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Dinkla, IJT; Janssen, DB

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Simultaneous Growth on Citrate Reduces the Effects of Iron Limitation during Toluene Degradation in *Pseudomonas*

I.J.T. Dinkla, D.B. Janssen

Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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ABSTRACT

Rhizoremediation has been suggested as an attractive bioremediation strategy for the effective breakdown of pollutants in soil. The presence of plant root exudates such as organic acids, sugars, and amino acids that may serve as carbon sources or biosynthetic building blocks and the limited bioavailability of iron may influence the degradation of pollutants in the rhizosphere. To test the effect of such compounds on hydrocarbon degradation, trace concentrations of yeast extract or mixtures of organic acids and amino acids were added to continuous cultures of *Pseudomonas putida* mt2 and *P. putida* WCS358 (TOL) growing on toluene. By addition of these compounds increased growth yields and higher specific growth rates on toluene were obtained. The effects of iron limitation on the substrate utilization pattern of both strains were tested by growing the strains on a mixture of toluene and the readily degradable carbon source citrate while the iron concentration was varied. Simultaneous use of both substrates under carbon-limited as well as iron-limited conditions was observed. Growth yields were less reduced and iron requirement was lower during iron-limited growth in the toluene + citrate grown cultures compared to cultures in which toluene was used as the sole carbon source. The kinetic properties of the cells for toluene degradation were less hampered by the lack of iron when citrate was used as an additional carbon source. The results indicate that the availability of low concentrations of natural organic compounds, such as produced in the rhizosphere, may positively influence the degradative performance of hydrocarbon-degrading bacteria.

Introduction

The rhizosphere provides environmental characteristics for microorganisms that differ from those of bulk soil. Rhizosphere bacteria benefit from plant root exudates that contain organic acids, sugars, amino acids, and phenolic compounds [19]. Additionally, plants with a deep and extensive root system provide ample colonization possibilities for bacteria and improve the soil porosity and air supply. In this way plants may aid in the removal of soil pollutants by excreting nutrients and supporting bacteria that degrade xenobiotic compounds [11, 33], which makes

Correspondence to: D.B. Janssen; E-mail: D.B.Janssen@chem.rug.nl

rhizoremediation a promising method for a fast and thorough cleanup of soils polluted with hydrophobic hydrocarbons.

Many different substrates, e.g., organic acids and sugars, are present at low concentrations in the rhizosphere [19]. Plant species and growth conditions determine the composition of root exudates, which, among many other compounds, can contain organic acids such as acetate and lactate [9] and amino acids such as glutamate and aspartate [30]. The presence of multiple carbon and energy sources may result in catabolite repression or simultaneous use of these compounds [3] (for an overview see [8, 15]). An example of catabolite repression was found in *Pseudomonas putida* mt2 in which expression of the toluene degradation pathway was inhibited by the presence of succinate [5]. In contrast, mixtures of sugars were found to be utilized simultaneously in *Escherichia coli* continuous cultures [17]. In chemostat cultures of *E. coli*, steady-state levels of glucose and 3-phenylpropionic acid were found to be lower during growth on a mixture of the two substrates compared to cultures in which only one of the substrates was used, and simultaneous use of both substrates occurred with a μ_{max} value that was higher than that for 3-phenylpropionic acid alone [14]. Thus, the simultaneous use of different carbon sources may influence the growth yield and may result in residual concentrations of pollutant that are higher or lower than what can be achieved if the compounds were degraded separately [2].

The limited knowledge of the effects of low concentrations of cosubstrates on the degradation of pollutants makes it difficult to predict whether catabolite repression or stimulated substrate removal will occur. To obtain more insight in the influence of degradable cosubstrates we investigated the effects of the addition of model root exudates on the degradation of toluene in continuous cultures of two strains that colonize plant roots *P. putida* mt2 [23] and *P. putida* WCS358 (TOL) [10].

As it is likely that degradation of a pollutant in the rhizosphere takes place under multiple-nutrient-limited conditions [7, 15], substrate preference may also be influenced by other limitation effects. Previous experiments with toluene-degrading cultures have shown that iron limitation may be an important factor in the degradation of pollutants that require iron-containing oxygenases for their metabolism [4, 30]. It was observed that the quantity of iron required for the formation of a certain amount of cell dry weight was much higher in cultures grown on toluene compared to cultures in which citrate was used as the sole carbon and energy source. This raises the question whether the degradation of a mixture of these compounds is influenced by the amount of iron that is available.

One of the systems that is well suited to study the effects of iron limitation on mixed substrate degradation is the pathway for the degradation of toluene and xylenes encoded by the pWW0 (TOL) plasmid [20, 26]. This well-studied pathway contains two catabolic operons, the upper operon and the *meta* operon, which both contain iron-binding and iron-free enzymes. This makes it possible to study the effects of the presence of citrate on the degradation capacities of the cells for toluene under conditions of iron sufficiency or iron limitation, and on the levels of individual enzymes in the toluene pathway using chemostat cultures. In this paper, we describe the simultaneous use of a mixture of citrate and toluene during iron limitation in such chemostat cultures of the root-colonizing strains *P. putida* mt2 and *P. putida* WCS358 (TOL).

