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Conjugative transfer of preferential utilization of aromatic compounds from *Pseudomonas putida* CSV86

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Abstract *Pseudomonas putida* CSV86 utilizes naphthalene (Nap), salicylate (Sal), benzyl alcohol (Balc), and methylnaphthalene (MN) preferentially over glucose. Methylnaphthalene is metabolized by ring-hydroxylation as well as side-chain hydroxylation pathway. Although the degradation property was found to be stable, the frequency of obtaining $Nap^{-}Sal^{-}MN^{-}Balc^{-}$ phenotype increased to 11% in the presence of curing agents. This property was transferred by conjugation to *Stenotrophomonas maltophilia* CSV89 with a frequency of 7×10^{-8} per donor cells. Transconjugants were $Nap^{+}Sal^{+}MN^{+}Balc^{+}$ and metabolized MN by ring- as well as side-chain hydroxylation pathway. Transconjugants also showed the preferential utilization of aromatic compounds over glucose indicating transfer of the preferential degradation property. The transferred properties were lost completely when transconjugants were grown on glucose or 2YT. Attempts to detect and isolate plasmid DNA from CSV86 and transconjugants were unsuccessful. Transfer of degradation genes and its subsequent loss from the transconjugants was confirmed by PCR using primers specific for 1,2-dihydroxynaphthalene dioxygenase and catechol 2,3-dioxygenase (C23O) as well as by DNA–DNA hybridizations using total DNA as template and C23O PCR fragment as a probe. These results indicate the involvement of a probable conjugative element in the: (i) metabolism of aromatic compounds, (ii) ring- and side-chain hydroxylation pathways for MN, and (iii) preferential utilization of aromatics over glucose.

Keywords Conjugative element · *Pseudomonas putida* CSV86 · Biodegradation · Carbon source preference · Naphthalene and methylnaphthalene metabolism

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Introduction

Aromatic hydrocarbons are ubiquitous and produced primarily by pyrolysis of organic matter. Next to glucosyl residue, the benzene ring is the most widely distributed unit of chemical structure in the biosphere. Besides their natural origin they are also synthesized in large quantity by man, used heavily in various industries and are major environmental pollutants (Gibson and Subramanian 1984; Goldman et al. 2001). Due to their recalcitrant nature, mutagenic and carcinogenic properties, the study of biodegradation of these compounds is important. Microbes can degrade various aromatic compounds due to their ability to evolve and adapt novel catabolic pathways (Nojiri et al. 2004). In environment, acquisition of genes by horizontal gene transfer

(HGT) is one of the driving forces behind microbial evolution. The elements responsible for HGT are conjugative plasmids, integrative plasmids, conjugative transposons, and integrative and conjugative elements, ICE lands; (Burrus et al. 2002; Nojiri et al. 2004; Osborn and Boltner 2002; Springael and Top 2004; Top and Springael 2003; van der Meer et al. 2001; van der Meer and Sentchilo 2003)]. These elements (10–500 kb) can exist either as linear or circular plasmids or integrated into the host chromosome at specific sites (Nojiri et al. 2004; van der Meer et al. 2001; van der Meer and Sentchilo 2003). Pseudomonads contain several of these diverse elements responsible for degradation of various aromatic compounds. Catabolic plasmids like pWW0, OCT, NAH are well characterized and shown to contain complete set of transfer and catabolic genes (Nojiri et al. 2004; Yen and Serdar 1988; Dennis 2005). Genes coding for degradation of naturally occurring compounds occur on IncP-2 or IncP-9 and for man made xenobiotics they are frequently found on broad-host-range IncP-1 plasmids (Nojiri et al. 2004).

Naphthalene and methylnaphthalenes are commonly occurring hydrocarbons in the environment (Mahajan et al. 1994). Involvement of plasmids (NAH7, pDTG1, pNL1, pND6-1) and class II transposons (Tn*4655*) in naphthalene degradation is well documented (Dennis and Zylstra 2004; Li et al. 2004; Nojiri et al. 2004; Tsuda et al. 1999; Yen and Serdar 1988). *Pseudomonas putida* CSV86 utilizes naphthalene (Nap), salicylate (Sal), benzyl alcohol (Balc), 1- and 2-methylnaphthalene (MN) [Fig. 1, (Basu et al. 2003; Mahajan et al. 1994)]. MN is metabolized by two pathways. The first route is the 'carbon source' pathway, involves ring-hydroxylation to yield MN-diol and enters TCA cycle *via* methylcatechol *meta*-pathway (Fig. 1B). The steps are similar to naphthalene metabolic pathway [(Davies and Evans 1964), Fig. 1A]. The second route is 'detoxification' pathway which involves side-chain hydroxylation and subsequent oxidation of methyl group to naphthoic acid (Fig. 1C). Strain CSV86 degrades Balc *via* catechol *ortho*-pathway (Basu et al. 2003). The steps are similar to MN side-chain hydroxylation pathway (Fig. 1C, D) and are probably carried out by the same set of enzymes (Basu et al. 2003). Strain degrades aromatics and organic acids faster compared to glucose (Basu and Phale 2006) and shows a unique

