Analysis of preference for carbon source utilization among three strains of aromatic compounds degrading *Pseudomonas*

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One sentence summary: The current work investigates the uniqueness of *Pseudomonas putida* CSV86 for its preference for aromatic compounds over simple carbon source in comparison with other *Pseudomonas*.

ABSTRACT

Soil isolates *Pseudomonas putida* CSV86, *Pseudomonas aeruginosa* PP4 and *Pseudomonas* sp. CSpp degrade naphthalene, phthalate isomers and carbaryl, respectively. Strain CSV86 displayed a diauxic growth pattern on phenylpropanoid compounds (veratraldehyde, ferulic acid, vanillin or vanillic acid) plus glucose with a distinct second lag-phase. The glucose concentration in the medium remained constant with higher cell respiration rates on aromatics and maximum protocatechuate 3,4-dioxygenase activity in the first log-phase, which gradually decreased in the second log-phase with concomitant depletion of the glucose. In strains PP4 and CSpp, growth profile and metabolic studies suggest that glucose is utilized in the first log-phase with the repression of utilization of aromatics (phthalate or carbaryl). All three strains utilize benzoate via the catechol 'ortho' ring-cleavage pathway. On benzoate plus glucose, strain CSV86 showed preference for benzoate over glucose in contrast to strains PP4 and CSpp. Additionally, organic acids like succinate were preferred over aromatics in strains PP4 and CSpp, whereas strain CSV86 co-metabolizes them. Preferential utilization of aromatics over glucose and co-metabolism of organic acids and aromatics are found to be unique properties of *P. putida* CSV86 as compared with strains PP4 and CSpp and this property of strain CSV86 can be exploited for effective bioremediation.

Keywords: aromatic compound degradation; benzoate; diauxic growth profile; phenylpropanoids; preferential utilization; *Pseudomonas*

INTRODUCTION

Aromatic hydrocarbons are a major concern worldwide because of their recalcitrant nature and harmful effects on biota. The source of these compounds in the environment can be from nature (petroleum products, lignin, plant exudates, vol- canoes, forest fire, etc.) or anthropogenic activities (fuel combustion, crude oil spillage, gasoline leakage, industrial activities, etc.). Toxicity of aromatic compounds (endocrine
disruptive, genotoxic, mutagenic and carcinogenic) coupled with continuous release in the environment makes their removal desirable. Microbial remediation serves as the most effective way for the complete removal of aromatic compounds.

Despite the fact that number of microorganisms have been isolated and identified, factors that limit the aromatic compound degradation in the environment include poor bioavailability due to high hydrophobicity, low solubility and adsorption onto the soil matrix (Cerniglia 1993; Lu, Zhang and Fang 2011). Additionally, the presence of simple and easily metabolizable carbon sources like glucose and organic acids tends to limit the utilization of complex and reduced aromatic compounds. When a mixture of different carbon sources is available at a concentration that does not limit their growth, organisms show either co-metabolism or preferred utilization of one of the carbon sources over others. Moreover, during preferential utilization, organisms tends to use the carbon source that provides proficient growth, at the same time suppressing genes involved in the catabolism of other available non-preferred carbon sources. Many organisms show selective carbon source utilization. The classic example is glucose–lactose diauxia in Escherichia coli (Monod 1942). The presence of glucose leads to inhibition of secondary carbon source utilization. This phenomenon is known as carbon catabolite repression (CCR) and is one of the major obstacles in the process of bioremediation. Pseudomonads prefer organic acids over glucose (referred to as reverse CCR; Collier, Hager and Phibbs 1996). Glucose and organic acids are reported to suppress utilization of aromatics by down-regulating enzymes involved in their catabolism (Holtel et al. 1994; Schleissner et al. 1994; Muller et al. 1996; Cases, Perez-Martín and de Lorenzo 1999; Santos et al. 2000; Rentz, Alvarez and Schnoor 2004; del Castillo and Ramos 2007; Rampioni et al. 2008). An interesting aspect of biodegradation would be the preferential utilization of xenobiotics/aromatics even in the presence of a simpler carbon source. Genetically modifying strains to gain this property can be attempted but poses a concern with respect to stability, viability of these strains and risk to the environment (Singh et al. 2011).

