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Competition in Chemostat Culture between *Pseudomonas* Strains That Use Different Pathways for the Degradation of Toluene

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***Pseudomonas putida* mt-2, *P. cepacia* G4, *P. mendocina* KR1, and *P. putida* F1 degrade toluene through different pathways. In this study, we compared the competition behaviors of these strains in chemostat culture at a low growth rate ($D = 0.05\ h^{-1}$), with toluene as the sole source of carbon and energy. Either toluene or oxygen was growth limiting. Under toluene-limiting conditions, *P. mendocina* KR1, in which initial attack is by monooxgygenation of the aromatic nucleus at the *para* position, outcompeted the other three strains. Under oxygen limitation, *P. cepacia* G4, which hydroxylates toluene in the *ortho* position, was the most competitive strain. *P. putida* mt-2, which metabolizes toluene via oxidation of the methyl group, was the least competitive strain under both growth conditions. The apparent superiority of strains carrying toluene degradation pathways that start degradation by hydroxylation of the aromatic nucleus was also found during competition experiments with pairs of strains of *P. cepacia*, *P. fluorescence*, and *P. putida* that were freshly isolated from contaminated soil.**

During the past decades, a broad variety of bacteria capable of degrading aromatic hydrocarbons have been isolated and characterized. A wide range of genera and species is represented; many individual compounds were found to be degradable through more than one pathway (for a review, see reference 22). For alkyl-substituted benzenes, two approaches in the initial degradation steps can be distinguished: a methyl group may be oxidized by a monooxygenase to the corresponding alcohol (TOL pathway), or the aromatic nucleus may be directly attacked by an oxygenase.

For the degradation of toluene, both approaches are known to be used by different strains. The most intensively studied strain that uses the first approach, i.e., harbors a TOL pathway, is *Pseudomonas putida* mt-2. The genetic information for this TOL pathway (see Fig. 1), through which *m*- and *p*-xylene may be degraded too, is situated on TOL plasmid pWW0. A large number of strains carrying almost identical TOL genes have since been reported (for a review, see reference 3). The second approach, the direct-ring attack, was also found to be applied frequently by toluene-degrading bacteria. The enzymatic steps in individual strains show considerable variation (Fig. 1). In *P. putida* F1, the aromatic nucleus is dioxygenated at the 2,3 position, yielding 3-methylcatechol (12, 23). In *P. cepacia* G4, 3-methylcatechol is also an intermediate, but two monooxygenation steps can be distinguished, at the *ortho* and the *meta* positions, respectively (21). In *P. mendocina* KR1, the initial monooxygenation (in the *para* position) is followed by a number of conversions, eventually yielding the intermediate 3,4-dihydroxybenzoate (29).

Little is known about the relative importance of these different pathways in the conversion of toluene in the environment. In polluted soils and groundwater, bacterial growth will often be limited by oxygen or the bioavailability of the carbon sources (18, 27). Also, when physical transport is not limiting biodegradation, the concentration of lipophilic compounds such as toluene may be very low in environments polluted with oil: the maximum solubility of toluene is as high as 5 mM, but the concentration of toluene in water equilibrated with gas-oil is no more than approximately 1 μM due to the tendency of toluene to partition in the oil phase (15). The success with which different aerobic toluene-degrading strains proliferate in the environment will therefore depend strongly on their ability to compete for toluene or oxygen.

In this study, we forced different toluene-degrading strains to compete in chemostat culture, with toluene as the sole carbon source under either toluene- or oxygen-limiting conditions. It was shown that strains which hydroxylate the aromatic nucleus as the first step of biodegradation outcompete strains that start by oxidation of the methyl group.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study and the aromatic compounds they may utilize are listed in Table 1. *P. cepacia* G4, *P. mendocina* KR1, and *P. putida* F1 were kindly donated by M. Reagin, K. M. Yen, and D. T. Gibson, respectively. *P. cepacia* M1C4, *P. cepacia* O2C1, *P. fluorescens* R2AT2, *P. fluorescens* A4C2, *P. putida* A3AT1, and *P. putida* M1D2 were isolated from polluted topsoil samples from different sites in The Netherlands. The strains were identified by using the biochemical criteria of Stanier et al. (24). Soil isolates were assumed to possess a TOL pathway when toluene-pregrown cells could also oxidize *p*-xylene and *m*-xylene but not benzene or ethylbenzene and were not able to utilize the last two compounds as growth substrates on mineral salts medium (MM medium) plates either. Strains were assumed to degrade toluene through a pathway involving oxygenation of the aromatic ring as an initial degradation step when toluene-pregrown cells could also oxidize benzene and ethylbenzene but not *p*-xylene and *m*-xylene. All strains were maintained on mineral medium agar plates supplied with toluene as the sole source of carbon and energy through the vapor phase (air in equilibrium with a solution of 10% [vol/vol] toluene in hexadecane).

