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Mineralization of 4-aminobenzenesulfonate (4-ABS) by *Agrobacterium* sp. strain PNS-1

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Abstract

A bacterial strain, PNS-1, isolated from activated sludge, could utilize sulphanilic acid (4-ABS) as the sole organic carbon and energy source under aerobic conditions. Determination and comparison of 16S r DNA sequences showed that the strain PNS-1 is closely related to the species of *Agrobacterium* genus. Growth on 4-ABS was accompanied with ammonia and sulfate release. TOC results showed complete mineralization of sulphanilic acid. This strain was highly specific for 4-ABS as none of the sulphonated aromatics used in the present study including other ABS isomers were utilized. Strain PNS-1 could, however, utilize all the tested monocyclic aromatic compounds devoid of a sulfonate group. No intermediates could be detected either in the growth phase or with dense cell suspensions. Presence of chloramphenicol completely inhibited 4-ABS degradation by cells pregrown on succinate, indicating that degradation enzymes are inducible. No plasmid could be detected in the *Agrobacterium* sp. Strain PNS-1 suggesting that 4-ABS degradative genes may be chromosomal encoded.

Abbreviations: 4-ABS – (4-aminobenzenesulfonate); GA – (Gentisate); MOHB – (m-hydroxy benzoate); PABA – (p-aminobenzoate); PCA – (Protocatechuate); PHBA – (p-hydroxybenzoate); PYA – (Pyrocatechuate)

Introduction

Sulphanilic acid (4-ABS) is an intermediate in the production of pharmaceuticals, pesticides and is a component of many azo dyes. The presence of a sulfonic acid group on the aromatic ring confers a xenobiotic character to 4-ABS as not many aryl sulfonates are known among natural compounds (Laskin & Lechevalier 1984). This, along with its polar nature, renders 4-ABS resistant to degradation by unadapted activated sludge and many bacterial species utilizing natural aromatics. This can be due to the lack of specific transport proteins required for the uptake of polar 4-ABS. During the last few years, mixed cultures and a few bacterial strains which can grow on 4-ABS, as the sole carbon and energy source, have been isolated (Thurnheer et al. 1986; Feigal & Knackmuss 1988, 1993; Perei et al. 2000; Coughlin et al. 2003; Tan et al. 2005). The isolation of bacterial strains utilizing different isomers of 4-ABS was reported by Thurnheer et al. (1986). The microbial degradation of 4-ABS by a two-component bacterial consortium was reported by Feigal and Knackmuss (1988, 1993) and Dangmann et al. (1996). One of the species, *Hydrogenophaga palleroni*, strain S1

(reclassified as *Hydrogenophaga intermedia*) (Contezen et al. 2000) was able to oxidize 4-ABS to catechol 4-sulfonate, which was further utilized by strain S1 as well as the other member of the consortium, *Agrobacterium radiobacter* strain S2. (Feigal & Knackmuss 1993; Dangmann et al. 1996). Later, a bacterial strain, S5, was isolated by continuous adaptation of *H. palleroni* S1, which could grow aerobically on an azo compound, 4 - carboxy - 4 sulfoazobenzene (Blumel et al. 1998). Recently, degradation of 4-ABS by an unidentified strain SAD4I and *Pseudomonas paucimobilis* has been reported (Perei et al. 2000; Coughlin et al. 2003). In the present paper, we describe the isolation of 4-ABS degrading bacterial strain PNS-1 and its characterization. Other studies with this strain have been reported elsewhere. (Singh et al. 2004).

Materials and methods

Isolation of strain PNS-1

Strain PNS-1 was isolated from a 4-ABS degrading enrichment culture developed with activated sludge, derived from the Kanpur city domestic wastewater treatment unit, as the inoculum. One ml of activated sludge was added to 100 ml of mineral medium (MM, composition given below) supplemented with 400 mg/l (2.3 mM) 4-ABS as growth substrate and the flask was kept in a rotary shaker (120 rpm) at 35 °C. When >80% of the 4-ABS was degraded, further enrichment was carried out by transferring 10% inoculum to fresh medium. After 20 such transfers, two strains, PNS-1 and PNS-2, were isolated from the enrichment culture.