Pseudomonas putida CSV86 utilizes aromatic compounds over glucose (Basu, Apte and Phale 2006; Shrivastava, Purohit and Phale 2011), whereas organic acids and aromatics are co-metabolized (Basu, Apte and Phale 2006). The genome sequence has revealed the presence of genes involved in the degradation of phenylpropanoid compounds (veratraldehyde, ferulic acid, vanillin and vanillic acid), which was confirmed functionally by growth analysis (Phale et al. 2013; Paliwal et al. 2014). Pseudomonas sp. have been reported to display the carbon source preference in the order organic acids > glucose > aromatic compounds. The questions raised in this study are (i) whether the preferential utilization of aromatics over glucose is applicable to all aromatics degraded by strain CSV86, and (ii) whether this property is unique to strain CSV86. Therefore, the utilization pattern of phenylpropanoid compounds in the presence of glucose was investigated. Soil isolates Pseudomonas aeruginosa PP4 and Pseudomonas sp. C5pp, which degrade phthalate isomers and carbaryl, respectively, were also analysed for carbon source preference.

The growth and metabolic studies suggest that the preferential utilization of aromatics over glucose is unique to strain CSV86. However, in strains PP4 and C5pp a diauxic growth response was observed with glucose utilization in the first log-phase and aromatics in the second log-phase.

MATERIALS AND METHODS

Organisms and growth conditions

Soil isolates Pseudomonas putida CSV86 (Mahajan, Phale and Vaidyanaathan 1994), Pseudomonas aeruginosa strain PP4 (Vamsee-Krishna, Mohan and Phale 2006) and Pseudomonas sp. C5pp (Swetha and Phale 2005) with the ability to degrade aromatics like naphthalene, benzoate, phthalate isomers and carbaryl were used in the present study. Here onward these isolates will be referred to as strains CSV86, PP4 and C5pp. The cultures were grown on minimal salt medium (MSM) (150 ml in a baffled 500 ml Erlenmeyer flask; Basu, Apte and Phale 2006) containing aromatic compounds (0.1%), glucose (0.5, 0.25, 0.05 or 0.025%), succinate (0.25 or 0.05%) or their combination at 30°C at 200 r.p.m. Growth profiles obtained using aromatic compound grown culture as inoculum are presented in the main paper, while profiles obtained with glucose grown culture as inoculum are given in the Supplementary data. Growth was monitored spectrophotometrically at 540 nm. Reducing sugar concentration in the medium was measured using dinitrosoisaliclylic acid (DNSA) reagent with glucose as the standard (Miller 1959).

Respiration rate analysis

Cells were harvested by centrifugation (7800 × g), washed twice with ice cold potassium phosphate buffer (50 mM, pH 7.5) and re-suspended [200 mg (wt wt) ml⁻¹] in the same buffer. The respiration rates were measured on various metabolic intermediates at 30°C using an Oxigraph (Hansatech, UK). The reaction mixture (2 ml) contained cells (8 mg), substrate (100 μM) and phosphate buffer (50 mM, pH 7.5). The observed rates were corrected for endogenous cell respiration (without substrate) and expressed as nmol O₂ consumed min⁻¹ mg⁻¹ cells.

Preparation of cell-free extract and enzyme assays

Cells grown under appropriate conditions were harvested and cell-free extract for strains CSV86 (Basu, Apte and Phale 2006), PP4 (Vamsee-Krishna, Mohan and Phale 2006) and C5pp (Swetha and Phale 2005) were prepared as described. Glucose 6-phosphate dehydrogenase (zwf; Lessmann, Schimz and Kurz 1975), catechol 1,2-dioxygenase (C12DO; Kojima et al. 1967), protocatechuate 3,4-dioxygenase (PDO; Fujisawa and Hayaishi 1968; Stanier and Ingraham 1954), carbaryl hydrolase (CH; Swetha and Phale 2005), 1-naphthol 2-hydroxylase (1-NH; Swetha and Phale 2005) and gentisate 1,2-dioxygenase (GDO; Suárez, Ferrer and Garrido-Pertierra 1995) were monitored spectrophotometrically (Lambda 35, Perkin Elmer, USA) as described. Protein estimation was carried out by the Bradford method using bovine serum albumin as the standard (Bradford 1976). The specific activities are expressed as nmol min⁻¹ mg⁻¹ of protein.