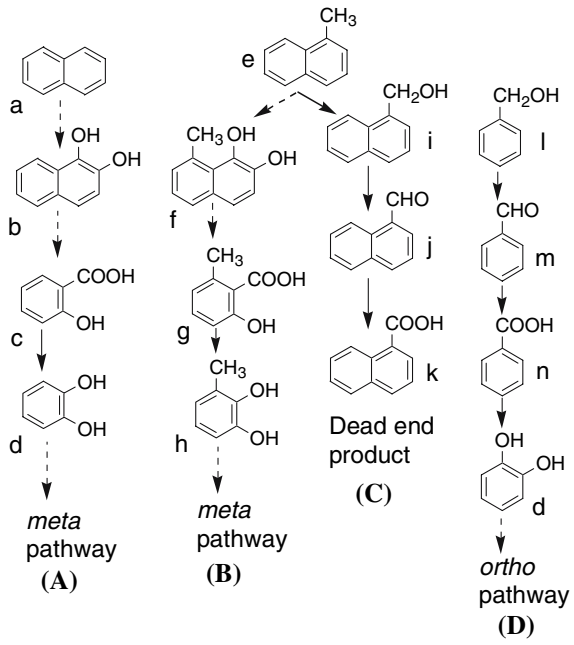

Fig. 1 Pathways for metabolism of (**A**) naphthalene; (**B**) methylnaphthalene ring-hydroxylation; (**C**) methylnaphthalene side-chain hydroxylation; and (**D**) benzylalcohol in *P. putida* CSV86. Various metabolites of the pathway are: a, naphthalene; b, 1,2-dihydroxynaphthalene; c, salicylate; d, catechol; e, 1-methyl naphthalene; f, 1,2-dihydroxy-8-methylnaphthalene; g, 3-methylsalicylate; h, 3-methyl catechol; i, 1-hydroxymethylnaphthalene; j, 1-naphthaldehyde; k, 1-naphthoic acid; l, benzyl alcohol; m, benzaldehyde; n, benzoic acid

preference for aromatic compound over glucose and co-metabolizes organic acids and aromatic compounds (Basu et al. 2006).

In the present study we assess the conjugal transfer of the phenotype to preferentially utilize aromatic hydrocarbons over glucose, from *P. putida* CSV86 to *Stenotrophomonas maltophilia* CSV89.

Materials and methods

Bacterial strains and growth conditions

Strains used in this study are given in Table 1. Strains were grown on Minimal Salt Medium [MSM, (Basu et al. 2003)] with appropriate carbon source (aromatic compound, 0.1% or glucose 0.25%; supplemented aseptically) or on rich medium, 2YT (Tryptone 16 g, yeast extract 10 g and NaCl 5 g per liter of distilled water). Agar plates were prepared by supplementing

Table 1 List of various strains used in the study

Strain[a]	Phenotype[b]	Details	Reference
S. maltophilia CSV89	Nap^-, Sal^-, MN^-, $Balc^-$, Am^S, Km^R, Pig^+	Blue-green pigment, Km^R due to pET27b	This study
P. putida CSV86	Nap^+, Sal^+, MN^+, $Balc^+$, Am^R, Km^S, Pig^-	Wild type, No pigment	Mahajan et al. (1994)
Derived from CSV86			
D7, D17	Nap^-, Sal^-, MN^-, $Balc^-$, Am^R, Pig^-	Glucose cured	This study
2YT6, 2YT8, 2YT39	Nap^-, Sal^-, MN^-, $Balc^-$, Am^R, Pig^-	2YT cured	This study
M9, M14	Nap^-, Sal^-, MN^-, $Balc^-$, Am^R, Pig^-	Mitomycin C cured	This study
Transconjugant T7	Nap^+, Sal^+, MN^+, $Balc^+$, Am^R, Km^R, Pig^+	Conjugation between CSV86 and CSV89	This study
Derived from T7			
T7D1, T7D4, T7D7, T7D9, T7D29, T7D40	Nap^-, Sal^-, MN^-, $Balc^-$, Am^R, Km^R, Pig^+	Glucose cured	This study
rT7-1, rT7-3, rT7-4	Nap^+, Sal^+, MN^+, $Balc^+$, Am^R, Km^R, Pig^+	Conjugation between CSV86 and T7D7	This study
P. putida RKJ1	Nap^+, Sal^+	Harbors pRKJ1	Samanta et al. (1998)

[a] *Pseudomonas putida* CSV86 and *Stenotrophomonas maltophilia* CSV89 referred as CSV86 and CSV89 throughout the manuscript