Methods

Bacterial strains

Pseudomonas putida mt2 (ATCC 33015) harbors the TOL plasmid pWW0 [1]. *Pseudomonas putida* WCS358 (TOL) is a transconjugant of *P. putida* WCS358 containing the self-transmissable TOL plasmid pWW0 obtained by A. Ooyevaar (University of Utrecht, The Netherlands).

Media

The growth medium was a mineral salts medium (MM) as described previously [22], but without addition of iron. Toluene or citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) was added to the medium as sole carbon and energy source. Organic acids and amino acids were autoclaved separately before addition to the medium. Iron was added to the medium as $FeCl_3$. In order to avoid contamination of media by residual iron, all glassware was soaked in 0.5 M EDTA solution and rinsed extensively with doubly distilled water. An iron concentration of up to 0.5 μM typically remained in routinely prepared solutions after these cleaning steps. Luria broth (LB) [28] was used to grow cells on rich medium. All media and carbon sources were of analytical grade.

Continuous Culture

Fermentors of 2.5 L working volume were used to grow the microorganisms in MM supplied with 20 mg/L yeast extract (MMY) or with vitamin solution [12] (MMV). The pH of the cultures was adjusted to 7 with autoclaved 1 M NaOH or

0.5 M H_2SO_4. The temperature was set at 28°C, and the impeller speed at 900 rpm. For growth on toluene, a flow of air was led through two bottles containing cooled (11°C) toluene via glass filters (P3, Elgebe, Leek, The Netherlands) prior to addition to the culture.

For growth on citrate, the MMY medium was supplemented with 15 mM citrate. An organic acids mixture consisting of 100 μM acetate, 100 μM lactate, 10 μM glutamate, and 10 μM aspartate was also used. Additionally, a stream of water-saturated air was continuously introduced into the cultures to supply sufficient oxygen. The flow rates of the toluene- and water-saturated air streams were controlled with mass flow controllers (type F201C-FA-11-V, Bronkhorst High-Tec BV, Veenendaal, The Netherlands). All gases were filter sterilized before addition to the culture. The outgoing gas stream was led through a water column under slight overpressure which facilitated the detection of possible leakage.

The chemostats were inoculated with toluene-pregrown batch cultures to a cell density of approximately 5 mg cell dry weight L^{-1}. The organisms were grown on toluene in fed-batch mode to a cell density of around 250 mg cdw L^{-1} before applying a dilution rate of 0.1 h^{-1}. The concentrations of toluene in the in- and outgoing gas streams were determined by gas chromatography. The detection limit of this method was a toluene concentration of 0.05 μM, and toluene removal rates were calculated using the mean value of at least 10 measurements. In order to check the purity of the cultures during operation of the chemostats, fermentor samples were regularly plated on LB agar plates, which were incubated at 30°C.

Determination of Maximal Specific Growth Rates

Values for the maximal specific growth rate μ_{max} were determined from washout curves. At each steady state a dilution rate (D) was used that was three- to fivefold higher than the expected μ_{max} value and the decay in biomass was determined by measurements of the optical density at 450 nm (OD_{450}) at 10-min intervals. Values for μ_{max} were calculated by

$$\mu_{max} = \frac{1}{t} \ln \frac{OD_{450,t}}{OD_{450,t=0}} + D$$

Iron and Carbon Determinations

For the determination of the iron content in culture supernatants and medium samples a colorimetric bathophenanthroline-based method was used [34]. Three equivalents of 4,7-diphenyl-1,10-phenanthroline disulfonic acid and 1 equivalent of Fe^{2+} stoichiometrically react to form a red colored complex, which strongly absorbs at 537 nm. The detection limit of this method was an iron concentration of 0.3 μM.

For determination of citrate in culture supernatants and medium samples an enzyme-based colorimetric analysis kit for citrate (Boehringer Mannheim, Mannheim, Germany) was used.

Culture Density

The culture density was estimated by measuring the optical density at 450 nm (OD_{450}) on a Pharmacia Novaspec II Rapid spectrophotometer and correlating these values to the corresponding cell dry weights. The latter were determined by centrifuging duplicate 100 mL samples of culture (15 min, 6000 *g*, 4°C), washing the pellets with the same volume of cold demineralized water, and drying the pellets to constant weight in a preweighed aluminum cup for 3 days at 80°C.

Preparation of Crude Cell Extracts

Cells from 150 mL culture samples were harvested by centrifugation (15 min, 6000 *g*, 4°C) and washed two times with ice-cold 0.1 M Tris-HCl (pH 7) containing 0.1 mM 1,4-dithiothreitol (TD buffer). After resuspension in a small volume of TD, cells were disrupted by sonication and centrifuged in an ultracentrifuge for 1 h at 150,000 *g* and 4°C in order to remove cell debris. The protein concentrations of the crude cell extracts were determined by the Bradford method using bovine serum albumin as a standard.

