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Genetic requirements for the expression of benzylamine dehydrogenase activity in *Pseudomonas putida*

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Abstract

Pseudomonas putida grows on benzylamine as a sole source of carbon/energy and nitrogen. Synthesis of an inducible benzylamine dehydrogenase (BMDH) depends on the specific RNA polymerase sigma factor σ^{54} and is subject to carbon source-dependent inhibition. The presence of TOL plasmid pWW0 harboring the genetic information for the catabolism of toluene exerts strong inhibition of induction of BMDH activity. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Pseudomonas putida*; Benzylamine dehydrogenase; σ^{54}; Catabolite repression; TOL plasmid

1. Introduction

Microbial degradation of aromatic amines proceeds via initial attack of either oxygen-dependent amine oxidases [1] or amine dehydrogenases employing intermediate electron acceptors [2–4]. An amine dehydrogenase enzyme of the latter class, oxidizing benzylamine to benzaldehyde (Fig. 1), has been reported for the soil bacterium *Pseudomonas putida* [3,5]. This organism possesses a TOL plasmid-encoded 'upper' and *meta* cleavage pathway for the catabolism of toluene, benzaldehyde, benzoate and derivative aromatic compounds [6], as well as an additional, chromosomally encoded pathway for benzoate degradation via an *ortho* cleavage route [7] (Fig. 1). We report here some features of the regulation of the expression of benzylamine dehydrogenase (BMDH) activity in *P. putida* and the interplay of expression of BMDH and TOL plasmid-carried *xyl* catabolic genes.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

P. putida strain KT2440 is a TOL plasmid-cured derivative of *P. putida* mt-2 [8]; its kanamycin-resistant mutant derivative KT2440 *rpoN* lacks the specific

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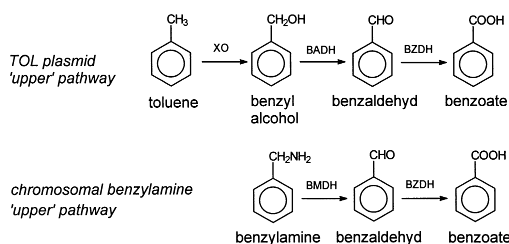
RNA polymerase sigma factor σ^{54} [9]. Strain KT2442RS is a rifampicin- and streptomycin-resistant variant of KT2440 (I. Möhler, unpublished). *P. putida* TOL plasmid pWW0 [10] and its derivative pWW0ΔPm [11], where a 0.4-kb *Pst*I fragment harboring the Pm promoter of the *xyl meta* pathway was replaced by an Ω-type transcription termination cassette encoding streptomycin resistance, have also been described. Cultures to be monitored for growth and benzylamine consumption were grown at 30°C in LB or modified M9 medium as described [12], but supplemented with 7.5 or 5 mM benzylamine instead of NH_4Cl, and additional carbon sources at a final concentration of 0.8% (w/v) as indicated. Rifampicin (50 μg ml^{-1}), kanamycin (100 μg ml^{-1}) and streptomycin (100 μg ml^{-1}) were added where required.

A)



B)

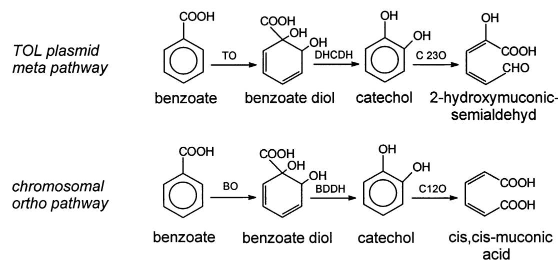


Fig. 1. Pathways for the catabolism of toluene and benzylamine in *P. putida*. A: Oxidation of the side chain ('upper' pathway). B: Cleavage of the aromatic ring via the TOL plasmid *meta* pathway or the chromosomal *ortho* pathway. BDDH, benzoate diol dehydrogenase; BMDH, benzylamine dehydrogenase; BO, benzoate dioxygenase; BZDH, benzaldehyde dehydrogenase; C12O, catechol 1,2-dioxygenase; C23O, catechol 2,3-dioxygenase; DHCDH, dihydroxycyclohexadiene carboxylate dehydrogenase; TO, toluate/benzoate dioxygenase; XO, xylene oxygenase.