Growth and metabolic analyses were performed independently at least four to five times with respective enzyme and cell respiration measurements in triplicate or quadruplicate. Within the set of experiments, the trends observed were similar and the best profiles are presented.

RESULTS AND DISCUSSION

Growth of Pseudomonas putida CSV86 on phenylpropanoid compounds

Pseudomonas putida CSV86 preferentially utilizes aromatic compounds (naphthalene, benzyl alcohol, benzoate) over glucose
and co-metabolizes organic acids plus aromatics; organic acid suppresses utilization of glucose (Basu, Apte and Phale 2006). Though the molecular mechanism of preferential utilization is not clear, based on earlier studies it has been attributed to repression of glucose metabolizing enzymes (zwf), inability of glucose to suppress aromatic degrading enzymes and modulation of glucose transport proteins (Basu, Apte and Phale 2006; Basu and Phale 2006; Basu et al. 2007; Shrivastava, Purohit and Phale 2011; Modak, Bhaumik and Phale 2014). The question raised is whether the preferential utilization of aromatic compounds over glucose is applicable to all aromatics including phenylpropanoid compounds degraded by strain CSV86. Hence, growth and metabolic studies were performed on cells grown on phenylpropanoid compounds (0.1%) plus glucose (0.25%) using phenylpropanoid compound-adapted culture as inoculum (Fig. 1). Strain CSV86 showed distinctive diauxic growth pattern on veratraldehyde plus glucose (Fig. 1A), ferulic acid plus glucose (Fig. 1B), vanillin plus glucose (Fig. 1C) and vanillic acid plus glucose (Fig. 1D) with distinct first log, second lag and second log-phase. The first log-phase of the diauxic growth profile coincides with the growth pattern on the respective single aromatic compound (Fig. 1A–D). The glucose concentration remained constant in the first log-phase and declined significantly during the second log-phase (Fig. 1A–D). In the veratraldehyde plus glucose condition, cells in the first log-phase showed higher respiration rates on veratraldehyde and protocatechu- ate and non-detectable respiration on glucose. During the second log-phase, cells showed significant increase in the respiration rate on glucose with concomitant decrease in the glucose concentration from the medium, suggesting glucose utilization (Fig. 1A). On ferulic acid plus glucose, the specific activity of PDO, an enzyme reported to be involved in ferulic acid metabolism (Narbad and Gasson 1998), showed gradual increase during the first log-phase (maximum activity at 12 h, 385 nmol min⁻¹ mg⁻¹; Fig. 1B). However, during the second log-phase, PDO activity decreased with a concomitant decrease in the glucose concentration in the medium. Similar growth and enzyme activity profiles were observed for vanillin plus glucose (Fig. 1C) and vanillic acid plus glucose (Fig. 1D). The growth analysis was also performed using glucose grown culture as inoculum for phenylpropanoid compounds plus glucose (see Supplementary data, Fig. S1A–D). Strain CSV86 showed a diauxic growth pattern on phenylpropanoid compounds plus glucose with similar metabolic pattern. The observed duration for the first lag-phase was significantly longer (ranging from 2 h for vanillic acid to 26 h for vanillin) than that for the aromatic grown inoculum. For example, on veratraldehyde plus glucose, the first lag-phase duration observed was of 8 h (glucose grown culture as inoculum) compared with 2 h for veratraldehyde grown cells as inoculum (see Supplementary data, Fig. S1A). In the first log-phase of the diauxic pattern, cells showed significantly lower zwf (22.1 nmol min⁻¹ mg⁻¹) activity and higher PDO (14 h, 541 nmol min⁻¹ mg⁻¹) activity. As the culture entered the second log-phase, it showed a gradual decrease in glucose concentration with concomitant increase in zwf activity (28 h, 102 nmol min⁻¹ mg⁻¹) as well as a decrease in PDO activity (see Supplementary data, Fig. S1A). This data demonstrate that lignin derived aromatic compounds (veratraldehyde, ferulic acid, vanillin and vanillic acid) are also preferred over glucose, in addition to naphthalene, benzoate, salicylate, benzyl alcohol and p-hydroxyphenylacetate (Basu, Apte and Phale 2006; Shrivastava, Purohit and Phale 2011) and this property is independent of the carbon source used for the inoculum preparation. On succinate plus phenylpropanoid, no diauxic growth pattern was observed (D).
activity (maximum activity at 10 h, 267.4 nmol min$^{-1}$ mg$^{-1}$) and concomitant decrease in the glucose concentration in the first log-phase. Furthermore, the PDO activity was maximum in the second log-phase (26 h, 314 nmol min$^{-1}$ mg$^{-1}$) (see Supplementary data, Fig. S3). Additionally, the duration observed for the second lag-phase was 2 h for terephthalate grown culture as inoculum compared with 10 h for glucose grown cells. This increased duration of the second lag-phase observed probably can be attributed to the continued presence of glucose in the medium and the time required for induction of terephthalate degrading enzyme. The residual PDO activity observed might be responsible for the shorter duration lag-phase observed when terephthalate adapted culture was used as inoculum (Fig. 2A). On terephthalate plus succinate, strain PP4 showed the diauxic growth pattern with a distinct second lag-phase at 9–11 h. The first log-phase of the diauxic growth profile coincided with that of succinate alone (Fig. 2B). The specific activity of PDO was lower in the first log-phase and increased significantly during the second log-phase. These results suggest that in strain PP4, glucose and succinate are preferentially utilized over terephthalate.