Medium and growth conditions

The growth medium consisted of the following constituents (g l^{-1} distilled water): $Na_2HPO_4 \cdot 2H_2O$ (2 g), KH_2PO_4 (1 g), NH_4Cl (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.25 g), K_2SO_4 (0.06 g) and $CaCl_2 \cdot H_2O$ (0.035 g) and 10 mg yeast extract. After sterilization, 1 ml of filter sterilized trace element solution was added (Kneimeyer et al. 1999). For ammonia release studies, NH_4Cl was omitted in the culture medium and the trace element solution without EDTA was used. For determination of sulphate release, $MgSO_4$ and K_2SO_4 were replaced with $MgCl_2$ and KCl, respectively. The required volumes of filter-sterilized 4-ABS (neutralized to pH 7.0 using NaOH) or other aromatic carbon sources were added to the medium from appropriate stock solutions. Liquid cultures were grown in 100 ml medium (taken in 250 ml Erlenmeyer flasks) at 35 °C on a rotary shaker (120 rev min^{-1}). For 4-ABS – agar plates, 800 mg l^{-1} 4-ABS and 20 g l^{-1} agar were added to MM.

For any given experiment, where different test conditions were used, the culture grown on 800 mg l^{-1} (4.6 mM) to late exponential phase was used as inoculum. Initial biomass optical density was generally below 0.08 at 555 nm when 10% (w/v) inoculum was used.

Uninoculated controls with the organic carbon source were always included in experiments. For measurement of biomass growth, inoculated medium without the carbon source was kept as a control.

4-ABS degradation

Kinetics of 4-ABS degradation and release of ammonia or sulphate were carried out by growing the strain PNS-1 with 4.6 mM 4-ABS in a medium devoid of either nitrogen or sulphate source. Aliquots were removed at different time intervals. The samples were centrifuged at $1150 \times g$ (3400 rpm) for 10 min. and filtered through a 0.45 μ membrane. 4-ABS as well as ammonia or sulphate concentrations were determined in the filtrate.

Induction of 4-ABS degradation

Strain PNS-1 was grown in two 250 ml flasks (100 ml medium) in MM with succinate (1.9 mM) upto an absorbance of 0.6–0.65 under conditions described above. Cells were harvested by centrifugation at 10,000 rpm at 4 °C and the cell pellet from one flask was resuspended in 100 ml sterile MM containing only 4-ABS (2.3 mM) whereas the other received 4-ABS (2.3 mM) and chloramphenicol (125 mg l^{-1}). The flasks were kept in shaking conditions (120 rpm) in an incubator. Degradation of 4-ABS with time was monitored. Two control flasks with 4-ABS pregrown cells instead of succinate were also included.

Utilization of aromatic compounds

The following monocyclic aromatic compounds were tested as growth substrates: 2-aminobenzene sulphonate (2-ABS), 3-aminobenzenesulphonate (3-ABS), 5-sulphosalicylate (5SA), p-hydroxybenzoic acid (PHBA), p-aminobenzoic acid (PABA), benzoate (B), m-hydroxy benzoate (mOHB), protocatechuate (3,4-dihydroxybenzoate or PCE) pyrocatechuate (2,3-dihydroxybenzoate or PYA), gentisate (2,5-dihydroxybenzoate or GA). Response of strain PNS-1 for utilization of these compounds was tested by growing the organism in MM supplemented with different compounds at an initial concentration of 800 mg l^{-1}. For these experiments, the culture grown on 800 mg l^{-1} 4-ABS (4.6 mM) to late exponential phase was used as the inoculum.

Characterization of isolated strains

Gram staining and biochemical tests were performed as per standard procedures (Cappuccino & Sherman 1999). The purity of the cultures was ascertained by plating on nutrient agar and microscopic observation.