[b] Nap, naphthalene; Sal, salicylate; MN, methylnaphthalene; Balc, benzyl alcohol; Am, Ampicillin; Km, Kanamycin; ^{R}, resistant; ^{S}, sensitive; Pig, production of blue-green diffusible pigment on 2YT; −, negative; +, positive

with 1.5% agar. Growth was monitored every hour by measuring the optical density (O.D.) at 540 nm. *Stenotrophomonas maltophilia* CSV89, formerly known as *Pseudomonas maltophilia* CSV89 (Phale et al. 1995), was made Kanamycin (Km) resistant by transforming pET27b (a non-conjugative plasmid) and maintained on 2YT-Km agar slants. *P. putida* CSV86 and *S. maltophilia* CSV89 (Km^R) hence onward will be referred as CSV86 and CSV89, respectively. Identity of CSV86, CSV89, T7, T7D7, 2YT39, M9 and M14 was confirmed by 16S rRNA sequence analysis.

Stability, curing and conjugation

The stability of degradation property was monitored by growing CSV86 on glucose or 2YT. For chemical curing, culture was grown on 2YT containing various concentrations of mitomycin-C (5, 10 and 20 μg/ml), acridine orange (25, 50 and 100 μg/ml) or ethidium bromide (50 μg/ml) on a rotary shaker at 30°C for 48 h. Appropriate dilutions were spread on 2YT agar plates. Isolated colonies were replica plated on 2YT, Sal and Nap plates. Nap^-Sal^- colonies were scored and reported as percent cured. The conjugation between 'donor' CSV86 and 'recipient' CSV89 was performed by liquid mating technique. Cells grown on 2YT plus antibiotic (Am 50 μg/ml for CSV86 and Km 50 μg/ml for CSV89) were harvested, washed gently and suspended in sterile 2YT (cell density, 6.4×10^8 cells/ml). Equal volume of cell suspensions were mixed together and incubated undisturbed at 30°C for 3 h. Transconjugants were selected on Sal-Km double selection plates and confirmed by growing them in MSM containing Nap-Km, Sal-Km, 1- or 2-MN-Km.

Molecular biology techniques

Plasmid isolation methods as described by Kado and Liu (1981); Hansen and Olsen (1978) and modified protocol (modification: no alkali denaturation and neutralization steps as described in the original method), Anderson and McKay (1983), El-Mansi et al. (2000), Mukhopadhyay and Mandal (1983); Wheatcroft and Williams (1981) and Sambrook et al. (1989) were performed to detect plasmid DNA from CSV86 and transconjugants. Pulse field gel electrophoresis (PFGE) was performed (Biorad CHEF mapper XA) on 0.9% agarose in 0.5 X (TBE

(Tris-borate, 45 mM and EDTA, 1 mM) for 18 h at 5 V/cm at 10°C with included angle of 120°, switch time of 15–120 s and linear ramp. Agarose-embedded DNA plugs were prepared from mid-log phase cells as described (Ravatn et al. 1998a). For restriction digestion, the agarose plugs (~20 µl) were washed with 0.1 X TE for 1 h. The plugs were further soaked in 300 µl of restriction buffer for 3 h. Restriction enzyme (150 U/100 µl of plugs) was added to fresh restriction buffer, and the agarose plugs were incubated overnight at 37°C. The gel was visualized under UV light after staining with ethidium bromide.

For PCR amplification and sequence analysis, total DNA from CSV86 and T7 prepared by modified protocol of Hansen and Olsen (Hansen and Olsen 1978) as described above, was used as template. Based on nucleotide consensus for catechol 2,3-dioxygenase (C23O) and 1,2-dihydroxynaphthalene dioxygenase (12DHNO) from various organisms, forward and reverse primers were designed and purchased from Microsynth (Balgach, Switzerland). Primer sequences are: *C23Of*—5′ ATG AAC AAA GGT GTA ATG CGC CC 3′; *C23Or*—5′ TCA GGT CAG CAC GGT CAT GAA TC 3′; *12DHNOf*—5′ ATG AGT AAG CAA GCT GCA GTT ATC G 3′; *12DHNOr*—5′ TTA GCT CAG TTT TAC ATC CAG GCC A 3′. Amplification was done by using Taq DNA polymerase (MBI Fermentas), T_m of 47°C for 12DHNO and 63°C for C23O. The PCR products were electrophoresed on 0.7% agarose gel. The fragments of interest were purified using Qiaex II gel extraction kit (Qiagen, Germany) and sequenced [Bioserve Biotechnologies (India) Pvt. Ltd].

To perform DNA–DNA hybridizations, *Eco*RI and *Eco*RV digested DNA was electrophoresed on agarose gel (0.9%) by FIGE and blotted on to Biodyne B, positively charged nylon membrane (Pall, USA) by procedure described by Sambrook (35). The DNA was fixed on the membrane with UV (254 nm, 140 mJ/cm^2) using UV-cross-linker (Amersham). Hybridization was performed with DIG-Easy-hybe (Roche applied sciences) at 42°C as described by the manufacturer for 20–24 h. DNA hybridization probes were labeled with Digoxigenin using a random-primer DNA labeling kit (Roche). PCR amplified 0.9-kb fragment of the C23O gene from CSV86 was used as DNA probe. Hybridized membranes were developed by anti-Digoxigenin-AP conjugate using the procedure described by the manufacturer.

