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Preferential Utilization of Aromatic Compounds over Glucose by *Pseudomonas putida* CSV86

Aditya Basu,[1] Shree K. Apte,[2] and Prashant S. Phale[1]*

Biotechnology Group, School of Biosciences and Bioengineering, Indian Institute of Technology—Bombay, Powai, Mumbai 400 076,[1] and Molecular Biology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085,[2] India

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Pseudomonas putida **CSV86, a naphthalene-degrading organism, exhibited diauxic growth on aromatic compounds plus glucose, with utilization of aromatics in the first log phase and of glucose in the second log phase. Glucose supplementation did not suppress the activity of degrading enzymes, which were induced upon addition of aromatic compounds. The induction was inhibited by chloramphenicol, suggesting that de novo protein synthesis was essential. Cells showed cometabolism of aromatic compounds and organic acids; however, organic acids suppressed glucose utilization.**

The most effective and economical way to remove aromatic pollutants is by means of microbial degradation (1). However, microorganisms in nature show a preference for a simple carbon source such as glucose, and unless it is completely depleted, the complex carbon sources such as aromatic compounds are not degraded (3). Therefore, the growth response on two carbon sources is diauxic, reflecting their sequential utilization. During the first growth phase, the simple carbon source is utilized and the enzymes required for the utilization of the second carbon source are repressed. This phenomenon of carbon catabolite repression (CCR) has been shown to occur in several microorganisms. A well-studied example is the repression of lactose utilization by glucose in *Escherichia coli* (30). Here CCR is mediated through cyclic AMP (cAMP) (7, 18). CCR in nonenteric bacteria such as pseudomonads is not clearly understood. Irrespective of the carbon source, the intracellular cAMP levels and adenylate cyclase remain constant, and external addition of cAMP does not alter the repression (20, 28). In pseudomonads, organic acids are found to suppress glucose uptake and its catabolizing enzymes (6, 16, 17, 23, 32). Enzymes necessary for the utilization of amide (29), histidine (20), protocatechuate (35), and xylene (4, 34) are suppressed in the presence of organic acids. Catabolite repression at the transcriptional level by glucose, gluconate, and organic acids has been reported for the enzymes involved in catechol and chlorocatechol degradation (13) and for those involved in methyl phenol degradation (15). Glucose is known to repress the enzymes responsible for benzyl alcohol degradation in *Pseudomonas putida* (5) and to delay induction of the phenylacetic acid transport system in *P. putida* U (26). Recently, repression of phenanthrene degradation in *P. putida* by a plant root extract and exudates containing glucose, acetate, and amino acids has been reported (22). Thus, preferential utilization of a simple carbon source represses the degradation of complex compounds, leading to their accumulation in nature, thereby locking off the carbon and aggravating pollution. Attempts have been made to engineer organisms for efficient utilization of aromatics in the presence of glucose (24). However, the stability and viability of such strains in nature poses a challenge.

We report that *Pseudomonas putida* CSV86 shows an unusual preference for aromatics when grown on an aromatic compound plus glucose. The strain cometabolizes aromatic compounds plus organic acids, and organic acids suppress glucose utilization.

Growth conditions, chemical estimations, and enzyme assays. *Pseudomonas putida* CSV86 (12) was grown on 150 ml mineral salt medium (MSM) (2) at 30°C on a rotary shaker. Aromatic compounds (0.1%), glucose (0.25%), or organic acids (0.25% succinate or citrate, or 0.1% pyruvate) were added aseptically as carbon sources either alone or in combination. Growth was monitored at 540 nm. Reducing sugar concentrations were estimated as described by Miller (14) using glucose as a standard. Salicylate was estimated by the ferric nitrate-HCl reagent (33) using salicylic acid as a standard. Cell extracts were prepared as described earlier (2). Protein was estimated as described by Lowry et al. (11) using bovine serum albumin as a standard. 1,2-Dihydroxynaphthalene dioxygenase (12DHNO) (31), benzyl