**Growth of Pseudomonas sp. CSV86, PP4 and C5pp on double carbon source**

*Pseudomonas* sp. C5pp utilizes carbaryl via salicylate and gentisate (Swetha and Phale 2005). The strain displayed a diauxic growth pattern on carbaryl plus glucose with distinct second lag-phase at 5–6 h when carbaryl adapted culture was used as an inoculum (Fig. 3A). As is evident from the glucose concentration, the utilization of glucose was complete after 5 h, the end of the first log-phase. The cell respiration rates on carbaryl, 1-naphthol, salicylate and gentisate (Fig. 3A) and enzymes involved in the carbaryl metabolism, viz. CH (115 nmol min$^{-1}$ mg$^{-1}$), 1-NH (273 nmol min$^{-1}$ mg$^{-1}$) and GDO (311 nmol min$^{-1}$ mg$^{-1}$), showed maximum activity (Fig. 3B) in the second log-phase (9 h) of diauxic growth. The experiment was performed with glucose adapted culture as inoculum (see Supplementary data, Fig. S4). The diauxic growth pattern was observed with significantly higher zwf activity (6 h, 450 nmol min$^{-1}$ mg$^{-1}$) in the first log-phase with concomitant decrease in the glucose concentration, whereas the GDO activity peaked in the second log-phase (12 h, 523 nmol min$^{-1}$ mg$^{-1}$) suggesting the utilization of carbaryl. On carbaryl plus succinate, strain C5pp showed a diauxic profile with distinct second lag-phase at 5–6 h (Fig. 3C). Enzymes involved in the carbaryl degradation showed increased activity in the second log-phase. Thus, based on growth and metabolic analysis, it appears that the strain C5pp prefers glucose and succinate over carbaryl.

**Growth of Pseudomonas aeruginosa PP4 on double carbon source**

*Pseudomonas aeruginosa* PP4 metabolizes phthalate isomers via protocatechuate (Vamshee-Krishna, Mohan and Phale 2006). Strain PP4 (terephthalate adapted cells as inoculum) on terephthalate plus glucose showed a diauxic growth profile with a distinct second lag-phase between 12 and 14 h. Utilization of glucose in the first log-phase was evident from the decrease in the concentration of glucose from the medium (Fig. 2A). This observation was further supported by the activity profile of PDO, an enzyme involved in the terephthalate degradation, which gradually increased and reached a maximum (20 h, 207 nmol min$^{-1}$ mg$^{-1}$) in the second log-phase. Similar results were observed when glucose adapted cells were used as inoculum. Cells showed a diauxic growth pattern with increase in zwf was observed (see Supplementary data, Fig. S2), indicating the co-metabolism.

In strain CSV86 a diauxic growth profile with distinct second lag-phase was observed on aromatics plus glucose even at higher concentration of glucose (0.25% and above; Basu, Apte and Phale 2006). In strains PP4 and C5pp the diauxic growth pattern was not as distinct (no clear second lag-phase was observed) at a glucose concentration of 0.25% in double carbon source media. Hence, the glucose concentration was reduced to 0.05% for strain PP4 and 0.025% for strain C5pp so as to dissect the diauxicity in the presence of aromatics.