Plasmid detection

Screening for plasmids in the strain PNS-1 was carried out by alkaline lysis method as described by Sambrook and Russel (2001) with slight modification. Plasmid screening was also carried out after chloramphenicol amplification (Sambrook & Rusell 2001).

Protocatechuate 3,4-dioxygenase assay

Cultures were grown on 800 mg l^{-1} aromatic carbon source upto late exponential phase and harvested by centrifugation at 12,000×g for 10 min. The cell pellet was then suspended in 5 ml of 50 mM phosphate buffer (pH 7.0). Crude extract was prepared by alternate freezing and thawing of cell suspension by plunging the vials into liquid nitrogen for 30 s and then placing it at 37 °C for 90 s. The thawing should be rapid in order to avoid crystal formation. The freezing followed by thawing was carried out three times. Extracts were stored on ice until use. Protocatechuate 3,4 dioxygenase activity was measured at 290 nm, as per the procedure described by Stainer & Ingraham (1954). The assay mixture consisted of 0.33 mM protocatechuate, 33 mM Tris-HCl buffer (pH 8.0) and 50–200 μl of crude extract in a total volume of 3.0 ml.

Analytical methods

Biomass growth was monitored by measuring the optical density in a UV-visible spectrophotometer (Shimadzu, Japan, model 160A) at 555 nm against distilled water. An optical density of 1.0 represented 340 mg cell dry weight per liter. Ammonia was measured colorimetrically by Nesslerization (APHA 1992). Sulphate in the culture filtrate was determined colorimetrically using barium chloroanilate as per the procedure described by Nortemann et al. (1986). 4-ABS and other aromatic substrates were estimated either by measuring the absorbance at their λ_{max} in UV-Spectrophotometer (Shimadzu, 160A) or by HPLC. For the detection of aromatic intermediate during 4-ABS degradation by strain PNS-1, culture filtrates, at different time intervals during the growth phase, were analysed by HPLC. A quantity of 20 μl of the sample was injected on a 4.6×250 mm C18 column (S.d. fine-chem Ltd., India). A mixture of methanol/deionized water/glacial acetic acid (60:38:2; v/v/v) was used as the solvent and flow rate was maintained at 0.5 ml min^{-1}. Another aqueous solvent system consisting of methanol (1%) and H_3PO_4 (0.1%) was also used. Analysis was also carried out with C8 column (S.d. fine-chem Ltd., India) using 40% methanol and 60% (0.05 M) phosphate buffer (pH 7.0). Emerging peaks were detected at 233 nm, 254 nm (for 4-ABS) and 274 nm using a UV-visible detector (Amersham Pharmacia Uppsala, Sweden). Organic carbon in the culture filtrates was analysed with a Shimadzu carbon analyzer (Model TOC – V_{CPN}) with biphthalate as a standard for dissolved organic carbon. Protein concentrations in cell extracts were estimated according to the modified procedure of Lowry et al. (1951) with bovine serum albumin as the standard. SDS-PAGE gel electrophoresis was carried out according to Laemmli (1970).

16S rDNA sequence determination and analysis

Genomic DNA was extracted and purified as described by Ausubel et al. (1992). PCR-mediated amplification of the 16S rDNA was carried out

using the universal primers 27f and 1525r according to Rainey et al. (1996). The same primers were also used for sequencing of the PCR products using the Bigdye terminator cycle sequencing ready reaction kit (Perkin–Elmer) with an ABI PRISM 377 DNA sequencer. Similarity searches were performed at NCBI using the BLAST program (Autschul et al. 1990), and a multiple alignment was constructed using ClustalX (Thompson et al. 1997). The sequence has been deposited in the NCBI GenBank PubMed database (http://www.ncbi.nlm.nih.gov/) with accession number AY00 762361.