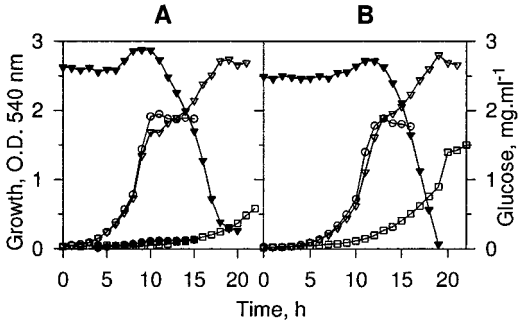

FIG. 1. Growth profile of *P. putida* CSV86 on 0.1% naphthalene plus 0.25% glucose (▽), 0.1% naphthalene (○), or 0.25% glucose (□). Cells grown on naphthalene (A) or glucose (B) were used as an inoculum. Solid symbols represent reducing sugar concentrations in the spent medium: ▼ for cells grown in naphthalene plus glucose and ● for naphthalene-grown cells.

* Corresponding author. Mailing address: Biotechnology Group, School of Biosciences and Bioengineering, Indian Institute of Technology—Bombay, Powai, Mumbai 400 076, India. Phone: 91 22 2576 7836. Fax: 91 22 2572 3480. E-mail: pphale@iitb.ac.in.

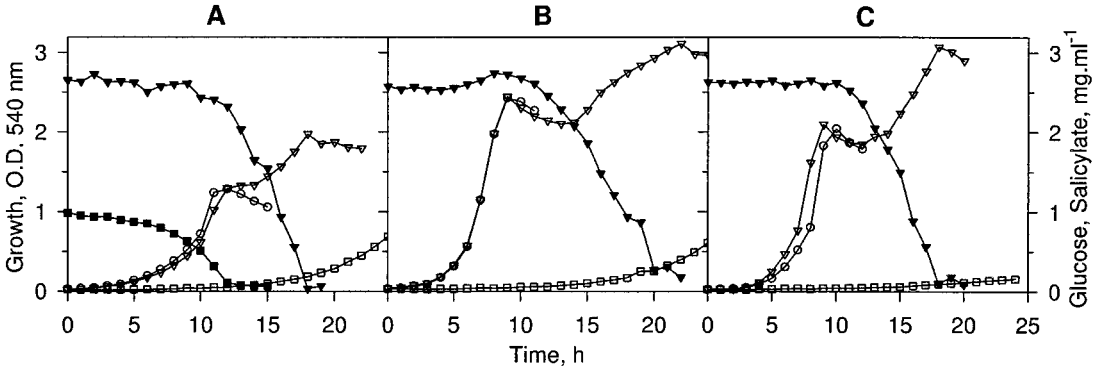

FIG. 2. Growth profile of *P. putida* CSV86 on 0.1% aromatics plus 0.25% glucose (▽), 0.1% aromatic compounds (○), or 0.25% glucose (□). Aromatics used were salicylate (A), benzyl alcohol (B), and benzoate (C). Cells grown on the respective aromatic compound were used as an inoculum. Measured salicylate (■) and glucose (▼) concentrations in the culture media are shown.

alcohol dehydrogenase (BADH) (27), catechol 1,2-dioxygenase (C12O) (8), catechol 2,3-dioxygenase (C23O) (19), and glucose 6-phosphate dehydrogenase (ZWF) (10) were monitored. Specific activities are expressed as nanomoles per minute per milligram of protein.

Growth profiles. *P. putida* CSV86 utilized naphthalene, methylnaphthalenes, benzyl alcohol, salicylate, and benzoate as the sole source of carbon and energy (2, 12). It also utilized glucose, glycerol, pyruvate, succinate, and citrate but failed to grow on gluconate, 2-ketogluconate, mannitol, or fructose. Pseudomonads sequester glucose as gluconate, and under carbon-limiting conditions the sequestered gluconate is used as a carbon source (9, 25). The inability of CSV86 to grow and respire on gluconate and 2-ketogluconate, the absence of gluconate oxidase activity (data not shown), and the presence of ZWF activity suggest that the organism utilizes glucose by the phosphorylative pathway and that the direct oxidative pathway is absent. Figure 1 depicts the growth profile on naphthalene plus glucose by using naphthalene- or glucose-grown cells (Fig. 1A or B, respectively) as an inoculum. Cells showed a diauxic (biphasic) pattern. The first growth phase of the diauxic profile overlapped with the naphthalene growth profile (Fig. 1), and the medium showed a characteristic olive-green color, indicating that naphthalene was utilized. As cells entered the second log phase, the glucose concentration declined (Fig. 1). Irrespective of the carbon source of the inoculum, cells grew slowly on glucose (Fig. 1). Varying the concentration of naphthalene (0.025 and 0.05%) or glucose (0.25, 0.5, or 1%) in a double-carbon-source medium yielded growth profiles with a shorter duration of the first log phase and a longer duration of the second log phase, respectively (data not shown). Cells showed a diauxic growth profile on salicylate plus glucose (Fig. 2A), benzyl alcohol plus glucose (Fig. 2B), and benzoic acid plus glucose (Fig. 2C) with utilization of salicylate (Fig. 2A) in the first and glucose (Fig. 2A, B, and C) in the second log phase. These results suggest that this strain utilizes aromatics in the first log phase and glucose in the second log phase.