2.2. Regulation of BMDH activity and monitoring benzylamine consumption

BMDH activities were determined in cells grown in 50-ml cultures of modified M9 medium as described above. Cells were harvested by centrifugation after 12 h of incubation and resuspended in 50 mM Na-phosphate pH 7. After passage through a French pressure cell the suspension of disrupted cells was centrifuged for 30 min (13 000 × *g*, 4°C), and samples of the supernatant fluid were used for BMDH assays: 10–200 μl crude extracts were added to 3 ml assay mixture containing 50 mM Na-phosphate pH 7, 0.5 mM phenazine methosulfate, 0.08 mM 2,6-dichlorophenolindophenol (DCPIP), 1 mM KCN, and 5 mM benzylamine, and the decrease in absorbance at 600 nm was monitored. The extinction coefficient for DCPIP at 600 nm is $\varepsilon_{600} = 21.5 \times 10^{-3}$ cm^{-1} μM^{-1}, 1 unit benzylamine dehydrogenase transforms 1 μmol benzylamine (equimolar turnover of 2,6-dichlorophenolindophenol) per minute. All BMDH activities indicated in Section 3 are the means of at least three independent experiments. Benzylamine concentrations in culture supernatant fluids were measured by HPLC analysis (Lichrospher RP8 5 μm column, solvent system methanol 5 mM/hexane sulfonic acid 1:1, absorbance monitored at 210 nm).

3. Results and discussion

3.1. Lag period of BMDH induction

Transfer of *P. putida* cells from cultures lacking benzylamine to minimal medium containing benzylamine always resulted in a long lag phase (about 10 h) before utilization of benzylamine commenced (see e.g. Fig. 4). While McClure and Venables [13] reported a similar 'genetic adaptation' of *P. putida* to aniline utilization to be correlated with the appearance of a novel plasmid [14], we found no evidence of plasmids in benzylamine-degrading *P. putida* strains (unpublished data). As one possible explanation of the observed lag period we considered that only a few cells in a culture population have the genetic potential for benzylamine utilization. To test this possibility, a *P. putida* overnight LB culture was washed, serially diluted in 50 mM Na-phosphate

pH 7 and spread in parallel on LB and succinate/benzylamine minimal plates. Since equal numbers of colonies were obtained on both media, it is concluded that obviously all viable *P. putida* cells (as judged by growth on LB plates) have the potential to utilize benzylamine (i.e. grow on succinate/benzylamine minimal plates). Another intriguing explanation for the observed lag effect would involve that, similar to the induction of the chromosomally encoded *P. putida ortho* cleavage pathway for benzoate degradation [15,6], a sufficiently high intracellular level of a slowly forming benzylamine-derived inducer must accumulate to allow for induction of BDMH synthesis.

3.2. Glucose-responsive inhibition of expression of BMDH activity

We have previously observed that degradation of benzylalcohol in *P. putida* is repressed by glucose [12]. To test whether such a 'catabolite repression' effect also inhibits benzylamine degradation, we monitored consumption of benzylamine by *P. putida* strain KT2442RS in cultures containing benzylamine and either succinate or glucose. After initial growth of all cultures (at the expense of a small casamino acid supplement added to obtain sufficient cell material for the subsequent enzyme assays, see footnote Table 1), a considerable growth lag period was observed, before in the succinate-, but not in the glucose-containing culture, consumption of benzylamine commenced and concomitantly, culture growth resumed (Fig. 2). Table 1 shows the respective BMDH activities of these cultures grown with various carbon sources. In the presence of benzylamine (either alone or with succinate) an approximately 10-fold induction of BMDH was obtained, while only about 15% of this level was reached in cultures containing glucose or gluconate. Thus, in addition to amine-responsive induction of BMDH activity, carbon source-dependent regulation exists.

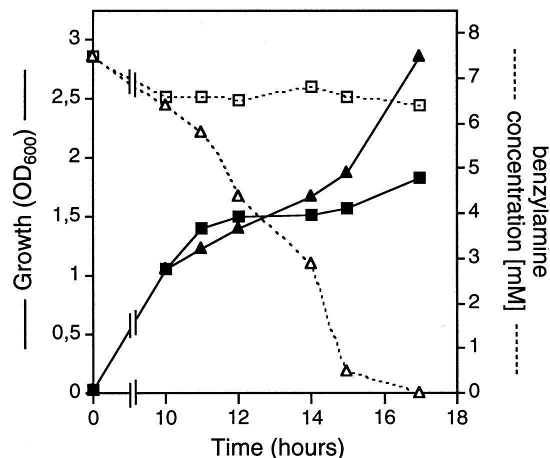


Fig. 2. Effect of additional carbon sources on growth (continuous lines) of and benzylamine utilization (dashed lines) by cells of *P. putida* strain KT2442RS in batch cultures containing benzylamine and either succinate (△,▲) or glucose (□,■); for more detailed culture conditions see footnote to Table 1.