Results

Enrichment and isolation of strain PNS-1

Enrichment was developed in batch culture with 4-ABS as the substrate under aerobic conditions. After at least 20 transfers in mineral medium supplemented with 400 mg l^{-1} 4-ABS, only two morphologically distinct types of colonies were observed in 24 h on nutrient agar plates, which led to the isolation of strains PNS-1 and PNS-2. Strain PNS-1 was cream colored, circular, smooth, opaque and 3 mm in size whereas strain PNS-2 was yellowish, circular and around 2 mm. Both strains were gram negative, rod shaped, catalase and urease positive, reduced nitrate to nitrite and utilized glucose as a carbon source for growth.

Sequencing of PCR-amplified 16S rRNA gene of strains PNS-1 and PNS-2 was carried out upto 1442 nucleotides, which represents around 97% of the complete gene. Sequence comparison showed that both strains PNS-1 and PNS-2 were identical and clustered with bacteria of α subclass of proteobacteria and closely with the genus *Agrobacterium* (Figure 1). All further studies were carried out with *Agrobacterium* sp. strain PNS-1.

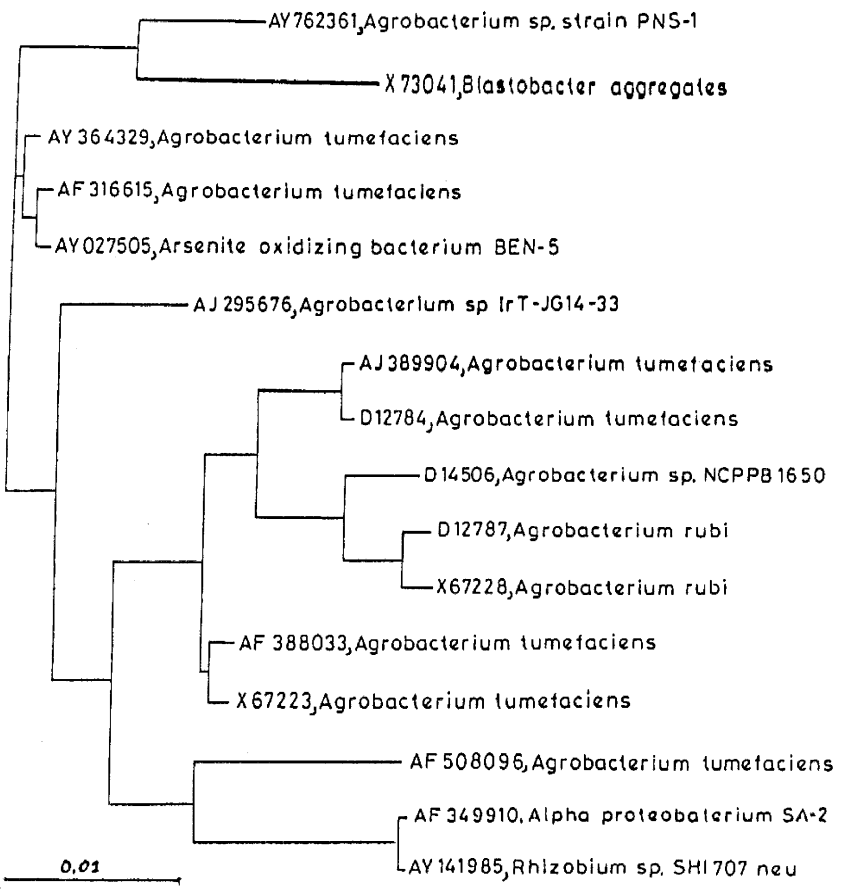

Figure 1. Unrooted dendogram showing the derived phylogenetic-taxanomic placement of strain PNS-1 among reference species. The scale indicates the evolutionary distance. The gene bank accession number is given in front of the species.