Media. The growth medium was MM medium described by Evans et al. (11), except that the concentrations of all nutrients

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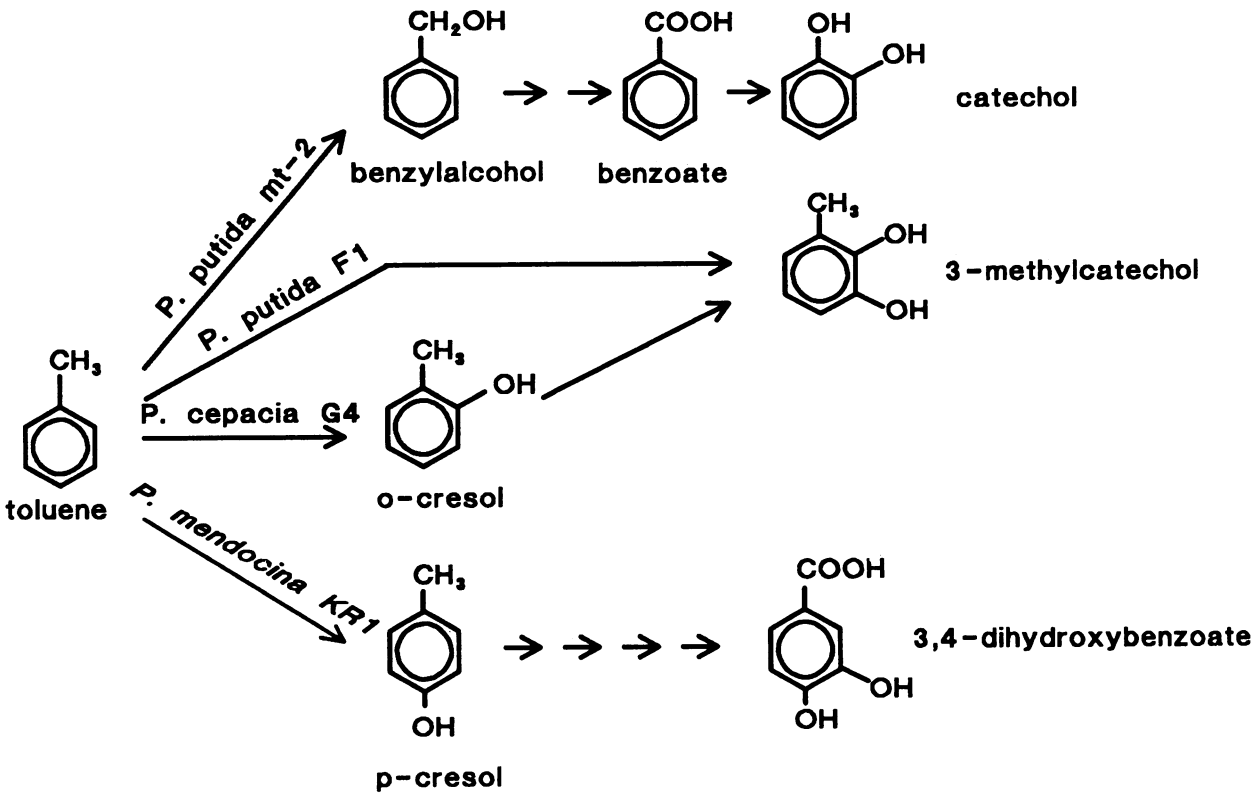

FIG. 1. Initial steps in different pathways for the bacterial degradation of toluene, as adapted from references 3, 12, 19, and 21.

were decreased to 5% of the published concentration and the complexing agent was omitted. The medium was supplemented with 50 mM K_2HPO_4, and the pH was set at 7.0. No antifoaming agent was used. All experiments were performed at 28°C.

Batch culture. Serum bottles (250 ml) with 50 ml of MM medium containing 2 mM succinate were inoculated with single colonies from agar plates. After growth overnight on a shaker (150 rpm), the batch culture was grown further with toluene as a C source (supplied through the gas phase by diffusion from an Eppendorf tube, suspended in the headspace and containing a 10% [vol/vol] solution of toluene in hexadecane; the lid of the tube had a hole 2 mm in diameter).

