contaminated soil (Figure 4). Other studies have demonstrated the effectiveness of these *Sphingomonas* strains for degrading pentachlorophenol in soil. Strain ATCC 33790 was inoculated at 6×10^6 cells g^{-1} into both a sand and a clay soil which had been spiked with 15.4 mg kg^{-1} PCP [12]. The cells removed approximately half of the PCP from the sand in 3 h, and almost complete disappearance of PCP from the clay was observed after 1 day. This illustrated the ability of the strain to remove PCP from soil with a high clay content. Colores *et al* [6] observed a significant increase in PCP mineralization up to 1200 mg kg^{-1} PCP when dry soil was inoculated with 6.3×10^6 *S. chlorophenolica* RA2 cells g^{-1}. Miethling and Karlson [27] observed a significant increase in the rate of PCP mineralization from a contaminated soil spiked with 30 mg kg^{-1} PCP compared to control soil. Inoculation of soil with *S. chlorophenolica* sp RA2 cells at 1×10^8 CFU g^{-1} resulted in essentially complete mineralization (80% recovery of $^{14}CO_2$) of the initial radiolabeled PCP in 1 month, compared with complete mineralization after 7 months for the uninoculated soil. The addition of cells immobilized either on polyurethane foam or in sawdust added to the soil had no effect on PCP mineralization. In the same soil spiked with 100 mg kg^{-1} PCP, inoculation with 1×10^8 CFU g^{-1} free cells, cells immobilized on polyurethane foam or cells with sawdust amendment led to complete PCP mineralization after 7 months. However, addition of sawdust to the soil prior to inoculation increased the rate of mineralization up to threefold. This rate was significantly higher than the other treatments.

Strain UG30 appears to be potentially useful for bioremediation of soil and compared well with soil studies using other PCP-degrading bacteria.

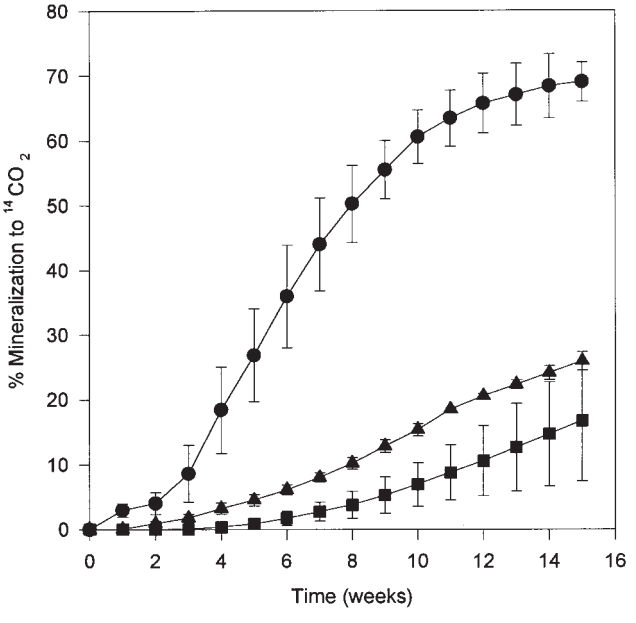

Figure 4 Mineralization of [U-^{14}C]PCP in soil by addition of 0.6 g sterile κ-carrageenan beads (■), skim milk powder (▲) or *Sphingomonas* sp UG30 cells encapsulated in κ-carrageenan amended with skim milk powder (●). Each point is mean ± SD for $n = 3$ [4].