Enzyme Assays

Benzyl alcohol dehydrogenase activity was measured by determining NAD reduction at 340 nm (ε_{NADH} = 6300 L mol^{-1} cm^{-1}). Reaction mixtures contained 20 mM of Tris-HCl (pH 7), 2 mM of NAD, 400 μM of benzyl alcohol, and 1–2 mg/mL of protein. Catechol-2,3-dioxygenase activity was measured by determining the formation of 2-hydroxy-6-oxohepta-2,4-dienoate (HMS) from catechol at 375 nm (ε_{HMS} = 36,000 L mol^{-1} cm^{-1}) [24]. Reaction mixtures contained 30 mM of Tris-HCl (pH 7.0), 500 μM of catechol, and 0.1–1 mg/mL of protein. Hydroxymuconic semialdehyde hydrolase activity was measured by determining the breakdown of HMS at 375 nm. Reaction mixtures contained 30 mM of Tris-HCl (pH 7.0), about 40 μM of freshly prepared HMS, and 1–2 mg/mL of protein. Preparation of the HMS was done as described [21].

Oxygen Uptake Measurements

Oxygen uptake experiments were carried out in order to estimate the activities of the membrane-bound toluene monooxygenase and the three-component benzoate-1,2-dioxygenase. Cells of 150-mL culture samples were harvested by centrifugation (15 min, 6000 *g*, 4°C), washed two times with ice-cold iron-free MM medium, and resuspended in 2 ml MM medium. Cells were added to a 1 mL stirred incubation vessel filled with air-saturated iron-free MM medium to a density of 0.3–0.75 mg/mL cell dry weight. The vessel was air-sealed and fitted with a fiber-optic oxygen sensor (Comte, Hannover, Germany). After determination of the endogenous oxygen consumption rate, a pulse of toluene or benzoate was added to the cell suspension to a final concentration of

Table 1. Effects of the addition of biosynthetic building blocks on the kinetics of toluene removal in chemostat cultures of *P. putida* mt2 and *P. putida* WCS358 (TOL)

P. putida mt2	Yield (g cdw g carbon removed^{-1})	μ_{max} (h^{-1})	V_{max} (μmol mg of cdw^{-1} min^{-1})	Toluene removal (%)
No addition	0.35	0.20	0.045	99.5
Vitamins	0.39	0.31	0.038	97.3
Low organic acids[a]	0.54	0.28	0.071	99.8
High organic acids[b]	0.51	0.28	0.065	99.7
Yeast extract[c]	0.68	0.29	0.085	99.9
P. putida WCS358 (TOL)	Yield (g cdw g carbon removed^{-1})	μ_{max} (h^{-1})	V_{max} (μmol mg of cdw^{-1} min^{-1})	Toluene removal (%)
No addition	0.36	0.19	0.054	97.4
Vitamins	0.32	0.28	0.050	99.3
Low organic acids[a]	0.48	0.37	0.062	99.8
High organic acids[b]	0.54	0.46	0.055	99.5
Yeast extract[c]	0.67	0.43	0.105	99.9

[a] Final concentrations of 1 μM glutamate and aspartate and 10 μM lactate and acetate
[b] Final concentrations of 10 μM glutamate and aspartate and 100 μM lactate and acetate
[c] Final concentration of 20 mg L^{-1}

about 2 mM. The difference between the oxygen consumption rates before and after the addition of substrate was used to calculate the specific oxidation rate of the substrate in μmol min^{-1} g of cdw^{-1}.

Estimation of Kinetic Parameters

The kinetic parameters (K_m and V_{max}) for toluene degradation were obtained from toluene depletion curves that were measured with cells taken from chemostat cultures [27]. At different steady states, 25-mL samples of the chemostat culture were added to a magnetically stirred (700 rpm) 120-mL stainless steel incubation vessel that was air-sealed and temperature controlled at 30°C. After adding a pulse of toluene to the reaction vessel, the depletion of toluene was measured by on-line analysis of the toluene concentration in the headspace by gas chromatography. Gas was continuously withdrawn from the headspace with a micro membrane pump (model NMP 02LU; KNF Neuberger GmbH, Freiburg-Munzingen, Germany). After passing a Valco 6-port sampling injector (Vici AG, Schenkon, Switzerland) to which a 35-μL sample loop was connected, the gas was injected back into the liquid phase of the incubation vessel. The content of the sample loop was injected every minute into a gas chromatograph (Chrompack, model CP 9001; Middelburg, The Netherlands) equipped with a CPsil 5 CB column (Chrompack). Stainless steel tubing and a glass-embedded magnetic stirrer were used in order to minimize adsorption of toluene.

The substrate depletion curves were fitted with a model in which a Michaelis–Menten-type equation and the gas-liquid mass transfer of substrate are incorporated with the gas and liquid phase concentrations (C_g and C_l) as variables. The dimensionless Henry coefficient (H) for toluene at 30°C is 0.27 [29]. The mass transfer coefficient (k_La) for toluene was determined to be 0.57 min^{-1} using a described procedure [33]. The volumes of the gas and liquid phases (V_g and V_l) were 0.025 and 0.095 L, respectively. The concentration of biomass (X) was determined in separate experiments. Since the measurements were carried out over short time periods (less than 30 min), bacterial growth could be neglected leading to a model consisting of two equations:

$$\frac{dC_g}{dt} = -k_L a \frac{V_l}{V_g}\left(\frac{C_g}{H} - C_l\right) \quad (1)$$

$$\frac{dC_l}{dt} = k_L a\left(\frac{C_g}{H} - C_l\right) - V_{max} X\left(\frac{C_l}{C_l + K_m}\right) \quad (2)$$

The parameters K_m, V_{max}, and the initial concentrations of toluene in the gas and liquid phases ($C_{g,0}$ and $C_{l,0}$) were varied to fit the numerically integrated equations (1) and (2) to the experimental data, using the Episode routine in Scientist for Windows 2.0 (Micromath Scientific Software, Salt Lake City, UT).