Determination of the maximal growth rate on toluene. Toluene-pregrown batch cultures (as described above) were diluted to an optical density at 540 nm (OD_{540}) of 0.05 in a 500-ml serum bottle containing 50 ml of MM medium and prewarmed at 28°C. The headspace of this bottle was flushed continuously (4 liters h^{-1}) with air containing 80 μmol of toluene $liter^{-1}$. This toluene-containing air stream was prepared in a buffer vessel by diluting toluene-saturated air (20°C)

TABLE 1. Bacterial strains

Strain	Growth[a] with given compound as sole carbon source						Reference(s)
	Benzene	Toluene	Ethylbenzene	*m*-Xylene	*p*-Xylene	*m*-Toluate	
Laboratory strain							
P. cepacia G4	−	+	+	−	±[b]	−	21
P. mendocina KR1	−	+	+	−	−	−	29
P. putida F1	+	+	+	−	−	−	12, 23
P. putida mt-2	−	+	−	+	+	+	3
Soil isolate							
P. cepacia M1C4	+	+	+	−	−	−	This study
P. cepacia O2C1	−	+	−	+	+	+	This study
P. fluorescens R2AT2	+	+	+	−	−	−	This study
P. fluorescens A4C2	−	+	−	+	+	+	This study
P. putida A3AT1	+	+	+	−	−	−	This study
P. putida M1D2	−	+	−	+	+	+	This study

[a] Growth was on MM agar plates.
[b] ±, weak growth.

TABLE 2. Physiological parameters of the laboratory strains grown in chemostat culture under toluene limitation at a dilution rate of 0.05 h^{-1}

Strain	Chemostat culture				μ_{max} on toluene at 28°C (h^{-1}) in batch culture
	OD_{540}	Dry wt (mg $liter^{-1}$)	Toluene concn (nM)[a]	Yield (g of dry wt g of $toluene^{-1}$)	
P. cepacia G4	0.14	38	390	0.16	0.19
P. mendocina KR1	0.20	83	<5	0.35	0.45
P. putida F1	0.37	105	10	0.44	0.38
P. putida mt-2	0.21	79	290	0.33	0.40

[a] Residual concentration in culture supernatant.

with pure air in the ratio 1:15. The batch was shaken at 150 rpm and 28°C, and the maximal growth rate was calculated from the increase in OD_{540} in time during the exponential growth phase.

Chemostat culture. Custom-made chemostats with a working volume of 110 ml (for details, see reference 10) were inoculated with 20 ml of a succinate-pregrown batch culture. The cultures were magnetically stirred with a 40-mm-long, Teflon-coated bar at 200 rpm. The culture was supplied with an MM medium containing 10 mM succinate as the sole carbon source at a dilution rate of 0.05 h^{-1}. After 7 days of growth, the nutrient medium containing succinate was replaced with an MM medium without any carbon source. From that time on, toluene was supplied through the gas phase: for carbon and energy limitation (C limitation), an airflow of 2 liters h^{-1} containing 9 μmol of toluene $liter^{-1}$ was bubbled through the culture; an additional (toluene-free) airflow of 6 ml h^{-1} was led through the culture to secure a sufficient oxygen supply. To reach oxygen-limiting conditions, the cultures were supplied with only one gas flow: 2 liters of a mixture of air and nitrogen h^{-1} in the ratio 1:6 containing 15 μmol of toluene $liter^{-1}$. Determination of dry weight was performed as previously described (10).

Analysis of residual concentrations of toluene in chemostat. Samples of 30 ml were taken from steady-state chemostat cultures in 35-ml glass sample bottles precooled to −5°C and filled with 3 ml of phosphoric acid (8.5%). The sample was subsequently extracted with 1 ml of CS_2 (Merck, pro analysi) containing 2.5 mg of D_{10}-ethylbenzene $liter^{-1}$ as an internal standard. After 30 min of shaking at 150 rpm, the CS_2 layer was separated and analyzed with a gas chromatograph-mass spectrometer (GC-MS) equipped with a CP Wax 52CB column and an ion trap detector.

Competition experiments. After 7 days of growth with toluene as the sole carbon source, the chemostats were assumed to have reached a steady state, and competition was started by mixing samples taken from two chemostats into a third chemostat of the same type. The ratio by which the cultures of the two strains were mixed was chosen to result in initially equal numbers of CFU when spread on peptone agar plates (0.2×10^8 to 1.3×10^8 CFU for toluene limitation and 0.1×10^7 to 1.5×10^7 CFU for oxygen limitation). Subsequently, the population composition was followed in time by spreading diluted samples from the chemostat on peptone agar plates. The different strains were distinguished on the peptone agar plates by colony morphology, size, and color. The validity of this method was checked for a limited number of colonies by testing for growth on different carbon sources: *P. putida* mt-2 was distinguished from the other strains by growth on *m*-toluate; *P. cepacia* G4, by growth on *o*-xylene; *P. putida* F1, by good growth on benzene; and *P. mendocina* KR1, by the absence of growth on any of these substrates.