Table 1
Effect of different carbon sources on benzylamine dehydrogenase activity in *P. putida* KT2442RS

Major source of carbon/nitrogen[a]	Benzylamine deydrogenase specific activity (mU mg^{-1})
Succinate/NH_4^+	8
Benzylamine/benzylamine	78
Succinate/benzylamine	73
Glycerol/benzylamine	53
Gluconate/benzylamine	15
Glucose/benzylamine	12

[a]Carbon sources added at 0.8%, benzylamine at 7.5 mM; 0.05% casamino acids added in all cases to obtain sufficient cell material even from cultures unable to utilize benzylamine for growth.

3.3. Synthesis of inducible BMDH activity requires RNA polymerase sigma factor σ^{54}

Since a number of substrate-induced catabolic pathways utilize RNA polymerase sigma factor σ^{54}, we tested whether benzylamine utilization and BMDH activity would be impaired in a σ^{54}-deficient *P. putida rpoN* mutant. Fig. 3 shows that wild-type strain KT2440 growing on succinate as carbon/energy source and benzylamine as the sole nitrogen source utilized benzylamine after the usual adaptation period, whereas the *rpoN* mutant metabolized only very little benzylamine. The corresponding BMDH activities were strongly reduced in the *rpoN* mutant strain grown under inducing conditions (both with benzylamine alone and with benzylamine+succinate) compared to the wild-type (Table 2), indicating that sigma factor σ^{54} is involved in the induction/synthesis of the inducible BMDH activity. The finding that synthesis of BMDH is both σ^{54}-

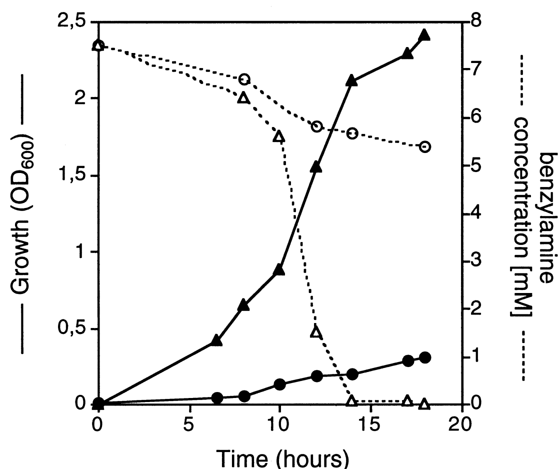


Fig. 3. Effect of an *rpoN* mutation on culture growth (continuous lines) of and benzylamine utilization (dashed lines) by *P. putida*. Batch cultures of wild-type strain KT2440 (△,▲) and derivative *rpoN* mutant (○,●) grown on benzylamine and succinate; for more detailed culture conditions see footnote to Table 1.

dependent and subject to a more general cellular carbon regulatory effect which is superimposed on the specific amine-responsive induction, parallels similar results obtained for other σ^{54}-dependent *P. putida* systems, e.g. the glucose-repressed σ^{54} *xyl* operon promoters [12,16] and the growth/energy status-responsive regulation of the σ^{54}-dependent phenol degradation operon promoter [17,18]. The apparent conformity of these different *P. putida* systems fosters the notion that a common carbon source- and/or growth/energy status-dependent regulatory mechanism is superimposed on, and overruling, the respective substrate-specific induction, and that this superimposed cellular regulation may specifically act on σ^{54}-dependent promoters as its primary targets.

3.4. Low expression of BMDH activity in bacteria containing TOL plasmid pWW0

Curiously, we noted poor growth of strain KT2440 in benzylamine medium after TOL plasmid pWW0 [10] had been introduced into it (Fig. 4A). In medium containing succinate as carbon source and benzylamine only as nitrogen source (Fig. 4B), both strains utilized benzylamine although the TOL plasmid-bearing strain grew more slowly. A corresponding pattern of BMDH activities in benzylamine- and in benzylamine/succinate-grown cells bearing the TOL plasmid was found (Table 2). The inhibitory effect of the TOL plasmid was largely absent in bacteria containing the TOL plasmid derivative pWW0ΔPm which lacks the *xyl meta* pathway promoter Pm and hence renders the cells unable to degrade (e.g. benzylamine-derived) benzoate through the TOL plasmid-encoded pathway [11]. This TOL plasmid-mediated inhibition of induction of BMDH synthesis resembles a similar regulatory interference effect by which in the presence of the TOL plasmid *meta* cleavage pathway for benzoate degradation induction of the chromosomally encoded benzoate *ortho* cleavage pathway is prevented; the more efficient