Metabolic studies

Biotransformation experiments were performed using 1- and 2-MN as substrate. The products were extracted with ethyl acetate and analyzed by TLC and GC-MS as described previously (Basu et al. 2003). Whole-cell O_2 uptake rates were measured using Oxygraph (Hansatech, UK) fitted with Clark's type oxygen electrode as described (Basu et al. 2003). The rates were corrected for endogenous respiration and expressed as nmol O_2 consumed min^{-1} mg^{-1}cells (wet wt.). Benzyl alcohol dehydrogenase (BADH), benzaldehyde dehydrogenase (BZDH), C12O and C23O were monitored in the cell-free extract as described earlier (Basu et al. 2003). Protein was estimated by the method of Lowry (Lowry et al. 1951) and specific activities were expressed as nmoles min^{-1} mg^{-1} protein. Reducing sugar was estimated as described by Miller (Miller 1959) using glucose as standard. Salicylate was estimated by ferric nitrate—HCl reagent (Trinder 1954) using salicylic acid as standard.

Results

Involvement of a conjugative element in aromatic metabolism

Pseudomonas putida CSV86 is Nap$^+$Sal$^+$MN$^+$Balc$^+$ [Table 1, (Basu et al. 2003; Mahajan et al. 1994)]. Irrespective of simple or rich media, ~1.2% of the cell population lost their ability to utilize aromatics and were Nap$^-$Sal$^-$ (6 out of 400 for glucose and 9 out of 820 for 2YT), rest were Nap$^+$Sal$^+$. Curing agents lead to increase in the frequency of occurrence of Nap$^-$Sal$^-$ in the population with increase in the concentration of curing agent. Compared to mitomycin-C (5%), acridine orange and ethidium bromide showed higher number of Nap$^-$Sal$^-$ colonies (11%). Phenotypes like Nap$^+$Sal$^-$ or Nap$^-$Sal$^+$ were not observed. Seven, Nap$^-$Sal$^-$ colonies obtained from curing experiment were chosen randomly for further analysis (Table 1). All seven strains failed to utilize Balc, Nap and MN (Table 1). Preserving these strains on agar slope or as glycerol stock was difficult. Liquid mating conjugation between *P. putida* CSV86 and *S. maltophilia* CSV89 yielded transconjugants with the frequency of transfer of 7×10^{-8} per donor

cell. The transconjugants, selected on salicylate (0.1%)-Kanamycin agar plates, were $Nap^{+}Sal^{+}MN^{+-}Balc^{+}Am^{R}Km^{R}$ in CSV89 strain as confirmed by 16S rRNA sequence analysis and their ability to produce blue-green diffusible pigment on 2YT. Of the seven transconjugants obtained, T7 was studied in detail. When grown on MSM supplemented with glucose (0.25%), T7 lost its ability to degrade aromatics and the obtained colonies were $Nap^{-}Sal^{-}MN^{-}Balc^{-}$ (100% loss, total 820 colonies screened), suggesting that the degradation property is unstable in transconjugant. Cured transconjugant strains, T7D7 and T7D9 were re-conjugated with CSV86. The observed transfer frequency was 4.5×10^{-8} per donor and transconjugants showed colony and degradation properties similar to T7 (rT7-1, rT7-3 and rT7-4, Table 1). Mating between CSV86 and D7 or D17 ($Nap^{-}Sal^{-}$ strains derived from CSV86) was unsuccessful, as $Nap^{-}Sal^{-}$ cells showed aggregation and were highly unstable leading to cell lysis. Similarly, mating between CSV86 and *P. putida* RKJ1 and other *Pseudomonas* strains was unsuccessful, indicating the specificity of transfer to CSV89. The identity of the parents as well as all derived strains was confirmed by 16S rRNA analysis.

To confirm the gain or loss of degradation property, metabolic studies were carried out. Transconjugant T7 showed: (i) growth on Nap, Sal, Balc, and MN; (ii) cell respiration on Nap, MN, Sal, catechol (Table 2); (iii) ability to transform 1-MN to 1-naphthaldehyde and 1-naphthoic acid (Table 3; similar results were obtained with 2-MN, data not shown); and (iv) cell-free extracts showed comparable activities of BADH, BZDH and C23O (Table 4). All observed metabolic properties of T7 were similar to that of CSV86 (Tables 2–4). Strain CSV89, T7D7 and few $Nap^{-}Sal^{-}$ strains derived from CSV86 failed to grow, respire or transform MN to naphthoic acid (Tables 2–4).