When assuming that during the competition experiment in chemostat culture at a fixed dilution rate, D, the ratio of the growth rates of strains A and B is constant ($C = \mu_{\text{strain } A}/\mu_{\text{strain } B}$), the change in the fractions p_A and p_B in time (t) can be described by the following equation (for a derivation, see references 9 and 10): $(p_B)^c/(1 - p_B) = [(p^0{}_B)^c/(1 - p^0{}_B)]\, e^{(1-C)D_t}$. $p^0{}_B$ represents the fraction of strain B at the start of the competition experiment. Values for C were generated by nonlinear least-squares fitting of the equation to the experimental data for the change of p_B in time (for details of the fitting procedure, see reference 9).

RESULTS

Maximal growth rate of laboratory strains on toluene. The growth rates of the strains were determined in batch cultures supplied with air containing 300 μmol of toluene $liter^{-1}$. The growth rates of the various strains in the exponential phase, which continued until an OD_{540} of ca. 0.5 was reached, were between 0.19 and 0.45 h^{-1} (see Table 2). The concentration of toluene in the culture fluid was 100 to 150 μM, which we assume to be growth saturating but well below the toxicity threshold for *P. putida* (315 μM according to reference 4).

Growth in chemostat culture. Under toluene limitation at a dilution rate of 0.05 h^{-1}, the toluene supplied was absorbed more than 80% by the cultures. The residual concentrations of toluene in the cultures were less than 500 nM for all strains (see Table 2). The OD_{540} and dry weights of the cultures correlated satisfactorily. The growth yield on toluene was the highest for *P. putida* F1 and the lowest for *P. cepacia* G4 (Table 2). Under oxygen limitation, the residual concentration of toluene varied between 16 and 25 μM, and under these conditions *P. cepacia* G4 showed the highest OD_{540} (results not shown).

Competition of laboratory strains. After 7 days of growth on toluene (12 generation times), the OD_{540} values of all axenic chemostat cultures were stable. At that time, competition was started by running a third chemostat filled with a mixture of two of these steady-state axenic cultures. In these one-to-one competition experiments under toluene-limiting conditions, *P. mendocina* KR1 outcompeted the other three strains. Under oxygen limitation, *P. cepacia* G4 was the most competitive strain. *P. putida* mt-2 was the least competitive strain under toluene limitation as well as under oxygen-limiting growth conditions (Table 3). Mathematical analysis of the experimental data for the population change in time yielded values for the ratio (C) of the growth rates of the competing strains (Table 3). One experiment (*P. cepacia* G4 against *P. putida* mt-2) yielded a negative value for C, implying a disappearance of *P. putida* from the culture at a rate faster than that of washout. This may be due to cell death or to loss of viability on agar plates. Figures 2 and 3 show examples of population shifts

TABLE 3. Competition experiments among laboratory strains growing on toluene in chemostat culture at a dilution rate of 0.05 h^{-1}

Strain 1	Strain 2	Toluene limitation		Oxygen limitation	
		Winning strain	C^a	Winning strain	C
P. mendocina KR1	*P. cepacia* G4	1	0.35	2	0.64
P. mendocina KR1	*P. putida* F1	1	0.25	—[b]	1.0
P. mendocina KR1	*P. putida* mt-2	1	0.0	1	0.09
P. putida F1	*P. cepacia* G4	1	0.67	2	0.76
		1[c]	0.0[c]		
P. putida F1	*P. putida* mt-2	1	0.20	1	0.56
		1[c]	0.03[c]	1[c]	0.57[c]
		1[c]	0.18[c]	1[c]	0.40[c]
P. cepacia G4	*P. putida* mt-2	1	−0.40	1	0.0

[a] C, ratio of growth rates of the losing and the winning strain.
[b] —, stable mixed culture.
[c] Duplicate experiments.

during competition experiments performed under toluene limitation and oxygen limitation.

Competition of soil isolates under toluene limitation. Three pairs of soil isolates were forced to compete under toluene limitation. The one-to-one competition experiments involved pairs of isolates belonging to the same species (*P. putida*, *P. fluorescens*, or *P. cepacia*) but harboring either a TOL pathway or a pathway involving a direct-ring attack. In all three competition experiments, the strains harboring a TOL pathway lost the competition (Table 4). The maximal growth rates of the soil isolates on toluene varied between 0.20 and 0.39 h^{-1} (Table 4). *P. putida* mt-2 was outcompeted by the soil isolate *P. putida* A3AT1, harboring a direct-ring attack pathway.