Toxicity assessment of soil bioremediated with *Sphingomonas* sp UG30 cells

Bioremediation of chemically contaminated soils often requires both soil toxicity data and chemical analysis of the soils for concentrations of pollutants. We used five bioassays to assess if toxicological properties of PCP-contaminated soil were affected during bioremediation [20]. The soil used was the same as that used in a previous study [4] and contained 350–370 mg kg^{-1} PCP and 21 000 mg kg^{-1} total petroleum hydrocarbons including 1224 mg kg^{-1} polyaromatic hydrocarbons. Bioremediation treatments tested in soil microcosms included amendment with 1000 ppm phosphorous and/or inoculation with strain UG30 either as free cells or encapsulated in κ-carrageenan. PCP degradation in each microcosm was monitored by chemical analysis. The solid-phase Microtox test, SOS-chromotest, lettuce seed germination, earthworm survival and sheep red blood cell (RBC) haemolysis assays were used to assess soil toxicity. PCP and TPH levels were reduced in all soil treatments after a 210-day incubation at room temperature. RBC lysis, the Microtox test, and the SOS-chromotest assays indicated reduced toxicity in most soil samples by day 210. In contrast, lettuce seed germination and earthworm survival indicated different assessments of toxicity in response to treatments. In soil amended with phosphorous, both seed germination and earthworm survival LC_{50} data indicated increased toxicity. However, in soil treated with encapsulated strain UG30 cells, earthworm survival was increased, while seed germination showed little change from the untreated soil. It is possible that more toxic intermediates were produced during PCP degradation by strain UG30, but by day 210 the toxicity appears to have been reduced by the bioremediation process. Interpretation of the data was also complicated by the presence of complex chemical mixtures, and the results cannot be attributed to the removal of

PCP alone. Some of the chemicals in the mixtures and/or their metabolites may also have contributed to soil toxicity.

Initial pathway of PCP biodegradation by *Sphingomonas* sp UG30

The initial pathway of PCP degradation by several *Sphingomonas* spp has been well characterized [9,31]. These microorganisms are unusual in that they use an oxygenolytic enzyme to initiate degradation of a compound as highly chlorinated as PCP. In the first reaction, PCP is hydroxylated to tetrachlorohydroquinone (TeCH) by PCP-4-monooxygenase encoded by the *pcpB* gene (Figure 5). The TeCH formed is reductively dechlorinated in two successive steps by TeCH dehalogenase (encoded by *pcpC*) to form 2,6-dichlorohydroquinone (2,6-DiCH) which is then converted to 6-chlorohydroxyquinol (6-CHQ or 6-chloro-1,2,4-benzenetriol) by 2,6-DiCH chlorohydrolase [21], mostly likely encoded by the *pcpA* gene [5]. The 6-CHQ or 6-chloro-1,2,4-benzenetriol is subjected to ring fission and metabolized as a carbon and energy source. We have evidence that the initial pathway of PCP degradation by strain UG30 may be identical to that shown in Figure 4. First, strain UG30 possesses two DNA sequences that hybridize to either *pcpB* or *pcpC* gene probes synthesized from *Sphingomonas chlorophenolica* ATCC 39723 DNA [22]. Second, we have cloned the *pcpB* gene from strain UG30 genomic DNA [25]. The *pcpB* gene and its translational product exhibit ≈90% sequence identity to their counterparts from other *Sphingomonas chlorophenolica* strains. Third, when expressed in *E. coli*, the strain UG30 pcpB protein converted PCP to TeCH as verified by GC-MS identification of this intermediate [25]. Fourth, we recently cloned the *pcpC* gene from strain UG30 (unpublished), and its sequence and that of its translational product also show a high level of identity to their counterparts from other *Sphingomonas chlorophenolica* strains. We have determined that strain UG30 metabolizes 6-chloro-1,2,4-benzenetriol through the *ortho* and not the *meta* pathway.