Isolation and detection of plasmid from CSV86 and T7 using various protocols was unsuccessful. This could be due to large size, sensitivity to shear stress and/or low copy number of the plasmid (if any), present in the strain. Therefore, cells were lysed in agarose plugs to reduce the shear and analyzed by PFGE. Such analysis yielded a single high molecular weight DNA, however we failed to detect any extra chromosomal DNA (data not shown). As a positive control, *P. putida* strain RKJ1 [harbors pRKJ1, 83 kb plasmid involved in naphthalene degradation, (Samanta et al. 1998)] showed a plasmid as well as genomic DNA bands.

To confirm the transfer of degradative genes and its subsequent loss from the transconjugants, PCR amplification of specific genes was carried out. Genes coding for 12DHNO ($\sim$302 aa, upper pathway enzyme) and C23O ($\sim$307 aa, lower pathway enzyme) of naphthalene pathway were selected. Using specific primers (see Material and methods), $\sim$0.9 kb PCR product corresponding to the expected size of 12DHNO and C23O genes was observed with CSV86 and T7 (Fig. 2A, B, lanes 1 and 3, respectively). Strain CSV89 and T7D7 failed to show any PCR product (Fig. 2A, B, lanes 2 and 4, respectively). Mitomycin-C cured $Nap^{-}Sal^{-}$ M9 and M14 strains, showed PCR product with 12DHNO primers (Fig. 2A lanes 6 and 7)

Table 2 Whole-cell oxygen uptake rates by various strains

Substrate	O_2 uptake rates[a] (nmol min^{-1} mg^{-1} cells) for different strains grown on respective carbon source						
	CSV86 Nap	CSV86 Glc	CSV89 Glc	T7 Nap	T7D7 Glc	M9 Glc	M14 Glc
1MN	3.3	tr[b]	tr	1	Tr	0.6	0.4
2MN	8.5	3.9	tr	6.9	Tr	0.5	0.5
Naphthalene	15.5	3.7	tr	9.8	Tr	tr	tr
Salicylate	2.3	0.9	tr	0.7	0.3	tr	tr
Catechol	23.9	5.6	0.3	22	0.4	tr	tr
Benzylalcohol	1.1	0.7	tr	0.4	Tr	tr	tr

1MN, 1-Methylnaphthalene; 2MN, 2-Methylnaphthalene; Glc, Glucose

[a] Oxygen uptake rates are corrected for endogenous cell respiration

[b] tr, values <0.3 nmol are reported as traces. Experiments repeated at least 3–4 times with triplicate readings, S.D ±5%

Table 3 Biotransformation of methylnaphthalene to naphthoic acid by various strains

Strain	TLC analysis[a]			Mass properties (m/z [molecular ion])	Identified as[c]
		R_f	Fluorescence property[b]		
P. putida CSV86					
	Spot I	0.97	Dark blue	142[M^+], 126, 115, 77	1-MN
	Spot II	0.93	Black non- fluorescent	156[M^+], 128, 101, 77	1-Nald
	Spot III	0.82	Bright blue	172[M^+], 155, 127, 115, 77	1-Nacid
S. maltophilia CSV89					
	Spot I	0.96	Dark blue	142[M^+], 126, 115, 77	1-MN
Transconjugant T7					
	Spot I	0.96	Dark blue	142[M^+], 126, 115, 77	1-MN
	Spot II	0.92	Black non- fluorescent	156[M+], 128, 101, 77	1-Nald
	Spot III	0.77	Bright blue	172[M+], 155, 127, 115, 77	1-Nacid
Cured strains: D7, D17, 2YT6, 2YT8, 2YT39, T7D1, T7D4, T7D7, T7D9, T7D29, T7D40					
	Spot I	0.98	Dark blue	142[M^+], 126, 115, 77	1-MN

[a] Solvent system used for TLC was Hexane:Chloroform:Glacial acetic acid (10:3:1, v/v) and GCMS conditions are as described earlier (Basu et al. 2003)

[b] UV fluorescence properties of the individual spots when exposed to the UV light (wavelength 254 nm)

[c] 1Nald, 1-naphthaldehyde; 1Nacid, 1-naphthoic acid

Table 4 Specific activity for enzymes of benzyl alcohol metabolism

Sp. activity (nmoles min^{-1} mg^{-1}) from various strains grown on						
Enzyme	CSV86		CSV89	T7		T7D7
	Balc	Ben	Ben[a]	Balc	Ben	Ben
BADH	485	5	Nd[b]	367	13	nd
BZDH	165	5	Nd	105	Nd	nd
C23O	70	47	Nd	74	54	nd
C12O	539	603	413	366	436	409

[a] Ben, Benzoate

[b] nd, enzyme activity could not detected. Experiments repeated at least three times with triplicate readings, S.D ±5%