Influence of growth rate on competition behavior. The influence of the dilution rate on the growth rate ratio C was studied for the combination *P. putida* mt-2-*P. putida* F1. At dilution rates of 0.1 and 0.2 h^{-1}, the growth rate ratios were 0.35 and 0.86, respectively. This indicated that the growth rate disadvantage of the TOL strain decreases when the toluene limitation becomes less stringent. This was also observed when

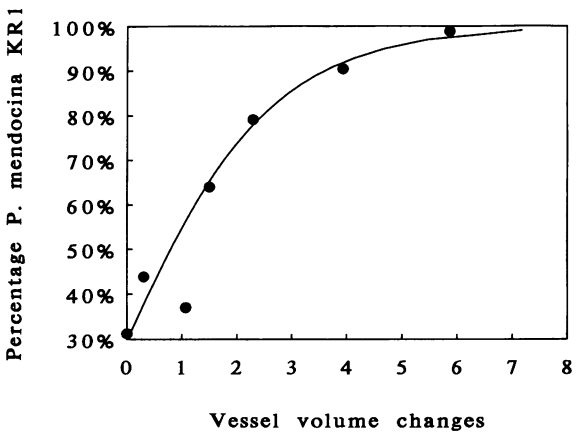

FIG. 2. Competition between *P. mendocina* KR1 and *P. cepacia* G4 in chemostat culture under toluene limitation at a dilution rate of 0.05 h^{-1} shown by the percentage of *P. mendocina* KR1 present (●). The solid line represents the theoretical change in population when a constant ratio (C) in the growth rates of the two strains of 0.35 is assumed.

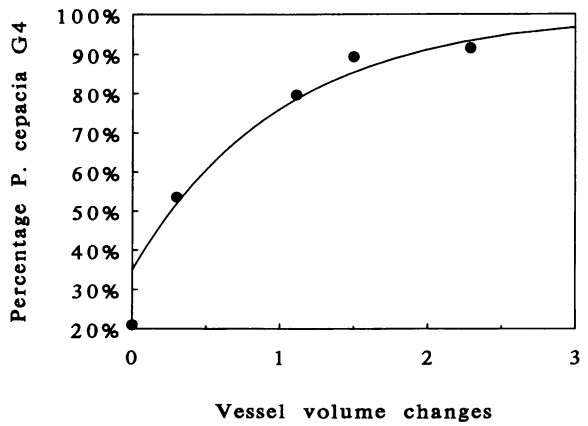

FIG. 3. Competition between *P. putida* mt-2 and *P. cepacia* G4 in chemostat culture under oxygen limitation at a dilution rate of 0.05 h^{-1} with toluene as the sole carbon source, shown by the percentage of *P. cepacia* G4 present (●). The solid line represents the theoretical change in population when a constant ratio (C) in the growth rates of the two strains of 0.0 is assumed, indicating that *P. putida* mt-2 disappears from the chemostat at the washout rate.

the *P. putida* isolates A3AT1 and M1D2 were forced to compete at dilution rates of 0.05 h^{-1} (C = 0.25) and 0.2 h^{-1} (C = 0.85 [Fig. 4]).

Reproducibility of competition experiments. A number of competition experiments were performed once or twice. The resulting values for the growth rate ratio C showed considerable variation. The qualitative outcome of the competition experiment, however, was always reproduced (Table 3).

DISCUSSION

In this study, we compared a number of toluene-degrading strains, either taken from laboratory collections or freshly isolated from oil-polluted soils, with respect to their maximal growth rate in batch culture and their competition behavior in chemostat culture under toluene and oxygen limitation.

The maximal growth rates of the laboratory strains with toluene as the sole source of carbon and energy were very similar (0.38 to 0.45 h^{-1}), except for *P. cepacia* G4, which grew significantly slower (μ = 0.19 h^{-1}). Few reference data on maximal growth rates on toluene are available. The maximal growth rate for *P. putida* mt-2 on toluene was earlier reported to be much lower (0.13 h^{-1} [28]), possibly because of a toxic

TABLE 4. Competition experiments in chemostat culture (dilution rate = 0.05 h^{-1}) under toluene limitation, involving soil isolates harboring either a direct-ring attack pathway or a TOL pathway

Direct-ring attack		TOL pathway		Winning strain	C^a
Strain 1	μ_{max} (h^{-1})[b]	Strain 2	μ_{max} (h^{-1})		
P. cepacia M1C4	0.25	*P. cepacia* O2C1	0.20	1	0.0
P. fluorescens R2AT2	ND[c]	*P. fluorescens* A4C2	ND	1	0.44
P. putida A3AT1	0.39	*P. putida* M1D2	0.27	1	0.25
P. putida A3AT1	0.39	*P. putida* mt-2	0.40	1	0.0
				1[d]	0.44[d]

[a] C, ratio of growth rates of the losing and the winning strain.
[b] μ_{max}, maximal growth rate on toluene in batch culture.
[c] ND, not done.
[d] Duplicate experiment.