Organic acids are known to suppress utilization of aromatics (3–5, 13, 15, 21, 22, 26, 34). On naphthalene plus succinate (Fig. 3A) and salicylate plus succinate (Fig. 3B), cells did not show a biphasic growth profile and utilized salicylate in the log phase. Other combinations, such as naphthalene plus pyruvate, salicylate plus organic acid, and benzyl alcohol plus organic acid, with cells grown either on aromatics or on organic acid as an inoculum gave similar results (data not shown). When grown on glucose plus succinate, cells showed a diauxic profile, with the first growth phase overlapping with the growth profile on organic acid alone, while the glucose concentration in the medium declined in the second log phase (Fig. 4). Inocula pre-

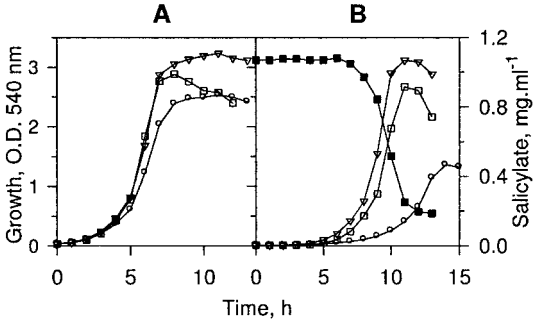

FIG. 3. (A) Growth profile of *P. putida* CSV86 on 0.1% naphthalene plus 0.25% succinate (▽), 0.1% naphthalene (○), or 0.25% succinate (□). (B) Growth profile of *P. putida* CSV86 on 0.1% salicylate plus 0.25% succinate (▽), 0.1% salicylate (○), or 0.25% succinate (□). ■, salicylate concentration in the medium. Naphthalene- or salicylate-grown cells were used as an inoculum.

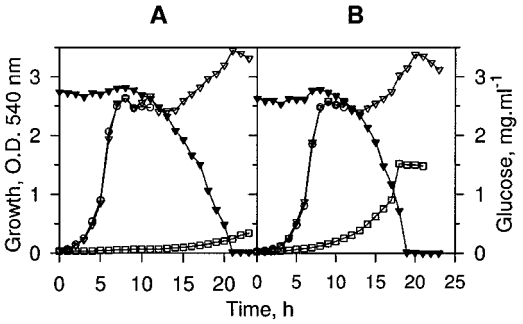

FIG. 4. Growth profile of *P. putida* CSV86 on 0.25% succinate plus 0.25% glucose (▽), 0.25% succinate (○), or 0.25% glucose (□). Cells grown on succinate (A) or glucose (B) were used as an inoculum. ▼, glucose concentration in the medium.