Results

Addition of Biosynthetic Building Blocks to Toluene-Grown Cultures

To investigate the effect of the presence of small amounts of biosynthetic building blocks, which serve as a model for plant root exudates, on the degradation of toluene by *P. putida* mt2 and *P. putida* WCS358 (TOL), yeast extract or mixtures of organic acids and amino acids were added to the growth medium. The organic acids and amino acids in the mixtures were selected on basis of their presence in root exudates [9, 30]. For both strains, growth yields (g cdw/g toluene removed) were almost doubled when a complex mixture of building blocks in the form of 20 mg/L yeast extract was added (Table 1). This concentration of yeast extract contributes less than 2.2% of the total organic carbon introduced into the fermenters. In the case of addition of a mixture of 10 μM acetate and lactate and 1 μM glutamate and aspartate an increase of the growth yield on

toluene of 54% for *P. putida* mt2 and 33% for *P. putida* WCS358 (TOL) was observed. Upon addition of a 10-fold higher concentration of the acids no significant further change was observed for *P. putida* mt2, as the increase in growth yield was 46%. This suggests that the lower concentrations of organic and amino acids supply the strain with sufficient building blocks. For the *P. putida* WCS358 strain, however, a further increase in growth yield of 50% was observed. This additional increase may be explained by a higher requirement of biosynthetic building blocks for optimal growth or by a less efficient uptake of these compounds. Although the addition of a vitamin solution did not result in a significant increase in growth yield, an increased maximal specific growth rate (μ_{max}) on toluene was obtained with both cultures. In *P. putida* mt2 the addition of the mixture of organic acids and amino acids or yeast extract resulted in a μ_{max} value similar to that found for the vitamin addition. In the *P. putida* WCS358 (TOL) strain, however, values for μ_{max} increased more than twofold when yeast extract or the organic acid mixture was added, again indicating that *P. putida* WCS358 (TOL) benefits more from cosubstrates than *P. putida* mt2.

Toluene-depletion experiments confirmed the positive effect of the addition of the mimicked root exudate mixtures on the kinetics of toluene degradation as it resulted in a clear increase of the maximal specific conversion rate V_{max} of the cells when yeast extract was present in the growth medium (Table 1). A similar but smaller increase of V_{max} was observed when either of the organic acid mixtures were supplied to the *P. putida* mt2 culture. For *P. putida* WCS358 (TOL) no significant increase of V_{max} was observed, indicating that degradation rates are less stimulated than biosynthetic rates by addition of the organic acid mixtures. No significant differences in K_m values could be detected using the toluene depletion experiments (data not shown).

To determine whether the improved kinetic parameters resulted in more efficient removal of toluene in the reactor, the in- and outgoing toluene concentrations were measured. The results show that the addition of organic acids or yeast extract to the growth medium increased the removal of toluene from the reactor from an incomplete degree of 97.3% or 99.3% at the steady state where only vitamins were added to an almost complete removal of more than 99.9%. The results indicate that the addition of low concentrations of certain root exudate components, which contribute to less than 2.2% (for yeast extract) and 0.2% (for the acid mixtures) of the total carbon budget, still can function as biosynthetic building blocks and cause increased growth yields, improved degradation kinetics for toluene, and more complete removal of toluene from continuous cultures.

Mixed Substrate Growth under Carbon Limitation

To investigate the effect of the presence of higher concentrations of an alternative carbon source on the degradation of toluene by *P. putida* mt2 and *P. putida* WCS358 (TOL), cells were grown in continuous culture on 10 mM toluene with the addition of 5 mM citrate. Continuous cultures grown on 10 mM toluene or 10 mM citrate as the carbon source were used as controls. Growth yields (g cdw/g carbon removed) and toluene removal rates of the cultures were determined (Table 2). By summarizing the amount of biomass that would be obtained on the separate carbon sources using individual growth yields, the expected growth yields on the toluene–citrate mixtures were calculated. Under carbon-limited growth the yield found for the toluene plus citrate grown cells was 10% higher than this expected yield. Both toluene and citrate were completely removed. Thus, the simultaneous presence of citrate as an alternative carbon source did not inhibit toluene removal but had a synergistic effect on the growth yield in continuous cultures of *P. putida* mt2 and *P. putida* WCS358 (TOL) growing under carbon limitation.