Table 2
Influence of an *rpoN* mutation and TOL plasmid determinants on benzylamine dehydrogenase activity in *P. putida*

Strain	TOL plasmids	Major source of carbon/nitrogen[a]	Benzylamine deydrogenase specific activity (mU mg^{-1})
KT2440	none	benzylamine/benzylamine	63
		succinate/NH_4^+	6
		succinate/benzylamine	78
KT2440 *rpoN*	none	benzylamine/benzylamine	3
		succinate/NH_4^+	5
		succinate/benzylamine	5
KT2440 (pWW0)	TOL plasmid pWW0,	benzylamine/benzylamine	6
	xyl 'upper; and	succinate/NH_4^+	7
	meta pathway	succinate/benzylamine	18
KT2440 (pWW0ΔPm)	TOL plasmid pWW0,	benzylamine/benzylamine	38
	no functional *xyl*	succinate/NH_4^+	14
	meta pathway	succinate/benzylamine	87

[a]See footnote to Table 1.

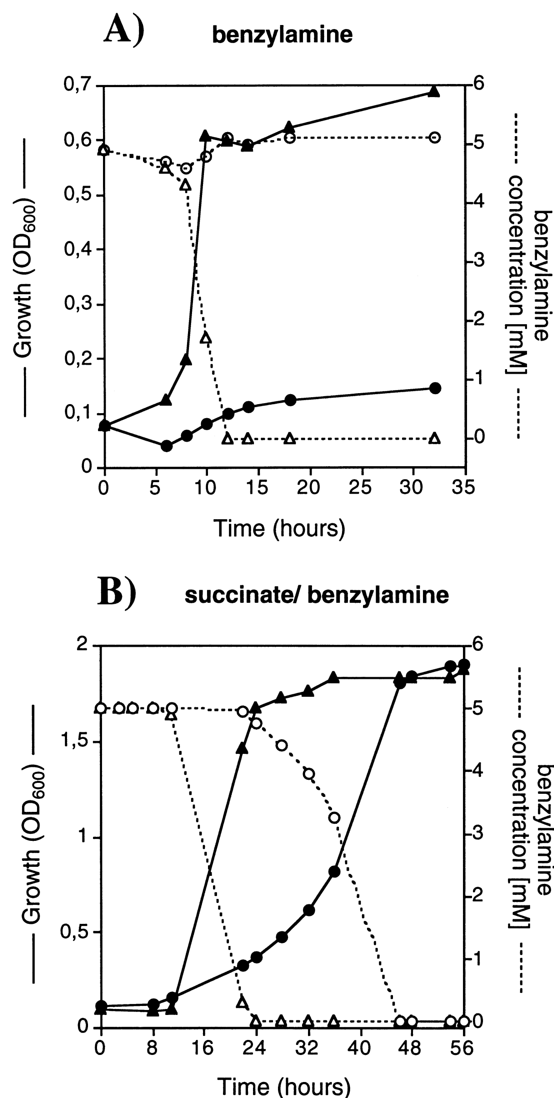


Fig. 4. Effect of TOL plasmid pWW0 on growth (continuous lines) and benzylamine utilization (dashed lines) in batch cultures of *P. putida* containing benzylamine as the sole source of carbon/energy and nitrogen (A), or succinate as carbon/energy source and benzylamine as the sole nitrogen source (B). Symbols used: △,▲ strain KT2440 devoid of TOL plasmid pWW0; ○,● strain KT2440 harboring wild-type TOL plasmid pWW0.

meta pathway immediately transforms all intracellular benzoate, which thus never attains intracellular concentrations sufficiently high to allow benzoate to be transformed by basal levels of *ortho* pathway enzymes. However, these initial *ortho* pathway enzymatic reactions would transform benzoate to *cis,cis*-muconate, the actual inducer that is required to efficiently switch on the *ortho* pathway [6,15,19]. It is possible that in a similar manner also the expression of benzylamine dehydrogenase activity requires intracellular accumulation of inducer (possibly also *cis,cis*-muconate), which is prevented by TOL plasmid *meta* pathway expression (Fig. 1).

The results reported here provide new insights into the genetic requirements for BMDH expression in *P. putida* and the interplay of this expression with TOL plasmid gene expression. It should be emphasized that this interplay of pathways and regulatory circuits is the normal situation in the strain studied: KT2440 is a TOL plasmid-cured derivative of *P. putida* mt-2 [8].

Acknowledgments

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