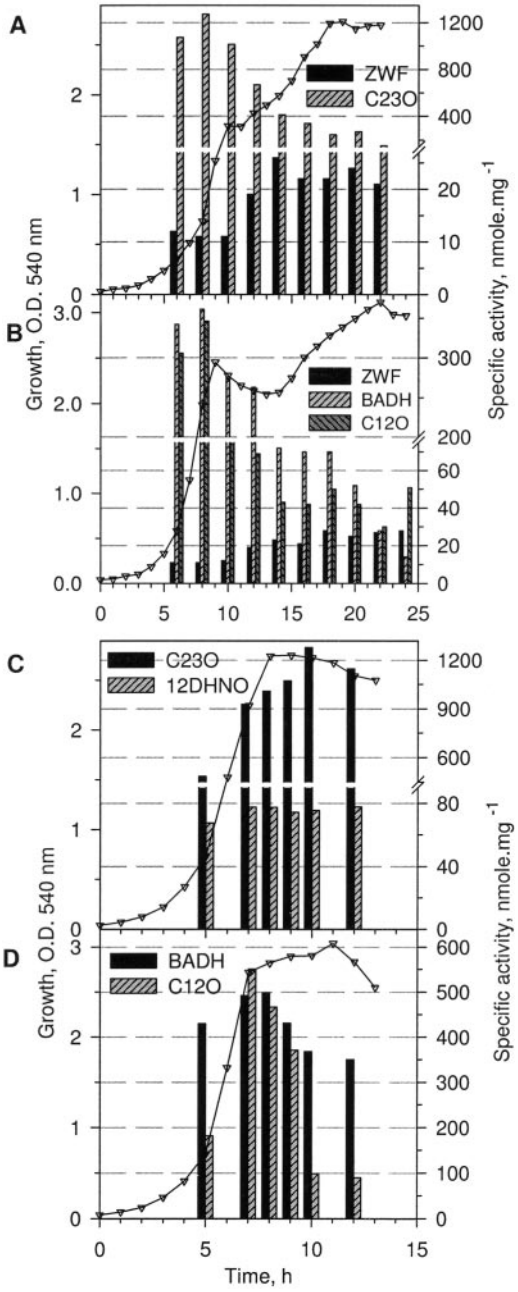

FIG. 5. Activity profiles of the enzymes involved in utilization of aromatics and glucose from cells grown on 0.1% naphthalene plus 0.25% glucose (A), 0.1% benzyl alcohol plus 0.25% glucose (B), 0.1% naphthalene plus 0.1% pyruvate (C), and 0.1% benzyl alcohol plus 0.25% succinate (D). Cells grown on the respective aromatic (for A and B) or organic acid (for C and D) were used as inocula. Growth profiles on double carbon sources are indicated by ▽ and specific activities of the enzymes by bars as indicated.