Biodegradation of *p*-nitrophenol (PNP) by *Sphingomonas* sp UG30

During studies on the PCP-degrading activity of *Sphingomonas* sp UG30, we became intrigued by the possibility that closely related PCP-degrading *Sphingomonas* strains may also degrade and mineralize nitrophenolic compounds. Since both chlorine and nitro substituents are electron-withdrawing, they pose a similar bioenergetic challenge to enzymes that mediate their release from aromatic compounds. Bacterial strains able to degrade one aromatic compound often can degrade similar aromatic compounds [8,14,15]. Furthermore, Xun *et al* [41] have shown that the PCP-4-monooxygenase from *S. chlorophenolica* ATCC 39723 exhibits broad substrate specificity and will hydroxylate the *para* position of halo-, nitro-, amino- and cyano-substituted phenols, forming hydroquinones and releasing halide, nitrite, hydroxylamine and cyanide, respectively. The strain ATCC 39723 PCP-4-monooxygenase preferentially hydroxylates the *para* position of phenolic substrates that are substituted at the *ortho* position relative to the -OH group [41]. The amino acid sequences of PCP-4-monooxygenases from several *Sphingomonas* spp are very similar to that of the strain ATCC 39723 enzyme [9,25], suggesting they may hydroxylate nitrophenols. Since minor amino acid sequence variations in very similar enzymes can result in dramatically different substrate specificities [13], we hypothesized that some of the similar PCP-4-monooxygenases may hydroxylate nitrophenolic substrates that do not possess an *ortho* substituent. We also postulated that once the nitro group was removed, the cells would degrade the hydroquinone products further, much as they degrade TeCH arising from PCP.

We examined the ability of several PCP-mineralizing strains (*Sphingomonas* sp UG30, and *S. chlorophenolica*

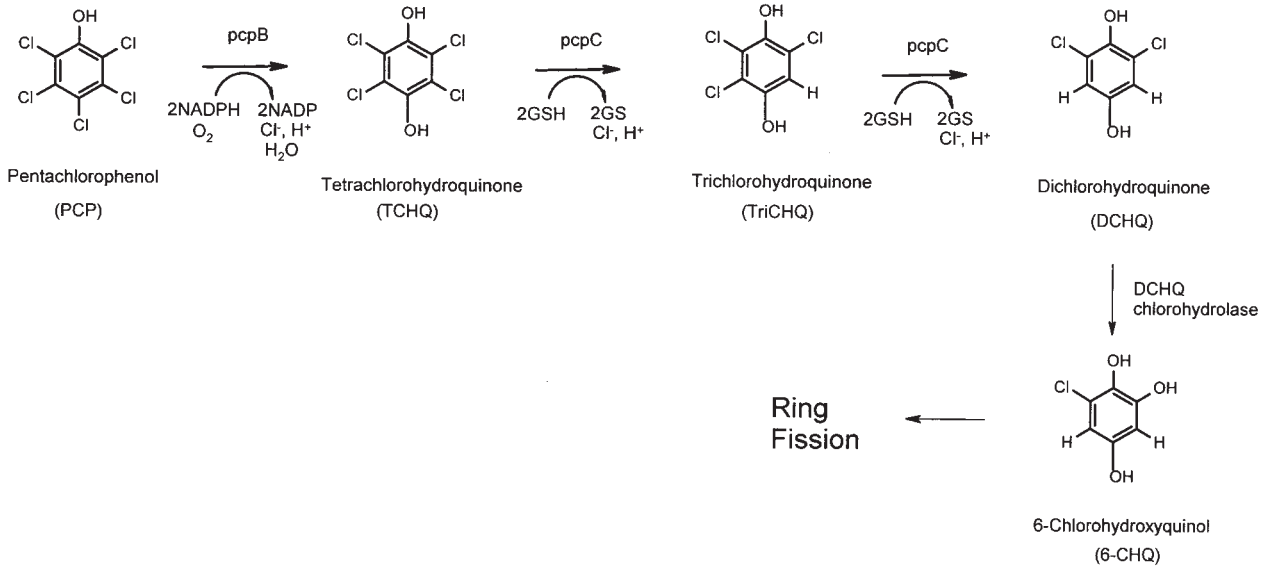

Figure 5 Proposed pathway of PCP degradation by *Sphingomonas* sp UG30.

ATCC 39723 and RA2) to degrade *p*-nitrophenol (PNP). All of the strains degraded PNP with nitrite release [23]. Using ^{14}C-labelled PNP, a significant amount of the PNP (15–22% of 140 μM) was mineralized to $^{14}CO_2$ by each strain in a minimal salts-glucose medium after 10 days. Mineralization of PNP by strain UG30 to CO_2 was not observed if glutamate was used instead of glucose in the minimal salts medium. However, nitrite was released by cells grown in minimal salts-glutamate medium containing PNP. Nitrite accumulated at a rate corresponding to PNP disappearance. Strain UG30 cells grown on glucose exhibited a transient accumulation of nitrite following which the nitrite was likely utilized as a nitrogen source.