Effects of Iron Limitation on Mixed Substrate Growth

Previous experiments have shown that the degradation of toluene results in a higher requirement and consumption of iron per amount of biomass formed than the degradation of citrate [4]. In *P. putida* mt2 the production of 1 g cell dry weight from toluene requires 1.81 μmol iron, compared to 0.40 μmol iron when citrate is the carbon source. This raises the question whether the degradation of a mixture of these compounds is influenced by the amount of iron that is available. To this end we determined the growth yield on a mixture of citrate and toluene while varying the iron:carbon ratios from an iron excess of 1.04 to an iron-limiting 0.03 μmol Fe/mmol carbon. The negative effects of iron limitation were reflected by a significant decrease in growth yield (g cdw/g carbon-substrate removed) when the iron/toluene ratio was lowered (Fig. 1). A decrease in growth yield of 35% was observed between the highest and lowest iron concentrations tested, while removal of citrate and toluene was still complete in these mixed substrate

Table 2. Growth yields and toluene removal in continuous cultures of *P. putida* mt2 and *P. putida* WCS358 (TOL) grown on single or mixed carbon sources in iron-excess and iron-limited conditions

	Carbon-limited growth (iron:carbon ratio >0.33×10^{-3})			Iron-limited growth (iron:carbon ratio >0.09×10^{-3})		
P. putida mt2 growth substrate(s)	Yield measured[a]	Yield expected[b]	Toluene removal (%)	Yield measured[a]	Yield expected[b]	Toluene removal (%)
Citrate	0.21			0.21		
Toluene	0.69		99.5	0.31		77.2
Toluene + citrate	0.48	0.44	99.9	0.31	0.25	99.9
	Carbon-limited growth (iron:carbon ratio >0.33×10^{-3})			Iron-limited growth (iron:carbon ratio >0.09×10^{-3})		
P. putida WCS358 (pWW0) growth substrate(s)	Yield measured[a]	Yield expected[b]	Toluene removal (%)	Yield measured[a]	Yield expected[b]	Toluene removal (%)
Citrate	0.24			0.24		
Toluene	0.71		99.9	0.35		96.3
Toluene + citrate	0.49	0.45	99.9	0.36	0.30	99.9

[a] The yield is defined as grams of cell dry weight formed per gram of carbon source consumed
[b] The theoretical yield was calculated on the basis of the yields determined for the single carbon sources

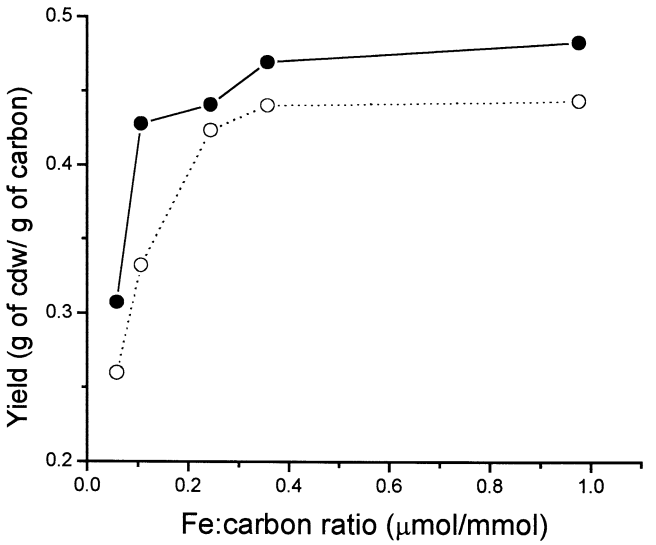

Fig. 1. Effects of the iron:carbon ratio (mmol/mol) on the growth yield in toluene–citrate-grown continuous cultures of *P. putida* mt2 (solid circles). Cell cultures were grown onmineral medium in a chemostat at a fixed dilution rate of 0.1 h^{-1}. The toluene and citrate concentrations were kept constant while the iron concentration was varied. Based on yieldsfound for growth on toluene and citrate separately, expected yields were calculated (open circles).

cultures (Table 2). In previous experiments in which cultures were grown on toluene as the sole carbon source at similar iron:toluene ratios, a much more severe decrease in growth yield of 55% was observed, combined with an incomplete removal of toluene [4]. In cultures grown on citrate as the sole carbon source no difference in growth yield was observed at the different iron:toluene ratios. At each iron/toluene ratio, the growth yield for the cultures growing on the mixture of citrate and toluene was compared to the theoretical yield that was calculated on basis of growth yields determined for growth on citrate or toluene separately (Fig. 1). At each iron:toluene ratio, the growth yield on the mixture was found to be 4% to 30% higher than the expected yield, showing that efficient degradation of toluene is less inhibited by iron limitation when citrate is present as an additional carbon source.

Iron Utilization during Mixed Substrate Growth of P. putida mt2 and P. putida WCS358 (TOL)

To determine whether the observed effects are caused by differences in iron uptake efficiency or iron utilization, the in- and outgoing iron concentrations were measured. The iron removal values calculated from these data showed that under iron excess conditions of 10 μM only part of the iron was taken up (Table 3). Removal rates varied from 41% for the toluene-grown cells to 90% for the cultures grown on the toluene and citrate mixture, which also had a higher density. In the toluene- and toluene–citrate-grown cultures iron removal increased to 100% under iron limitation conditions. The amount of iron consumed per gram cell dry weight formed was calculated for cells grown on the different substrates. Under all iron excess conditions, iron consumption levels (amounts of iron taken up per gram cell dry weight formed) were more than two-fold higher than the expected minimal requirements calculated from

Table 3. Effects of iron addition on iron utilization by *P. putida* mt2 during growth on 10 mM toluene or a mixture of 10 mM toluene and 5 mM citrate