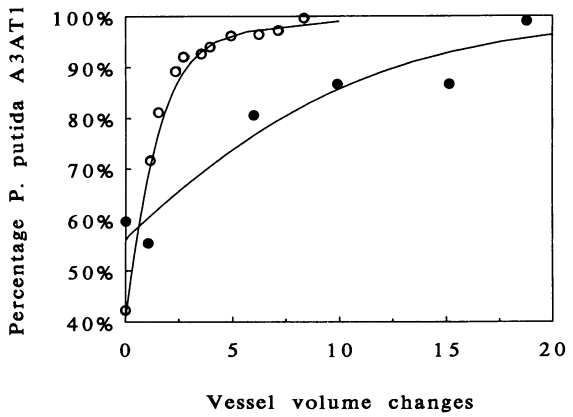

FIG. 4. Influence of dilution rate on the competition between *P. putida* A3AT1 (harboring a direct-ring attack pathway) and *P. putida* M1D2 (harboring a TOL pathway) in chemostat culture under toluene limitation. The symbols represent the percentage of *P. putida* A3AT1 present at dilution rates of 0.05 (○) and 0.2 (●) h^{-1}. The solid lines represent the theoretical change in population when constant ratios (C) in the growth rates of the two strains of 0.25 (D = 0.05 h^{-1}) and 0.85 (D = 0.2 h^{-1}) are assumed.

effect of the toluene concentrations supplied. The maximal growth rates of the soil-isolated strains of *P. putida* and *P. cepacia* were, on the average, slightly lower than those of the laboratory strains. The growth yields on toluene were similar to those of other toluene-degrading strains (reviewed in reference 1).

Under toluene limitation, *P. mendocina* KR1 showed the best competitive behavior, while *P. cepacia* G4 was the best performing laboratory strain under oxygen limitation. *P. putida* mt-2 was outcompeted by the other three strains under both conditions. As pointed out by Harder et al. (14), the outcome of a competition experiment between different bacterial strains in chemostat culture is mainly determined by the relationship between the growth rate (μ) and the residual concentration of the limiting nutrient (s); the strain with the highest specific growth rate at the actual concentration of the growth-limiting nutrient outcompetes the other one. As far as we know, no specific uptake systems for nonpolar compounds such as toluene exist; passive diffusion into the cell membrane is the most likely way of uptake. Therefore, we assume, in pursuit of what Button (5, 6) stated in general for nonpolar compounds, that the rate-controlling enzyme of the degradation pathway determines the specific affinity (the slope of the μ versus s curve near the origin). In this light, it seems justified to interpret the results of the competition experiments between the laboratory strains in terms of differences between the toluene degradation pathways that they harbor.

When we compare the first steps of the different toluene degradation pathways in thermodynamic terms, it becomes obvious that conversion of toluene to one of the three isomers of cresol is energetically more favorable than conversion to benzyl alcohol (on the basis of the values of the standard free energy of formation given in reference 25). According to the Marcus theory, a relation exists between the standard free-energy change and the reaction rate (8). Whether or not this is reflected in a relatively low affinity of xylene monooxygenase (which performs the conversion of toluene to benzyl alcohol) and hence underlies the relatively poor performance of *P. putida* mt-2 is uncertain. Detailed kinetic studies on this or similar oxygenases have not yet been performed, probably because of the difficulties encountered during measurement of their in vitro activity (26, 31). An additional explanation for the inferior competitiveness of *P. putida* mt-2 may lie in nonspecific reactions, such as conversion of tryptophan, known to be performed by xylene monooxygenase (30).

That strain-specific characteristics other than the degradation pathway influence the outcome of competition, however, may not be excluded. For example, the extent of accumulation of toluene in the cell membrane (by a factor of ca. 300 [16]) may be influenced by the membrane composition. Further, it has been observed previously that the level of expression of the toluene degradation pathway may have a strong effect on the affinity for toluene (16) and may override mechanistic aspects of the pathway.