Two aerobic pathways have been described for the initial degradation of PNP by bacteria [37]. In the first pathway, represented by a *Moraxella* sp [38], PNP is hydroxylated by PNP-4-monooxygenase which replaces the nitro group with an -OH group with subsequent release of nitrite (Figure 6, pathway 1). Ring fission of the hydroquinone proceeds via formation of hydroxymuconic semialdehyde, maleylacetic acid and β-ketoadipate. In the second pathway, found in *Arthrobacter* sp JS443 [17], PNP is hydroxylated by a monooxygenase to yield the major product 4-nitrocatechol or the minor product 4-nitroresorcinol (Figure 6, pathway 2). Following nitrite release mediated by a 4-nitrocatechol monooxygenase, these products are converted to 1,2,4-benezenetriol which is further broken down by *ortho*-cleavage [17].

We initially hypothesized that the PCP-4-monooxygenase may be involved in the initial transformation of PNP by *Sphingomonas* spp. In this scenario, PNP would be degraded according to the pathway used by *Moraxella* sp where PNP is *para*-hydroxylated to yield hydroquinone (Figure 6, pathway 1). However, our experimental evidence

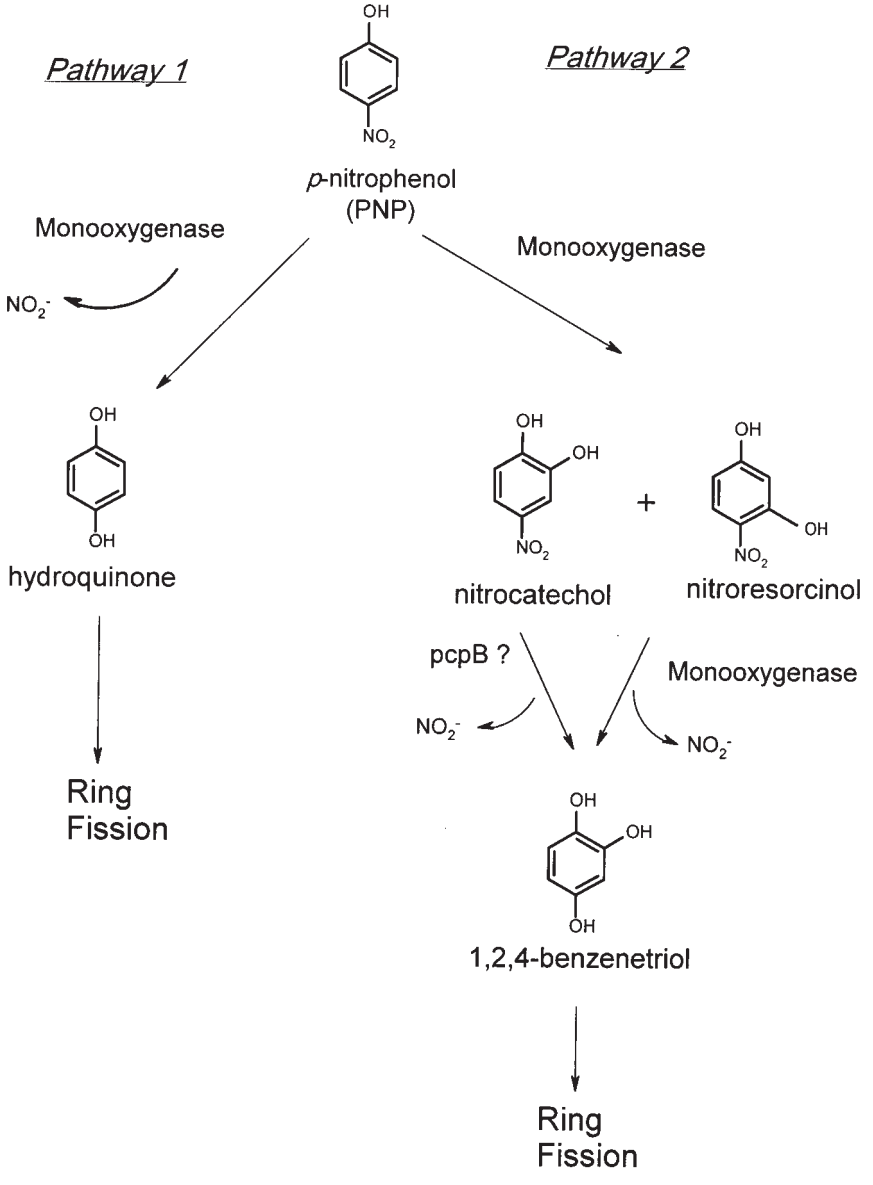

Figure 6 Two aerobic pathways of PNP degradation by bacteria. The proposed pathway for *Sphingomonas* sp UG30 is pathway 2. The involvement of the *pcpB* gene product in nitrocatechol (major) and PNP (minor) transformation is indicated.