Kinetics of 4-ABS degradation

Figure 2(a) shows the growth curve for culture of PNS-1 along with substrate consumption and ammonium release. In inoculated controls, in the absence of 4-ABS, no growth was observed. The mean doubling time in the exponential growth phase was 3.2 h. This was marginally higher as compared to the growth of the culture in MM supplemented with NH_4Cl as nitrogen source (data not shown). Final concentration of ammonium in the culture filtrate was around 76% of the initial concentration of 4-ABS. The balance was probably incorporated into the cell material, as 4-ABS was provided as the only source of nitrogen. Sulphate was another product of 4-ABS degradation and its release in the culture was around 95% of the initial 4-ABS concentration (Figure 2b). Mineralization of 4-ABS was ascertained by determining the TOC of the culture medium prior to and after growing the strain on 4-ABS at two different initial concentrations. It was observed that residual TOC in the culture filtrate (0.45 μ filtered) were 9.8 mg l^{-1} and 11.6 mg l^{-1} when the organism was grown on an initial concentration of 2.3 mM (166 mg TOC l^{-1}) and 4.6 mM (322 mg TOC l^{-1}) 4-ABS, respectively. In uninoculated flasks, the change in TOC was less than 2.5%.

No aromatic metabolites could be detected either in the growth phase or when dense cell suspensions were incubated with 4-ABS in the presence or absence of aromatic ring cleavage inhibitors like α,α bipyridyl (Chapman & Hopper 1968; Spain & Gibson 1991).

Induction of ABS degradation

To determine, whether utilization of 4-ABS was inducible or constitutive, cells of *Agrobacterium* strain PNS-1, grown on succinate, were collected, washed and resuspended in fresh medium. Duplicate cultures received 4-ABS (400 mg l^{-1}) in the presence or absence of 125 mg chloramphenicol l^{-1} to inhibit protein synthesis. Substrate utilization was monitored. As a control, similar experiments were performed with 4-ABS grown cells. Results are presented in Figure 3. When precultured on 4-ABS, cells of *Agrobacterium* sp. strain PNS-1 degraded 4-ABS immediately whereas preculturing on succinate led to a lag phase of a few hours. The

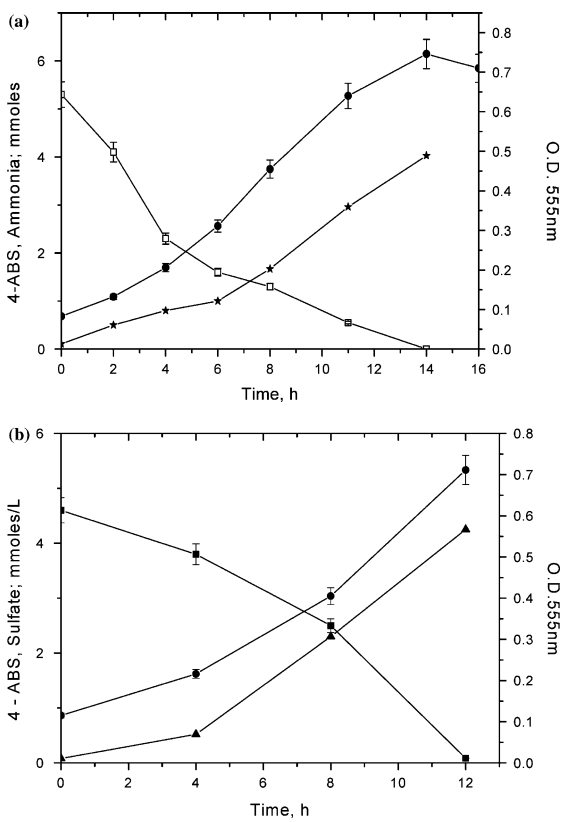

Figure 2. (a) 4-ABS degradation (□), release of ammonium (★) and increase in absorbance at 555 nm (biomass) (●). (b) Sulfate release during 4-ABS degradation. Sulfate (●), 4-ABS (■) and increase in absorbance at 555 nm (biomass) (▲).