The residual concentration (s) of toluene in axenic chemostat cultures of the four laboratory strains correlated fairly well with the results of the competition experiments. The best competitor under toluene limitation, *P. mendocina* KR1, had the lowest s (<5 nM), while the s of the second best competitor, *P. putida* F1, was somewhat higher (10 nM) but still much lower than that of the worst competitor, *P. putida* (290 nM). Only in the culture of *P. cepacia* G4 is the s of 390 nM inexplicably high in the light of its win over *P. putida* mt-2. The absolute values should, however, be interpreted with care, as during sampling complete inactivation (by phosphoric acid) of the cells is probably not reached faster than within 1 or 2 s. When we take into account that the conversion rate of toluene in the chemostat cultures is ca. 50 nM s^{-1}, we must assume that a significant portion of the actual s in the chemostat is converted during sampling. This leads to an underestimation, which will probably be the most significant for the lowest values measured (*P. mendocina* KR1 and *P. putida* F1). Most previous studies on different toluene-degrading strains mention Monod constants in the 400 to 4,000-nM range (for a review, see reference 1). When assuming maximal growth rates similar to the strains used in our study (ca. 0.4 h^{-1}), these Monod constants would theoretically result in values of s at a dilution rate of 0.05 h^{-1} in the 50- to 500-nM range. For (batch-grown) cells of *P. putida* F1, a Monod constant of 680 nM was measured (19), corresponding to an expected s at D = 0.05 h^{-1} of approximately 80 nM. The lower value in our study (10 nM) may be partly explained by toluene conversion during sampling and partly by adaptation processes known to take place during steady-state chemostat cultivation under carbon and energy limitation (13). In environments that are adapted to low concentrations of toluene, such as raw seawater, Monod constants for toluene uptake as low as 5 nM were found (7). Groundwater bacteria were found to degrade toluene to a concentration below 12 nM (2).

In despite of its relatively low maximal growth rate on toluene (Table 2), *P. cepacia* G4 outgrew the other laboratory strains under oxygen-limiting conditions. Interpretation of the competition experiments under oxygen limitation is complicated by the fact that oxygen is needed both for the functioning of the oxygenation steps in the toluene degradation pathways and as a terminal electron acceptor in the electron transport chain. However, the Michaelis constants for oxygen of purified oxygenases (0.3 to 2 mg of O_2 $liter^{-1}$ [20]) are generally higher than those reported for the electron transport chain (0.01 to 0.04 mg of O_2 $liter^{-1}$ [17]). Therefore, we expect that the outcome of the competition experiments was determined mainly by the affinities of the oxygenases for oxygen.

Soil-isolated *P. cepacia*, *P. fluorescence*, and *P. putida* strains harboring a TOL pathway lost competition experiments under toluene limitation to isolates from the same species that harbor a direct-ring attack pathway. In combination with the poor

competition behavior of the laboratory strain, *P. putida* mt-2, it seems justified to conclude that the direct-ring attack approach is favorable for the degradation of low concentrations of toluene. The widespread occurrence in the environment of strains harboring TOL pathways might be attributed to their unique ability to degrade dialkyl-substituted benzenes such as *m*-xylene, *p*-xylene, and 3-ethyltoluene, and even a trimethyl benzene such as pseudocumene (3), rather than to their ability to degrade toluene.