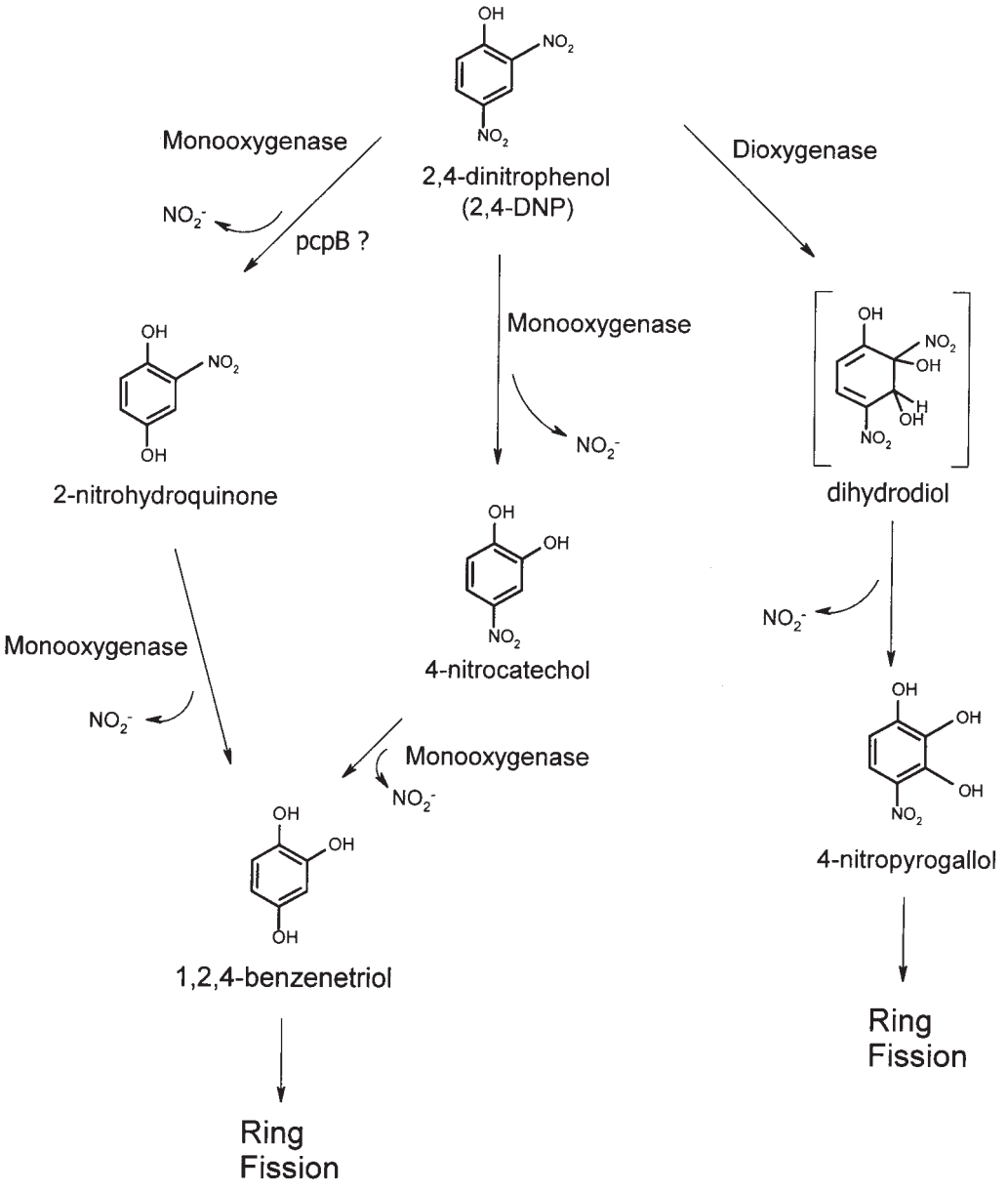

Figure 7 Various proposed aerobic pathways of 2,4-DNP degradation by bacteria. The pathway taken by *Sphingomonas* sp UG30 is proposed to be that on the left branch, where the *pcpB* gene product transforms 2,4-DNP into 2-nitrohydroquinone, which is subsequently converted to benzenetriol prior to ring fission.

suggests otherwise. First, thin layer chromatographic (TLC) analysis of metabolites produced from PNP by strain UG30 revealed accumulation of 4-nitrocatechol as an intermediate [23]. Second, PNP degradation by strain UG30 was not induced by prior exposure to PCP, a condition that induces PCP-4-monooxygenase [36]. In contrast, PNP induces the transformation of both PNP and PCP by UG30 [23]. These results suggest that UG30 degrades PNP by the alternate route (Figure 6, pathway 2). To resolve the above and to determine if PCP-4-monooxygenase is involved in PNP metabolism, the strain UG30 *pcpB* gene was cloned into *E. coli* BL21 cells. On expression, the recombinant pcpB protein readily hydroxylated 4-nitrocatechol to form 1,2,4-benzenetriol, with nitrite release [25]. In contrast, PNP was hydroxylated only to a slight extent. These results confirm the PCP-4-monooxygenase of strain UG30 preferentially *para*-hydroxylates phenols substituted in the *ortho* position with an electron withdrawing group, as seen in the same enzyme from *S. chlorophenolicus* ATCC 39723 [41].