Carbon source	Added iron (μM)	% of iron removed[a]	Iron consumption[b] (μmol Fe g cdw^{-1})	Theoretical minimal requirement[c] (μmol Fe g cdw^{-1})
Toluene	10	41	4.6	1.81
	1	100	1.81	1.81
Toluene + citrate	10	90	8.80	1.57
	0	100	0.82	1.63

[a] The percentage of iron removal was determined by measuring the iron concentration in the in- and outgoing growth medium
[b] The iron consumption is defined as the amount of iron taken up per gram of cell dry weight formed
[c] The minimal amount of iron that was expected to be consumed was calculated on basis of iron consumption values from previous experiments on single carbon sources

Table 4. Kinetic parameters of toluene degradation by *P. putida* mt2 and *P. putida* WCS358 (pWW0) using continuous culture cells grown on a toluene/citrate mixture with addition of 0–10 μM iron[a]

	P. putida mt2			*P. putida* WCS358 (pWW0)		
Carbon source	Iron:carbon ratio	K_m (μM)	V_{max} (μmol mg of cdw^{-1}min^{-1})	Iron: carbon ratio	K_m (μM)	V_{max} (μmol mg of cdw^{-1} min^{-1})
Toluene	0.36×10^{-3}	2.4	0.102	0.63×10^{-3}	15.3	0.105
	0.09×10^{-3}	4.2	0.026	0.05×10^{-3}	5.8	0.073
Toluene + Citrate	0.36×10^{-3}	1.24	0.038	1.04×10^{-3}	0.63	0.063
	0.11×10^{-3}	3.86	0.032	0.05×10^{-3}	7.08	0.054

[a] Values were obtained by numerical fitting of data from toluene depletion experiments using freshly harvested cells in batch incubations

iron consumption values determined during iron-limited growth on the separate substrates. During iron-limited growth, when no iron was added to the cultures growing on the toluene and citrate mixture, however, the iron consumption was lower than the expected value. Thus, the simultaneous use of toluene and citrate lowered the amount of iron required for the formation of biomass compared to biomass production from the substrates separately.

Effects of Iron Limitation on Kinetic Parameters for Toluene Degradation in Mixed Substrate Cultures

Above we have shown that trace amounts of carbon sources may positively influence the kinetics of toluene removal. To investigate whether iron limitation alters the overall kinetics of toluene removal during growth on the toluene and citrate mixture, the kinetic parameters for toluene degradation were determined at different iron concentrations. Cells were taken from steady states with iron:toluene ratios ranging from 0.05 to 0.74 μmol Fe/mmol toluene. Values for the maximal specific substrate conversion rate (V_{max}) and the substrate affinity constant (K_m) were obtained by least-squares fits of toluene depletion measurements that were carried out with resting cell suspensions. The values obtained for K_m showed no significant difference between the steady states tested (Table 4). The maximal specific conversion rates for toluene (V_{max}) were lower for the cells grown on the toluene plus citrate mixture than for toluene-grown cultures. Upon iron limitation a significant decrease of V_{max} was observed for cells from the cultures grown on toluene as well as on the toluene–citrate mixture. In the case of cells cultured on the toluene–citrate mixture, however, the decrease of 16% and 14% for *P. putida* mt2 and *P. putida* WCS358(pWW0) was much less severe than that for the toluene-grown cells, where V_{max} values dropped by 75% and 30%, respectively. Although the presence of citrate reduces the V_{max} for toluene under iron excess, its presence reduces the loss of the absence of iron during iron-limited growth.

Iron Limitation Impacts on the TOL Pathway Enzyme Activities during Mixed Substrate Growth

Previous observations with cultures growing on toluene have shown that iron limitation specifically reduced the activities of the iron-containing oxygenases as well as that of the iron-free *meta*-pathway hydroxy muconic semialdehyde hydrolase (HMSH) encoded by the TOL plasmid. The

Table 5. Effects of iron limitation on the activity of the TOL pathway enzymes in *P. putida* mt2 during growth on toluene or toluene and citrate mixtures

		Upper pathway		*Meta* pathway		
	[Fe] (μM)	TMO (U/g [dry weight] of cells)[a]	BADH (U/g cfe)[b]	B12O (U/g [dry weight] of cells)[a]	C23O (U/g cfe)[b]	HMSH (U/g cfe)[b]
Toluene	10	490	74	2037	2417	39
	1	216	68	357	61	9
% reduced		56%	<10%	82%	97%	77%
Toluene + citrate	10	1220	154	1860	2228	35
	1	638	1968	635	827	23
% reduced		48%	Increase	66%	63%	34%