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REFERENCES

1. **Alvarez, P. J. J., P. J. Anid, and T. M. Vogel.** 1991. Kinetics of aerobic biodegradation of benzene and toluene in sandy aquifer material. Biodegradation **2:**43–51.
2. **Arvin, E., B. Jensen, J. Aamand, and C. Jørgensen.** 1988. The potential of free-living ground water bacteria to degrade aromatic hydrocarbons and heterocyclic compounds. Water Sci. Technol. **20:**109–118.
3. **Assinder, S. J., and P. A. Williams.** 1990. The TOL plasmids: determinants of the catabolism of toluene and the xylenes. Adv. Microb. Physiol. **31:**1–69.
4. **Bringmann, G., and R. Kühn.** 1980. Comparison of the toxicity thresholds of water pollutants to bacteria, algae, and protozoa in the cell multiplication inhibition test. Water Res. **14:**231–241.
5. **Button, D. K.** 1985. Kinetics of nutrient-limited transport and microbial growth. Microbiol. Rev. **49:**270–297.
6. **Button, D. K.** 1993. Nutrient-limited microbial growth: overview and recent advances. Antonie van Leeuwenhoek **63:**225–235.
7. **Button, D. K., B. R. Robertson, and K. S. Craig.** 1981. Dissolved hydrocarbons and related microflora in a fjordal seaport: sources, sinks, concentrations, and kinetics. Appl. Environ. Microbiol. **42:**708–719.
8. **Connors, K. A.** 1990. Chemical kinetics: the study of reaction rates in solution, p. 209–229. VCH Publishers, Inc., New York.
9. **Duetz, W. A., and J. G. Van Andel.** 1991. Stability of TOL plasmid pWW0 in *Pseudomonas putida* mt-2 under non-selective conditions in continuous culture. J. Gen. Microbiol. **137:**1369–1374.
10. **Duetz, W. A., M. K. Winson, J. G. Van Andel, and P. A. Williams.** 1991. Mathematical analysis of catabolic function loss in a population of *Pseudomonas putida* mt-2 during non-limited growth on benzoate. J. Gen. Microbiol. **137:**1363–1368.
11. **Evans, C. G. T., D. Herbert, and D. W. Tempest.** 1970. The continuous cultivation of microorganisms. 2. Construction of a chemostat. Methods Microbiol. **2:**277–327.
12. **Gibson, D. T., M. Hensley, H. Yoshioka, and T. J. Mabry.** 1970. Formation of (+)-cis-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from toluene by *Pseudomonas putida*. Biochemistry **9:**1626–1630.
13. **Harder, W., and L. Dijkhuizen.** 1983. Physiological responses to nutrient limitation. Annu. Rev. Microbiol. **38:**1–23.
14. **Harder, W., J. G. Kuenen, and A. Matin.** 1977. Microbial selection in continuous culture. J. Appl. Bacteriol. **43:**1–24.
15. **Kappeler, T., and K. Wuhrman.** 1978. Microbial degradation of the water-soluble fraction of gas oil. Water Res. **12:**327–333.
16. **Law, A. T., and D. K. Button.** 1986. Modulation of affinity of a marine pseudomonad for toluene and benzene by hydrocarbon exposure. Appl. Environ. Microbiol. **51:**469–476.
17. **Longmuir, I. S.** 1954. Respiration rate as a function of oxygen concentration. Biochem. J. **57:**81–87.
18. **Mikesell, M. D., R. H. Olsen, and J. J. Kukor.** 1991. Stratification of anoxic BTEX-degrading bacteria at three petroleum-contaminated sites, p. 351–362. *In* R. E. Hinchee and R. F. Olfenbuttel (ed.), *In situ* bioreclamation. Butterworth-Heineman, Boston.
19. **Robertson, B. R., and D. K. Button.** 1987. Toluene induction and uptake kinetics and their inclusion in the specific-affinity relationship for describing rates of hydrocarbon metabolism. Appl. Environ. Microbiol. **53:**2193–2205.
20. **Shaler, T. A., and G. M. Klečka.** 1985. Effects of dissolved oxygen concentration on biodegradation of 2,4-dichlorophenoxyacetic acid. Appl. Environ. Microbiol. **51:**950–955.
21. **Shields, M. S., S. O. Montgomory, P. J. Chapman, S. M. Cuskey, and P. H. Pritchard.** 1989. Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. Appl. Environ. Microbiol. **55:**1624–1629.
22. **Smith, M. R.** 1990. The biodegradation of aromatic hydrocarbons by bacteria. Biodegradation **1:**191–206.
23. **Spain, J. C., and D. T. Gibson.** 1988. Oxidation of substituted phenols by *Pseudomonas putida* F1 and *Pseudomonas* sp. strain JS6. Appl. Environ. Microbiol. **54:**1399–1404.
24. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159–271.
25. **Stull, D. R., E. F. Westrum, Jr., and G. C. Sinke.** 1969. The chemical thermodynamics of organic compounds, p. 685–687. J. Wiley & Sons, Inc., New York.
26. **Suzuki, M., T. Hayakawa, J. P. Shaw, M. Rekik, and S. Harayama.** 1993. Primary structure of xylene monooxygenase: similarities to and differences from the alkane hydroxylation system. J. Bacteriol. **173:**1690–1695.
27. **Thomas, J. M., M. D. Lee, M. J. Scott, and C. H. Ward.** 1989. Microbial ecology of the subsurface at an abandoned creosote waste site. J. Ind. Microbiol. **4:**109–120.
28. **Vecht, S. E., M. W. Platt, Z. Er-El, and I. Goldberg.** 1988. The growth of *Pseudomonas putida* on m-toluic acid and on toluene in batch and chemostat cultures. Appl. Microbiol. Biotechnol. **27:** 587–592.
29. **Whited, G. M., and D. T. Gibson.** 1991. Separation and partial characterization of the enzymes of the toluene-4-monooxygenase catabolic pathway in *Pseudomonas mendocina* KR1. J. Bacteriol. **173:**3017–3020.
30. **Wubbolts, M. G., P. Reuvekamp, and B. Witholt.** TOL plasmid specified xylene oxygenase is a wide substrate range monooxygenase capable of olefin epoxidation. Enzyme Microb. Technol., in press.
31. **Yeh, W. K., D. T. Gibson, and T. N. Liu.** 1977. Toluene dioxygenase: a multicomponent enzyme system. Biochem. Biophys. Res. Commun. **78:**401–410.