Sphingomonas PCP-4-monooxygenases differ in several respects from the PNP monooxygenase from *Bacillus sphaericus* JS905 [18]. First, *Sphingomonas* PCP-4-monooxygenases are single component monomeric enzymes [25,30], while the PNP monooxygenase from *B. sphaericus* JS905 is a two-component enzyme consisting of a flavoprotein reductase and an oxygenase [18]. Second, *Sphingomonas* PCP-4-monooxygenases convert *p*-substituted polychlorophenols directly to chlorohydroquinones [31,41] instead of chlorobenzoquinones. While chlorobenzoquinone may be an intermediate in the reaction catalyzed by *Sphingomonas*

PCP-4-monooxygenase, it has not been detected in any studies to date and if it is formed, it is presumed to be reduced non-enzymatically to chlorohydroquinone. In contrast, PNP monooxygenase from *B. sphaericus* JS905 first oxidizes 4-nitrocatechol to benzoquinone, which is reduced enzymatically by the reductase component to a hydroxyquinone structure or 1,2,4-benzenetriol [18]. Third, PNP monooxygenase catalyzes both the hydroxylation of PNP and the oxidative release of nitrite from 4-nitrocatechol, while *Sphingomonas* PCP-4-monooxygenase does not convert PNP to 4-nitrocatechol and its main action in the PNP pathway appears to be the oxidative removal of nitrite from 4-nitrocatechol. We propose that the main route of PNP metabolism in strain UG30 is via pathway 2 (Figure 6), and that *Sphingomonas* PCP-4-monooxygenase is involved in the conversion of 4-nitrocatechol to 1,2,4-benzenetriol [25].