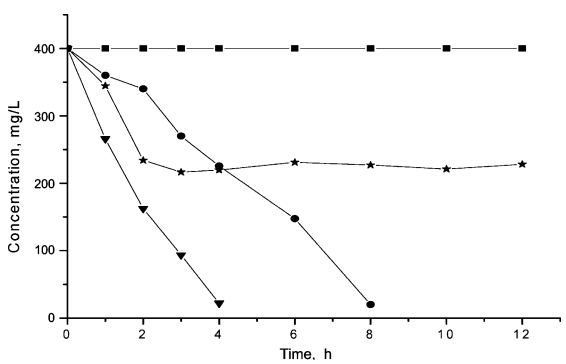

Figure 3. (▲) Degradation of 4-ABS by 4-ABS precultured cells, (★) Degradation of 4-ABS by 4-ABS degrading precultured cells in the presence of Chloramphenicol (125 mg l^{-1}), (●) Degradation of 4-ABS by succinate precultured cells and (■) Degradation of 4-ABS by succinate precultured cells in the presence of chloramphenicol (125 mg l^{-1}).

addition of chloramphenicol to cells precultured on succinate resulted in a total inhibition of 4-ABS degradation. 4-ABS grown cells were still able to degrade 4-ABS even in the presence of chloramphenicol. However complete degradation was not observed due to cell inactivation. Plating of suitable diluted aliquot on nutrient agar showed that around 45% viability was retained in the presence of chloramphenicol as compared to the control flask without inhibitor.

Growth on various monocyclic aromatic carbon sources

Among the sulfonated aromatics that were tested as growth substrates (2-ABS, 3-ABS and 5SA), *Agrobacterium* sp. strain PNS-1 could utilize only 4-ABS for growth. Growth and degradation of PHBA, PCA, PABA were almost immediate, whereas with B, mOHB, GA and PYA, a long lag phase extending upto 8–10 h was observed (data not shown). Crude extracts of 4-ABS, PHBA, PCA, PABA grown cells were assayed for protocatechuate 3,4-dioxygenase which is one of the key enzymes for aromatic compound degradation. No activity could be detected when the organism was grown on succinate, which was used as a control. Results presented in Table 1 shows that maximum activity was observed in PHBA grown cells, although PCA is the substrate for the enzyme. ABS also induced PCA dioxygenase activity. However, its activity was lower as compared to PHBA.

Table 1. Protocatechuate 3, 4-dioxygensae activity in *Agrobacterium* sp. strain PNS-1 grown on different carbon sources

Compounds	Specific activity[a] (μmole of PCA/min/mg Protein)
PHBA	1.67 ± 0.40
PCA	0.42 ± 0.26
PABA	0.28 ± 0.03
4-ABS	0.13 ± 0.03
MOHB	0.06 ± 0.01
GA	0.06 ± 0.01
PYA	0.04 ± 0.01
Sodium succinate	ND[b]

[a]Specific activity values of protocatechuate 3, 4 - dioxygensae are means of four independent experiments $\pm$S.E.M.
[b]Not detectable.

Discussion

4-aminobenzenesulfonic acid is a constituent of many azo dyes, pharmaceuticals and a few pesticides (Linder 1985). In this study, a new 4-ABS degrading bacterial strain, from an enrichment culture developed from activated sludge drawn from domestic wastewater treatment unit, is reported.

Determination and comparison of 16S rRNA sequences shows that this strain is more closely related to the species of *Agrobacterium* genus. Sequence comparison of the genes of 16S rRNA showed that the strain PNS-1 shared only 98% and 96% (Figure 1) identities with *A. tumefaciens* and *A. radiobacter*, respectively. Hence, it may be inferred that the isolated strain does not belong to these species. There have been reports on the isolation of *Agrobacterium* sp. from activated sludge treating domestic and industrial wastewaters and (Lauff et al. 1993; Dangmann et al. 1996; White et al. 1996). Strains of *A. radiobacter* appear to be the most frequently reported ones (Drysdale et al. 1999; Singh et al. 2004). *A. radiobacter* (strain S2) reported by Feigel and Knackmuss (1993) could not grow on 4-ABS as growth substrate. *Agrobacterium* sp. strain PNS-1 isolated in the present study could utilize 4-ABS as the sole carbon and energy source.