**Growth of Pseudomonas sp. strains CSV86, PP4 and C5pp on benzoate plus glucose**

Benzoate is used as the carbon source by strains CSV86, PP4 and C5pp via the catechol ‘ortho’ ring-cleavage pathway. The growth profile and metabolic studies were performed on benzoate plus glucose so as to avoid any possible disparity due to difference in the aromatic carbon source. Strain CSV86 showed a diauxic growth pattern with a gradual decrease in the glucose concentration in the second log-phase. The C12DO activity was significantly higher (8 h, 249.6 nmol min$^{-1}$ mg$^{-1}$) in the first log-phase. The zwf activity was maximum (17 h, 27.8 nmol min$^{-1}$ mg$^{-1}$) in the second log-phase (Fig. 4A). Similar profiles were observed when an equimolar concentration of benzoate (5.5 mM or 0.067%) and glucose (5.5 mM or 0.1%) was used.
Figure 3. Growth, cell respiration and enzyme activity profile of Pseudomonas sp. C5pp. (A and B) The growth profile (filled circles) on carbaryl (0.1%) plus glucose (0.025%). Open circles represent the glucose concentration in the medium. (C) The growth profile (filled triangles) on carbaryl (0.1%) plus succinate (0.05%) and succinate (0.025%, open triangles) alone. Cells grown on carbaryl (0.1%) were used as inoculum. Respiration rates on carbaryl (car, open bar), 1-naphthol (1-noh, hatched bar), salicylate (sal, grey bar) and gentisate (gen, black bar) are depicted in panel (A). The specific activities of various enzymes involved in carbaryl degradation, viz. carbaryl hydrolase (CH, open bar), 1-naphthol 2-hydroxylase (1-NH, grey bar) and gentisate dioxygenase (GDO, black bar), are depicted in panels (B) and (C).

(Fig. 5). Cells showed maximum activity for C12DO (10 h, 362 nmol min⁻¹ mg⁻¹) with significantly low activity of zwf in the first log-phase. In second log-phase, significant increase in the zwf activity (20 h, 109 nmol min⁻¹ mg⁻¹) was observed. These results suggest that benzoate is utilized over glucose.

In strains PP4 and C5pp, the diauxic growth pattern (no distinct second lag-phase) was observed with the decrease in the glucose concentration within the first 4–6 h and maximum zwf activity in the initial (first) log-phase (Fig. 4B and C). The C12DO activity was maximum in the late (second) log-phase (Fig. 4B and C). Trends observed in the growth profiles of all three strains were similar irrespective of the inoculum used (benzoate or glucose adapted culture; see Supplementary data, Fig. S5A–C). Thus, the above result suggests that CSV86 utilizes benzoate over glucose, whereas strains C5pp and PP4 glucose over benzoate.

The growth profile and metabolic analysis from strains PP4 and C5pp on double carbon source displayed the repression of aromatic compound utilization in the presence of glucose as well as succinate. This observation is similar to the repression of aromatic compound utilization by simpler carbon compounds like glucose and organic acid as reported in other Pseudomonads (Hoit et al. 1994; Schleissner et al. 1994; Muller et al. 1996; Rentz, Alvarez and Schnoor 2004; Collier, Hager and Phibbs 1996;

Thus, P. putida CSV86 is the only Pseudomonas sp. so far to display the preferential utilization of aromatics over glucose. This strain has been reported to be plasmid free with the property of stable aromatic degradation (Basu and Phale 2008). It degrades various aromatic compounds via catechol (ortho and meta ring-cleavage pathway) or the protocatechuate route (Basu, Dixit and Phale 2003; Shrivastava, Purohit and Phale 2011). However it lacks genes/enzymes involved in metabolism of gentisic acid (one of the three key intermediate besides catechol and protocatechuate in the metabolism of aromatic compounds). Thus, strain CSV86 can be genetically/metabolically engineered for the degradation of gentisic acid as well as various higher polycyclic aromatic hydrocarbons. The unique property of Pseudomonas putida CSV86 to degrade aromatics preferentially over glucose and co-metabolize them in the presence of organic acids makes this strain an ideal candidate for effective bioremediation of aromatics even in the presence of a simple carbon source, thus evading carbon catabolite repression.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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Conflicts of interest. None declared.

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