[a] Toluene monooxygenase (TMO) and benzoate-1,2-dioxygenase (B12O) activities are described by the rate of oxygen consumption (μmoles per min per g [dry weight] of cells, equivalent to units per g [dry weight] of cells)
[b] Benzyl-alcohol dehydrogenase (BADH), catechol-2,3-dioxygenase (C23O), and HMS hydrolase (HMSH) activities are described by the rate of substrate conversion (μmol min per g protein in cell free extract, equivalent to units per g protein in cell free extract)

activities of two upper- and three *meta*-pathway enzymes encoded by the TOL plasmid were measured to determine the influence of citrate as second growth substrate on their expression levels, and to investigate the effects of iron limitation during growth on the toluene/citrate mixture. The addition of citrate resulted in a decrease in activity of about 50% for the upper-pathway enzymes but did not alter the activities of the *meta*-pathway enzymes under iron-excess conditions. The reduction in activity of the iron-containing toluene monooxygenase (TMO), benzoate-1,2-dioxygenase (B12O), and catechol-2,3-dioxygenase (C23O) and the 2-hydroxy muconic semialdehyde hydrolase (HMSH) found during iron-limited growth was much stronger when toluene was used as the sole carbon source compared to growth with the toluene/citrate mixture. Where activities for the catechol-2,3-dioxygenase (C23O) dropped by 97% during growth on toluene, the reduction in enzyme activity was only 63% in cells grown on toluene and citrate simultaneously (Table 5). Surprisingly, the activity of the benzyl-alcohol dehydrogenase (BADH) increased strongly during simultaneous utilization of citrate and toluene under iron-limited conditions. Thus, the activities of the TOL pathway enzymes were enhanced by the presence of citrate as a secondary carbon source during iron-limited growth on toluene.

A more detailed investigation of the citrate–toluene-grown cultures in which the iron:carbon ratios were varied between 0.03 and 1.04 μmol Fe/mmol carbon showed that the effect of the additional carbon source on the expression of the upper and *meta* pathways was not the same in the two strains (Fig. 2). Similar trends could be observed in both strains for the upper-pathway enzymes. Activities of the toluene monooxygenase decreased upon iron limitation as was also observed previously for toluene-grown cells. Activities of the benzyl-alcohol dehydrogenase showed an increase in activity at iron:carbon ratios below 0.16 μmol Fe/mmol carbon that was not observed previously. In *P. putida* WCS358 (TOL) a reduction in *meta*-pathway enzyme activities was observed upon iron limitation comparable to previous observations with toluene-grown cells. In the case of the *meta*-pathway enzymes of *P. putida* mt2, this trend was not observed as a sudden increase in activity was determined at iron:carbon ratios below 0.11 μmol Fe/mmol carbon, suggesting different regulation of *meta*-pathway expression in both strains.

Discussion

The effects of the presence of alternative growth substrates in an environment during the degradation of an aromatic hydrocarbon can be important for the kinetics of biodegradation of these compounds. The results above show that simultaneous use of two carbon sources under carbon-limited growth in continuous cultures as also observed for several other carbon sources [3, 8, 14, 17] (for a review see [15]), proceeds with high efficiency in the case of the harmful hydrocarbon toluene and the readily degradable carbon source citrate. The effect of the addition of a second carbon source on the degradation of harmful hydrocarbons has been reported previously [14, 16], but did not include data on the effect of additional limitations, e.g., iron limitation, on substrate utilization. Our experiments in which citrate was used as the alternative carbon source in combination with toluene showed that simultaneous use of both toluene and a readily degradable carbon source can