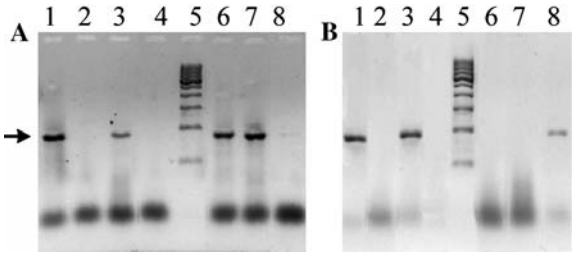

Fig. 2 Agarose gel electrophoresis of PCR product profile using (**A**) 12DHNO and (**B**) C23O primers. Lanes: 1, *P. putida* CSV86; 2, *S. maltophilia* CSV89; 3, Transconjugant T7; 4, T7D7; 5, Marker (500 bp ladder); 6, M9; 7, M14 and 8, 2YT39. Arrow indicates the PCR product (0.9 kb)

but failed to show product with C23O primers (Fig. 2B lanes 6 and 7), while 2YT39 Nap^-Sal^- strain showed product with C23O primers only (Fig. 2A, B lane 8). This may be due to partial loss or mutations in the genes by the curing agents, thus making them aromatic degradation negative. PCR products for 12DHNO and C23O from CSV86 and T7 were purified and sequenced partially (first 400 nt). The respective partial nucleotide sequences obtained from T7 and CSV86 were identical. Derived amino acid sequence of PCR products was used as query to search the protein RefSeq database (NCBI) using BLASTP (NCBI) with

default values for all other parameters. Search resulted in hits for sequences for C23O and 12DHNO enzymes from various organisms. For example, the query for amino acid sequence derived for PCR product of 12DHNO for CSV86 and T7 showed identity (130/141) and similarity (137/141) with *nahC* from *P. putida* NCIB 9816-4 and *Pseudomonas* sp. ND6. The query search for PCR product of C23O for CSV86 and T7 showed identity (135/141 and 127/141) and similarity (139/141 and 137/141) with *xylE* and *nahH* from *P. putida* and *P. putida* NCIB 9816-4, respectively.

DNA–DNA hybridization was carried out with *Eco*RI and *Eco*RV digested DNA of CSV86, CSV89 T7, and T7D7 using DIG labeled C23O PCR fragment as probe (Fig. 3). Using labeled probe, a single hybridizing band was observed with CSV86 and T7 (Fig. 3B). Recipient, CSV89 and cured transconjugant, T7D7 failed to hybridize with the probe (Fig. 3B) indicating the absence or loss of gene in the respective strains.

Preferential utilization of aromatics by transconjugant T7

Growth profile of T7 on benzoic acid plus glucose was diauxic with a distinct second lag phase at ~10–11 h. During first growth phase concentration of glucose in the medium remained constant which

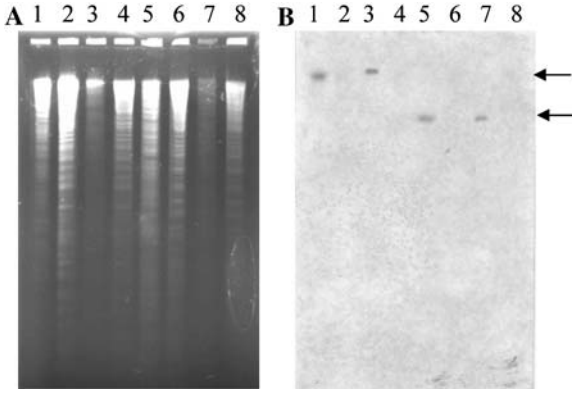

Fig. 3 Electrophoretic pattern of total DNA digested with restriction enzyme (**A**) stained with ethidium bromide, (**B**) southern hybridization with Dig-labeled C23O gene amplified from CSV86. Lanes 1–4 were digested with *Eco*RI and 5–8 were digested with *Eco*RV. Lane 1 and 5- CSV86; lane 2 and 6- CSV89; lane 3 and 7- T7; and lane 4 and 8- T7D7. Arrow indicates the DNA band labeled with probe after hybridization