Growth of strain PNS-1 on 4-ABS was accompanied with almost stoichiometric release of sulphate and 76% ammonium. More than 95% dissolved organic carbon removal, after 4-ABS consumption, indicated complete mineralization. No metabolic intermediates could be detected under the experimental conditions used in this study either during the active growth phase or with dense cell suspensions even in the presence of α, α bipyridyl. Feigel and Knackmuss (1988, 1993) have reported the accumulation of catechol-4-sulfonate during degradation of 4-ABS with *H. palleroni* strain S1 and have proposed a degradation pathway for 4-ABS by a co-culture comprised of *H. palleroni* strain S1 and *Agrobacterim radiobacter* strain 2 (Feigal & Knackmuss 1993). It is possible that 4-ABS is degraded by a similar pathway in *Agrobacterium* sp. strain PNS-1.

Agrobacterium strain PNS-1 could utilize all monocyclic aromatic compounds devoid of sulfonic acid group, used in this study, as growth substrate. However, degradation of sulphonated

aromatics was restricted to only 4-ABS. Such an observation has been reported for other 4-ABS degrading bacterial isolates (Thurnheer et al. 1986; Perei et al. 2000). Protocatechuate is one of the key intermediates in the degradation of various aromatic compounds. Aromatic ring cleavage of PCA by *ortho* or *meta* pathway is initiated by PCA 3,4-dioxygenase and PCA 4,5-dioxygenase respectively. It is reported that sulfonated substrates induced only PCA 3,4-dioxygenase in *H. palleroni* (strain S1) and *A. radiobacter* (strain S2) as well as in the mixed culture (Fiegel & Knackmuss 1993). From sulfocatechol degrading *A. radiobacter* strain S2, two different PCA 3,4-dioxygenase have been isolated. PCA 3,4-dioxygenase I, did not oxidize catechol-4-sulfonate, whereas PCA 3,4-dioxygenase II could oxidize catechol-4-sulfonate as well as PCA (Hammer et al. 1996). PCA 3,4-dioxygenase I was more efficiently induced as compared to PCA 3,4-dioxygenase II in strain S2 by the gratuitous inducer catechol-4-sulfonate (Fiegel & Knackmuss 1993). Dense cell suspensions of *Agrobacterium* sp. strain PNS-1 did not produce a yellow color indicative of meta cleavage, when incubated with either 4-ABS or PCA. Studies on PCA 3,4-dioxygenase showed that this is an inducible enzyme as it was not detectable in succinate-grown cells. PHBA and PCA were better inducers as compared to 4-ABS.

Expression of genes, which code for the degradation of xenobiotics like chlorobenzoate and nitroaromatics is generally inducible rather than constitutive (Bayle & Borbour 1984; Jain et al. 1994; Haggblom & Young 1999). Observation that 4-ABS was not degraded by succinate grown cells of *Agrobacterium* sp. strain PNS-1 in the presence of chloramphenicol also shows that degradative enzymes are not constitutive, but are produced only in the presence of 4-ABS. Our attempts to detect plasmid in the isolate, using standard procedures, were not successful. Large catabolic plasmids may not be detected under these conditions. However, it may be mentioned that the *ortho* cleavage pathway is usually chromosomally encoded while the genes for *meta* cleavage are located on plasmid, although there are exceptions (Hewetson et al. 1978; Kivisaar et al. 1989; 1990; van der Meer et al. 1992). There appears to be no report on the involvement of a plasmid for 4-ABS degradation. The genes for two different PCA 3,4-dioxygenases are encoded by genomic DNA in *Agrobacterium radiobacter* strain S2 (Contezen & Stolz 2000). 2-ABS degradation, which involves a *meta* cleavage pathway, has been shown to be associated with a plasmid in *Alcaligenes* sp. strain O-1 (Jahnke et al. 1990).

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