Biodegradation of other mono or dinitro-substituted phenols by *Sphingomonas* sp UG30

We also examined the ability of strain UG30 cells to oxidatively metabolize several dinitro-substituted phenolic compounds [42]. We observed that 2,4-dinitrophenol (2,4-DNP) and 4,6-dinitro-*o*-cresol (DNOC) were degraded, as monitored by nitrite release and an accompanying decrease in optical density (410 nm), indicating substrate disappearance. Oxidative metabolism was not observed with 2-, or 3-nitrophenol, 2,6-dinitrophenol or the herbicide dinoseb (2-*sec*-butyl-4,6-dinitrophenol). In mineralization studies using ^{14}C-labelled 2,4-DNP, we found that strain UG30 cells mineralized up to 20% of 103 μM 2,4-DNP within 5 days depending upon concentrations of glucose and glutamate in the medium. Glutamate represses PNP mineralization; however, mineralization of 2,4-DNP is enhanced by moderate levels of glutamate (0.4–4.0 g L^{-1}). Under higher glutamate conditions (4.0 g L^{-1}), only about 50% of the theoretical nitrite accumulated from 2,4-DNP metabolism, while a stoichiometric relationship was observed with PNP. Ethyl acetate phase partitioning and radiological thin layer chromatography indicated that less than 5–10% of the initial 2,4-DNP remained after 5 days incubation. Much of the 2,4-DNP (30–50% initial radioactivity) was transformed to an uncharacterized polar metabolite(s) that was not recovered by ethyl acetate phase partitioning of culture supernatants, and one major unidentified nonpolar metabolite (15–25% initial radioactivity) accumulated.

E. coli strain BL21(pBX2) expressing the *Sphingomonas* UG30 *pcpB* gene degraded both 2,4-DNP and DNOC, with release of nitrite corresponding to substrate depletion (Zablotowicz *et al*, unpublished). GC-MS showed that 2-nitrohydroquinone and 2-methyl-6-nitro-hydroquinone acccumulated in *E. coli* BL21 (pBX2) cultures incubated with 2,4-DNP and DNOC, respectively. These results suggested that the strain UG30 PCP-4-monooxygenase participates in the initial catabolism of both of these compounds and suggest that oxidative mechanisms are involved in 2,4-DNP (Figure 7) and DNOC catabolism by UG30. In the previously described ^{14}C-labelled 2,4-DNP studies with strain UG30, 2-nitrohydroquinone was rarely observed and then only early during incubation. Theoretically, 2-nitrohydroquinone can also be hydroxylated at the 3, 5, or 6 position by a monooxygenase and the nitrobenzenetriol product can be subjected to ring fission, as proposed during PNP metabolism by strain UG30 (Figure 6). Ring cleavage prior to release of the second nitro group is consistent with the stoichiometry of nitrite release and the accumulation of unmineralized polar metabolites. This route is similar to that of PCP where one chlorine is proposed to remain bound to the aromatic ring prior to ring fission (Figure 5).

The general aerobic pathway(s) for 2,4-DNP degradation by bacteria is not well characterized. As for the UG30 strain, we have evidence that PCP-4-monooxygenase is involved in 2,4-DNP degradation; however, pathways for PNP, 2,4-DNP and DNOC still need to be characterized. Recent studies [43] indicated that strain UG30 can transform the dinitroaniline herbicide trifluralin and the dinitrophenol herbicide dinoseb via aromatic nitroreduction. GC-MS analysis indicated that only one of the nitro groups from both trifluralin and dinoseb was reduced by strain UG30. Dinoseb was not an effective substrate for metabolism by strain UG30. However, the amino-derivative of dinoseb was not evaluated. Although strain UG30 is not as effective in microaerophilic reduction of these herbicides as are other *Enterobacter* and *Pseudomonas* strain [43], it is interesting that multiple mechanisms can be observed in *Sphingomonas* isolates such as UG30 for nitroaromatic transformation.