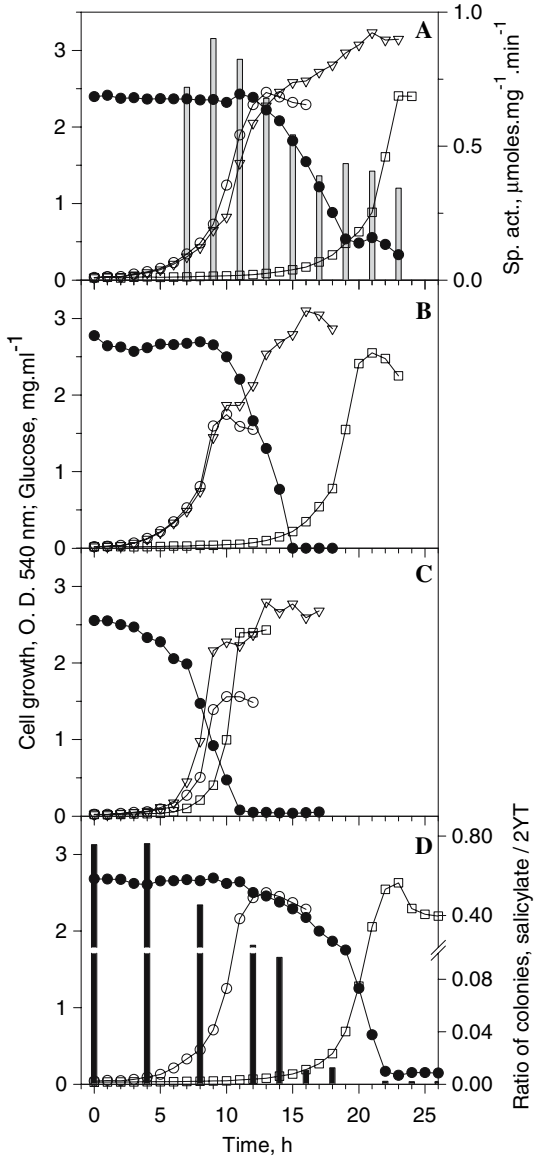

Fig. 4 Growth profile of (**A**) transconjugant, T7 and (**B**) cured transconjugant, T7D7 on double carbon source benzoic acid (0.1%) plus glucose (0.25%) (▽); benzoic acid (0.1%) (○); and glucose (0.25%) (□). Cells grown on benzoic acid were used as inoculum. (**C**) Growth profile of transconjugant T7 on naphthalene (0.1%) plus glucose (0.25%) (▽); naphthalene (0.1%) (○); and glucose (0.25%) (□).C23O activity is represented by gray bars. Cells grown on naphthalene were used as inoculum. (**D**) T7 on glucose, with inoculum prepared on naphthalene (□) and glucose (○). Black bars represent the ratio of colonies obtained on salicylate and 2YT plates. Reducing sugar concentration in the spent medium is represented by (●).Growth profile and glucose utilization pattern for CSV89 was identical to that of T7D7 strain (data not shown)

was rapidly consumed as cells entered the second log phase (Fig. 4A). Strain T7D7 also showed a diauxic growth pattern on benzoate plus glucose with distinct second lag phase at ~9–11 h and glucose utilization in the first growth phase (Fig. 4B), the profile was similar to CSV89 (data not shown). When grown on naphthalene plus glucose, T7 showed a diauxic growth pattern (Fig. 4C) with first growth phase overlapping with the naphthalene growth profile and the medium showing characteristic olive-green color. Glucose was consumed rapidly as cells entered the second log phase. The activity of C23O was maximum in the first log-phase which declined subsequently in the second log-phase indicating that naphthalene was utilized in the first growth phase (Fig. 4C). Similar diauxic growth profiles for T7 with utilization of aromatic compounds in the first and glucose in the second growth phase were observed on salicylate plus glucose and benzyl alcohol plus glucose (data not shown). T7 also showed diauxic growth profile with utilization of organic acids like succinate and pyruvate in the first phase of the growth and glucose utilization in the second growth phase when both organic acid plus glucose were provided as the carbon source. These profiles were similar to that observed for CSV86 (Basu et al. 2006). T7 pre-grown on naphthalene when inoculated on glucose had a long lag phase of about 12 h. Samples (1 ml) were withdrawn at (0, 4, 8, 12, 14, 16, 18, 20, 22, 24, and 26 h) serially diluted and plated on salicylate-MSM and 2YT agar plates. The colonies obtained were counted and the ratio of colonies on salicylate to 2YT was plotted against time (Fig. 4D). Results indicated that during the long lag phase the cells retained their hydrocarbon degrading property, however as the ability gradually diminished the cells entered the log phase utilizing glucose. It is interesting to note that the specific growth rate of T7 on glucose with inoculum grown on glucose or naphthalene is identical, although the naphthalene grown cells have a longer lag phase.

Discussion

Pseudomonas putida CSV86 utilizes naphthalene, methylnaphthalene and benzyl alcohol as the sole source of carbon and energy (Basu et al. 2003; Mahajan et al. 1994). This strain showed higher specific growth rate on aromatics as compared to glucose (Basu and Phale 2006). When grown on double carbon sources like aromatic compounds plus glucose, CSV86 showed a diauxic growth profile with preferential utilization of aromatics (Basu et al. 2006).