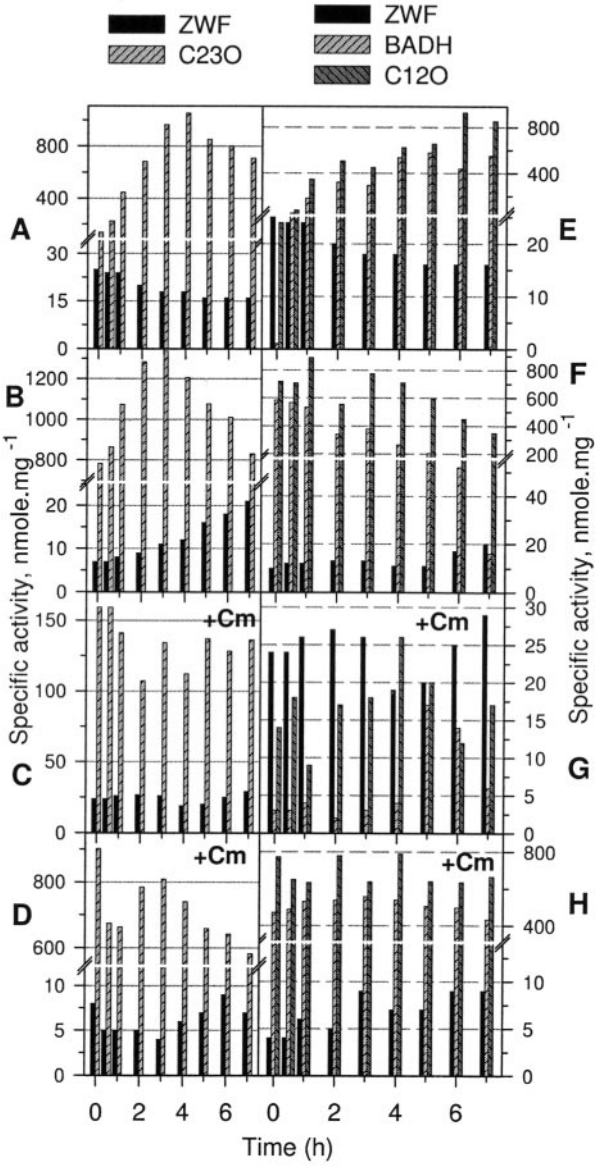

FIG. 6. Induction of key enzymes involved in the metabolism of aromatics and glucose in response to a second carbon source. Cells were grown to mid-log phase on glucose (0.25% for 18 h), naphthalene (0.1% for 6 h), or benzyl alcohol (0.1% for 6 h), and then the desired second carbon source (given below after the word "plus") was added either in the absence or in the presence of chloramphenicol (Cm; 100 μg/ml) at 0 h. Enzyme activities (bars) were monitored in the cell extracts. (A) Glucose plus naphthalene; (B) naphthalene plus glucose; (C) glucose plus naphthalene and Cm; (D) naphthalene plus glucose and Cm; (E) glucose plus benzyl alcohol; (F) benzyl alcohol plus glucose; (G) glucose plus benzyl alcohol and Cm; (H) benzyl alcohol plus glucose and Cm. Note that different scales are used on the *y* axis for each panel.

pared either on succinate (Fig. 4A) or on glucose (Fig. 4B) did not alter the organism's preference for organic acid utilization. Glucose plus pyruvate and glucose plus citrate gave similar profiles (data not shown). Suppression of glucose utilization by organic acids has been reported for several pseudomonads (3, 6, 17, 30, 32).

Enzyme activity profiles. On naphthalene plus glucose, cells showed maximum activity of C23O in the first log phase and ZWF activity in the second log phase (Fig. 5A). Similarly, cells grown on benzyl alcohol plus glucose showed maximum activity of BADH and C12O in the first log phase and ZWF activity in the second log phase (Fig. 5B). The activities of 12DHNO

and C23O (Fig. 5C) and of BADH and C12O (Fig. 5D) were maximal in log-phase cells grown on naphthalene plus pyruvate and benzyl alcohol plus succinate, respectively. Similar results were observed with other combinations (data not shown). The activity of ZWF from succinate-grown cells was low (specific activity, 7). Cells grown on glucose plus succinate showed significantly higher ZWF activity (specific activity, 21) in the second log phase of the diauxic growth profile than in the first log phase (specific activity, 11). These results indicated that succinate suppressed ZWF activity.

When naphthalene or benzyl alcohol was provided as the second carbon source to mid-log-phase glucose-grown cells, the activities of C23O (Fig. 6A), BADH, and C12O (Fig. 6E) increased significantly and the activity of ZWF declined marginally. When glucose was added as the second carbon source to aromatic-grown cells, the activities of C23O (Fig. 6B) and of BADH and C12O (Fig. 6F) reached a maximum by 3 and 1 h, respectively. The increase in ZWF activity was followed by a concomitant decrease in the aromatic compound-degrading enzymes. Addition of a protein synthesis inhibitor, chloramphenicol (Cm), along with the second carbon source showed no further increase (induction) of aromatic- or glucose-utilizing enzymes when aromatics or glucose was added as the second carbon source (Fig. 6C, D, G, and H). These results suggest that addition of glucose failed to induce ZWF or suppress the aromatic degradation enzymes and that the increase in enzyme activity (induction) was due to de novo synthesis of proteins.

The data presented clearly show that *P. putida* CSV86 preferentially utilizes aromatic compounds over glucose. This is a novel property and has not been reported so far. This strain cometabolizes aromatics plus organic acids, and organic acids suppress glucose utilization. Preferential utilization of aromatics by this strain could be due to either (i) inability of glucose to suppress the aromatic-degrading enzymes, (ii) modulation of glucose uptake, (iii) suppression of glucose utilization enzymes by aromatics ("reverse CCR") or organic acids produced during the degradation of hydrocarbon, or (iv) the combination of all or some of these events. The unusual carbon source preference by *P. putida* CSV86 provides opportunities for bioremediation of aromatic compounds even in the presence of simple carbon compounds such as glucose and organic acids in the environment.

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