Biodegradation of a mixture of PCP and PNP

Sphingomonas sp UG30 can mineralize up to 250 μg ml^{-1} PCP or up to 500 μg ml^{-1} PNP in liquid media when either compound is present individually. The question arises as to how it will metabolize a mixture of PCP and PNP. We investigated this aspect using free and encapsulated cells in broth as well as in soil column bioreactors. Strain UG30 was able to mineralize (70% of $^{14}CO_2$ recovered) a PCP and PNP mixture (30 μg ml^{-1} of each compound) in a liquid medium. However, no mineralization of either compound was observed when higher concentrations (100 μg ml^{-1} each or greater) were used, although some nitrite was released in both cases [1]. Soil columns were designed containing either 100 or 225 mg kg^{-1} of each compound and inoculated with and without free or encapsulated cells at 1×10^8 CFU g dry $soil^{-1}$. Columns were perfused with phosphate buffer for 20 days. Using this perfusion system, enhanced degradation of PCP and PNP mixtures was observed as compared to similar statically incubated soil samples as determined by HPLC analysis. At the lower concentrations of PNP and PCP (100 mg kg^{-1}), complete degradation of PNP was observed in the soil perfusion columns within 24 h, and this was not affected by inoculation with strain UG30. In contrast, all PCP was degraded within 7 days in columns inoculated with encapsulated cells, while about 30% of the PCP remained in uninoculated columns. In treatments where 225 mg kg^{-1} PCP and PNP levels were used, some PNP and PCP remained in the columns regardless of inoculation. A low extent of PNP degradation was observed in some soil columns (about 25% of experimental units), where all the PNP was degraded rapidly after 14–16 days in both inoculated and uninoculated soil columns. Although no benefit of

inoculation with strain UG30 was observed, soils perfused with phosphate buffer exhibited 50–75% enhanced degradation of both compounds. Apparently, when challenged with multiple contaminants the strain is unable to transform these compounds if their concentrations are above a threshold concentration of about 30 mg kg^{-1}.

Enumeration of *Sphingomonas* sp UG30 in soil

We have described a method to enumerate PCP-degrading bacteria in soil samples [24]. The efficiency of a modified most-probable-number/polymerase chain reaction (MPN/PCR) protocol was compared with the traditional MPN/[^{14}C]PCP mineralization assay [7] to quantify the density of PCP-degrading *Sphingomonas* sp UG30 cells inoculated in an agricultural soil. A 753-bp tetrachlorohydroquinone dehalogenase gene (*pcpC*) fragment of strain UG30 was used for the MPN/PCR amplification. The MPN/PCR protocol had a minimal detection limit of 3 cells g dry $soil^{-1}$. Southern hybridization was performed to confirm the specificity of the PCR detection assay. A good correlation was established between the MPN/PCR estimations and initial inoculum densities ranging from 30 to 6.3×10^9 cells per g of soil. However, the MPN/^{14}C-PCP mineralization protocol underestimated the inoculum density by 70 to 740-fold. Survival of strain UG30 cells in soil was monitored by the MPN/PCR assay. The cell density decreased from 1.8×10^8 to 1.9×10^5 cells g $soil^{-1}$ in the first 20 days in soil and then stabilized at 1.9×10^4 cells g $soil^{-1}$ after 50 days. When the soil was autoclaved prior to inoculation, cell density remained at 6.8×10^7 cells g $soil^{-1}$ after 50 days. The method is useful in gaining much-needed information on survival of specific microorganisms in soils. This type of information is essential when one wishes to correlate microbial activities and numbers and determine the survival of both naturally occurring and genetically engineered microorganisms in environmental samples. It also illustrates the use of direct DNA extractions from soil samples and PCR in studying survival of *Sphingomonas* cells in soil.

Concluding remarks

The ability of the PCP-degrading *Sphingomonas* sp UG30 to degrade various nitrophenolic compounds represents new knowledge which may be exploited for treatment of nitrophenol-containing wastes. *Sphingomonas* spp may or may not be as enzymatically versatile as the genus *Pseudomonas*. However, evidence indicates that some species may be effective PCP degraders. As more information is forthcoming on this interesting genus of bacteria, they may be better used in environmental biotechnology applications where PCP and nitrophenolic compounds need to be degraded.

In the future, new *Sphingomonas* spp will be isolated from diverse environmental samples. They may be capable of degrading chloro- and nitrophenols and other xenobiotic compounds. These new isolates will help build the data base of information on sphingomonads that is relatively limited. As more information on *Sphingomonas* ecology, physiology/biochemistry and genetics becomes available, molecular biology techniques will be easier to use with this genus and will yield new and interesting knowledge.

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