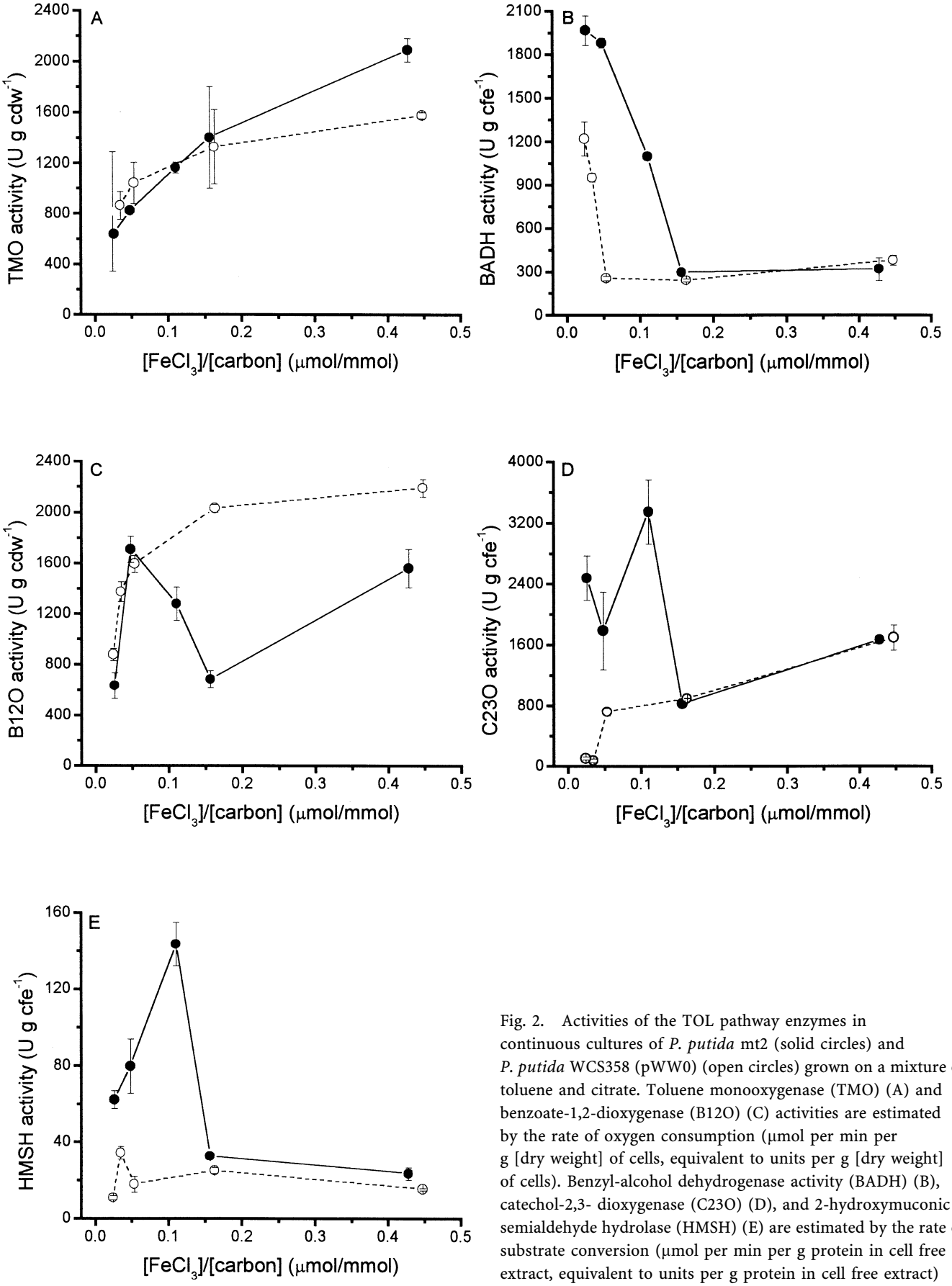

Fig. 2. Activities of the TOL pathway enzymes in continuous cultures of *P. putida* mt2 (solid circles) and *P. putida* WCS358 (pWW0) (open circles) grown on a mixture of toluene and citrate. Toluene monooxygenase (TMO) (A) and benzoate-1,2-dioxygenase (B12O) (C) activities are estimated by the rate of oxygen consumption (μmol per min per g [dry weight] of cells, equivalent to units per g [dry weight] of cells). Benzyl-alcohol dehydrogenase activity (BADH) (B), catechol-2,3- dioxygenase (C23O) (D), and 2-hydroxymuconic semialdehyde hydrolase (HMSH) (E) are estimated by the rate of substrate conversion (μmol per min per g protein in cell free extract, equivalent to units per g protein in cell free extract)

occur under iron limitation. The kinetic performance of the cells for toluene degradation was better maintained during iron-limited growth when citrate was available as an additional carbon source. This suggests that iron limitation is not as severe as during growth on toluene as the sole carbon source. The beneficial effect of citrate on the degradation of toluene during growth under iron-limited conditions may be explained by the low amounts of iron that are required for the formation of biomass from this carbon source [4]. The observation that the iron requirements of the cells during the degradation of the mixture of toluene and citrate were even lower than could be expected on the basis of the utilization of the separate carbon sources suggests that the cells may have economized their iron use by sharing iron-containing enzymes, e.g., those of the Krebs cycle, for the degradation of both substrates. This allows the formation of more biomass during iron-limited growth in the mixed substrate cultures. The chelating properties of citrate [25] may further aid in increasing the yield by saving energy for the production of siderophores that otherwise have to be produced for the uptake of sufficient amounts of iron [13, 18].

As a result of the high amounts of biomass that were formed during iron-limited growth in the mixed substrate cultures, more toluene could be converted. During iron-limited growth, despite the lower values for the maximal specific conversion rate V_{max} (μmol mg of cdw^{-1} min^{-1}) in cells grown on the toluene/citrate mixture, 30.4 and 43.9 μmol of toluene L^{-1} min^{-1} were converted by *P. putida* mt2 and *P. putida* WCS358 (TOL) in the mixed substrate cultures, whereas the conversion capacity of the toluene cultures was only 14.0 and 17.0 μmol toluene L^{-1} min^{-1}, respectively.

Determination of the effects of iron limitation on the enzymes of the TOL pathway showed trends similar to those during growth on toluene in *P. putida* WCS358 (TOL) [4]. For *P. putida* mt2, the activities of the *meta*-pathway enzymes suddenly increased as the iron concentration dropped. This may be the result of an increased expression of the *meta*-pathway that was previously observed in *P. putida* mt2 cells growing under nitrogen, sulfate, or phosphate limitation, and that is possibly due to a low energy status of the cells [6]. The difference in *meta*-pathway regulation can be explained by the fact that the TOL plasmid occurs naturally in *P. putida* mt2 but is believed to be integrated in the chromosome of the transconjugant *P. putida* WCS358 (TOL) (A. Ooyevaar, personal communication).

Besides acting as alternative carbon sources, many organic and amino acids present in trace concentrations in root exudates may influence the growth rate and degradative capacity of microorganisms in the rhizosphere. The addition of lactate, acetate, aspartate, and glutamate as model compounds for root exudates to toluene-grown continuous cultures has shown that an increase in yield and specific growth rate on toluene can be obtained. As these compounds did not significantly contribute to the total carbon supply, these effects suggest that they act as biosynthetic building blocks saving energy-consuming conversion steps from toluene to necessary cellular compounds. The results presented suggest that during carbon or iron limitation in the rhizosphere, the presence of root exudates may be beneficial to pollutant removal by acting as biosynthetic building blocks rather than competing as a growth substrate.

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