When grown on rich medium like 2YT or on simple carbon source like glucose, only 1% of the population lost its ability to degrade aromatic compounds and was $Nap^{-}Sal^{-}Balc^{-}MN^{-}$, indicating that the aromatic degradation property was stable in CSV86. As reported for other aromatic degrading organisms (Chakrabarty 1972; Dunn and Gunsalus 1973; Friello et al. 1976; Fujii et al. 1997; Mesas et al. 2004; Rheinwald et al. 1973), well known curing agents like mitomycin-C, acridine orange and ethidium bromide resulted in the significant increase (11%) in the $Nap^{-}Sal^{-}Balc^{-}MN^{-}$ population. Compared to CSV86, cured $Nap^{-}Sal^{-}$ colonies were large, translucent with uneven margin, produced yellow-red diffusible pigment and were cold sensitive. Changes in the cell size, cell morphology, colony morphology and pigment production due to loss of plasmid have been reported in *Pseudomonas, Klebsiella* and *E. coli* (Curiale and Mills 1982; El-Mansi et al. 2000; Rosas et al. 1983). These observations suggest that in CSV86 along with the loss of aromatic degradation property, few other vital functions are also lost or modified thus making cured cells unstable.

Degradation property of *P. putida* CSV86 could be transferred to *S. maltophilia* CSV89 by conjugation with a low frequency (7×10^{-8} per donor). Similar low frequency of transfer has been reported for *clc* element of *Pseudomonas* strain B13 (Ravatn et al. 1998b). Several attempts to transfer aromatic degradation property from CSV86 to other strains of *Pseudomonas* were failed. Obtained transconjugants were $Nap^{+}Sal^{+}MN^{+}Balc^{+}Am^{R}Km^{R}$. Pigment production on 2YT and 16S rRNA sequence analysis confirms the transfer from CSV86 to CSV89. When grown on 2YT or glucose all transconjugants lost the ability to degrade aromatics indicating that the degradation property is not stable. Metabolic studies like O_2 uptake, biotransformation and enzyme activity (Tables 2–4) demonstrate that T7 metabolizes naphthalene via catechol meta cleavage; methylnaphthalene via ring-hydroxylation 'carbon source' as well as side-chain hydroxylation 'detoxification'; and benzyl alcohol via catechol ortho

cleavage pathway. These metabolic steps were identical to that reported for CSV86 (Basu et al. 2003; Mahajan et al. 1994). Strains CSV89 and T7D7 failed to metabolize these aromatics (Tables 2–4). Curing, conjugation and metabolic studies suggest that all aromatic degradation genes including ring- and side-chain hydroxylation pathway genes for methylnaphthalene were transferred and lost together giving 'all or none' metabolic phenotype suggesting that aromatic degradation property is probably located on a single conjugative element. This conjugative element is stable in CSV86 but unstable in CSV89.

Pseudomonas putida CSV86 utilizes aromatic compounds in preference to glucose (Basu et al. 2006) and *S. maltophilia* CSV89 utilizes glucose in preference to benzoate (data not shown). Growth profile of transconjugant, T7 on aromatic compounds plus glucose was diauxic with utilization of aromatic compounds in the first growth phase (Fig. 4). The enzyme activity (C23O) and glucose utilization profiles were similar to CSV86 (Basu et al. 2006). Strain T7D7 ($Nap^{-}Sal^{-}Balc^{-}MN^{-}$) derived from T7 showed a diauxic growth profile on benzoate plus glucose, similar to that observed for CSV89, with utilization of glucose in the first growth phase (Fig. 4). Growth, enzyme activity and substrate utilization profiles of T7 and T7D7 indicate that the property of preferential utilization of aromatics was transferred from *P. putida* CSV86 to *S. maltophilia* CSV89.

Pseudomonads are very versatile microorganisms and reported to harbor large plasmids (conjugative, non-conjugative, integrative and linear) and conjugative transposons (Nojiri et al. 2004; Springael and Top 2004; Top and Springael 2003; Tsuda et al. 1999). Several large catabolic plasmids responsible for degradation of aromatic compounds have been isolated and characterized (Nojiri et al. 2004; Yen and Serdar 1988). There are few reports showing the conjugative transfer of aromatic degradation property but failed to detect extra-chromosomal DNA (Ka and Tiedje 1994; van der Meer et al. 2001; Weisshaar et al. 1987). Physical isolation and detection of plasmid from *P. putida* CSV86 and transconjugant T7 was unsuccessful. Large bacterial plasmids are difficult to detect or isolate reproducibly due to very low copy number (probably 1 or 2), high susceptibility to shear and/or may not exist as a plasmid due to integration into the host chromosome (Williams et al. 2004). *P. putida* CSV86 showed conjugative transfer of preferential utilization of aromatic compounds. PCR amplification of 12DHNO ($\sim$302 aa, upper pathway enzyme) and C23O ($\sim$307 aa, lower pathway enzyme) of naphthalene pathway was carried out. PCR product ($\sim$0.9 kb) corresponding to the expected size of 12DHNO and C23O genes was observed with CSV86 and T7; while CSV89 and T7D7 failed to show any amplification (Fig. 2) suggesting the absence and loss of transferred genes, respectively. DNA–DNA hybridization yielded a single hybridizing band in T7 and CSV86 and none in CSV89 or T7D7. Based on results obtained with *P. putida* CSV86, we hypothesize that in strain CSV86, a conjugative element is involved in the preferential degradation of aromatic compounds over glucose.

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