Stability of TOL plasmid pWW0 in *Pseudomonas putida* mt-2 under non-selective conditions in continuous culture

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Pseudomonas putida mt-2, harbouring the TOL plasmid pWW0, was grown in chemostat culture under succinate-, sulphate-, ammonium- or phosphate-limitation at different dilution rates. The fraction of mutant cells lacking the plasmid-encoded enzymes for the degradation of toluene and xylene (TOL⁻ cells), was determined. Genetic analysis revealed that all TOL⁻ cells isolated harboured partially deleted plasmids, lacking the TOL catabolic genes. The growth-rate advantage of the TOL⁻ cells was quantified from the kinetics of their increase as a fraction of the total population. At a dilution rate of 0.1 h⁻¹ no growth-rate advantage of TOL⁻ cells was found when phosphate or ammonium were limiting. Under sulphate-limitation, ingrowth of TOL⁻ cells was evident but did not follow a straightforward pattern. Under succinate-limitation the growth-rate advantage was the highest, particularly at low dilution rates (about 50% at $D = 0.05$ h⁻¹). In phauxostat culture, at the maximal growth rate, the growth-rate advantage of TOL⁻ cells was less than 1%. The specific activity in TOL⁺ cells of the plasmid-encoded enzyme catechol 2,3-dioxygenase was relatively high at a low growth rate.

Introduction

Many bacterial strains, particularly those of the genus *Pseudomonas*, are capable of degrading xenobiotic compounds; the catabolic enzymes involved are often encoded on large plasmids. These so-called catabolic plasmids (for a review see Sayler *et al.*, 1990) provide their hosts with the advantage of a broader growth-substrate spectrum. To be competitive in a certain niche, however, the advantage must be big enough to compensate for the costs of maintenance and expression of the catabolic plasmid. These considerations may be important for proliferation of strains harbouring catabolic plasmids and may explain why certain xenobiotics persist in biological systems even though their biodegradability has been proven possible.

To obtain more insight on the burden which the maintenance and expression of a plasmid may impose on the host cell, a frequently used method is to monitor the persistence of the plasmid-harbouring strain in continuous culture conditions. Most work has been done with strains harbouring plasmids encoding antibiotic resistance (e.g. Melling *et al.*, 1977; Godwin & Slater, 1979; Wouters *et al.*, 1980). Also, the persistence of genetically engineered plasmids has been subject to considerable

research in recent years (e.g. Seo & Bailey, 1985; Bentley *et al.*, 1990).

Most studies done on the stability of catabolic plasmids were concerned mainly with growth on benzoate (e.g. Keshavarz *et al.*, 1985; Williams *et al.*, 1988; Stephens & Dalton, 1988). The strong selection against TOL-plasmid harbouring cells observed during growth on benzoate is caused, however, by differences in the degradation pathway between the wild-type strain and mutant cells.

In this study we determined the stability of the low-copy-number TOL plasmid pWW0 (117 kb) in *Pseudomonas putida* mt-2 under non-selective conditions (succinate as the carbon and energy source) in continuous culture at different limitations and dilution rates. Two experimental approaches were employed. Firstly, in persistence experiments, succinate was fed to a continuous culture containing 100% TOL-plasmid-bearing cells; the kinetics of ingrowth of spontaneous mutant cells that lack the plasmid-encoded enzymes (TOL⁻ cells) were then examined. Secondly, the growth-rate advantage of these TOL⁻ cells was further quantified in competition experiments after seeding them into a culture of wild-type cells.

Methods

Nomenclature. The nomenclature and symbols used in this paper are the same as in the preceding paper (Duetz *et al.*, 1991); in addition, C is the ratio of the growth rates of the TOL⁺ and TOL⁻ populations and $t_{95\%}$ is defined as the time at which the percentage of TOL⁺ cells falls below 95%.

Bacterial strain. *Pseudomonas putida* mt-2 (ATCC 33015) harbouring the TOL plasmid pWW0 was a gift of P. A. Williams (Bangor, UK). It was maintained in agar tubes on a mineral medium with *m*-toluate as the sole carbon and energy source (C-source) at 4 °C. TOL⁻ strains were maintained in the same way but with succinate as the sole C-source.

Media. The growth medium was a mineral salts solution according to Evans *et al.* (1970), except that the concentrations of all nutrients were decreased to 25% of the published concentration, supplemented with 5 mM-*m*-toluate, 5 mM-*m*-methylbenzyl alcohol or 10 mM-succinate, when carbon-limited, or twice those concentrations for other nutrient limitations. For ammonium-limitation the NH₄Cl concentration was 1.88 mM, for phosphate-limitation the NaH₂PO₄ concentration was 150 µM, and for sulphate-limitation the Na₂SO₄ concentration was 30 µM. For all limitations the optical density at 540 nm was approximately 0.7 (dry wt approx. 400 mg l⁻¹) at a dilution rate of 0.1 h⁻¹. Nitrilotriacetate (NTA) was used as a chelating agent. Except for phosphate limitation, the pH of chemostat cultures was maintained at 7.0 using media containing 50 mM-KH₂PO₄/K₂HPO₄ buffer, silicone antifoaming agent (BDH) was added to the growth medium at a concentration of 62 µl l⁻¹. The medium for growth in phauxostat culture was identical but contained 15 mM-succinate and had a pH of 6.4.

Continuous culture. For chemostat culture at the lowest dilution rate ($D = 0.05$ h⁻¹) and under phosphate-limitation 500 ml fermenters (500 series, L & H Engineering) were used. The culture was stirred at 1500 r.p.m. The pH was maintained at 7.0 ± 0.05 by automatic addition of NaOH (1.0 M). The temperature was maintained at 28 ± 1 °C.

All other chemostat experiments were done in 100 ml glass fermenters, shaped like an Erlenmeyer flask, with a small headspace volume and stirred magnetically at 550 r.p.m. Medium entered at the bottom of the vessel through a needle with an internal diameter of 1.0 mm. Air was supplied through the same needle at 100 ml min⁻¹. The fermenter was equipped with two stainless steel baffles and was kept at 28 °C.

P. putida mt-2 was grown on succinate at a non-limiting rate by making use of a phauxostat as described in the preceding paper (Duetz *et al.*, 1991). Whenever wall growth was observed, the complete cell suspension was transferred to a new sterile fermenter vessel of the same type, without interrupting the addition of medium for more than 1 min.

Competition experiments. Competition experiments were done in the following way. A TOL-bearing strain and a derivative with a partially deleted plasmid were grown separately in two 500 ml chemostats with succinate as the sole C-source, at a dilution rate of 0.5 h⁻¹. After 2 d, when both chemostats were assumed to have reached a steady state, a 100 ml chemostat was inoculated with a 90 ml sample from the chemostat with the TOL⁺ strain and a 10 ml sample from the chemostat with the TOL⁻ strain. The dilution rate of this 100 ml chemostat was set at 0.5 h⁻¹ while the dilution rates of the two mother cultures were lowered to 0.2 h⁻¹. After a further 2 d, a second 100 ml chemostat ($D = 0.2$ h⁻¹) was inoculated in the same way and so on for dilution rates of 0.1 and 0.05 h⁻¹.

Assays. Assays for cell density, the percentage of TOL⁺ cells in a population, the statistical calculation of 95% reliability intervals and the genetic characterization of TOL⁻ cells were done as described in the preceding paper (Duetz *et al.*, 1991).

Assay for catechol 2,3-dioxygenase. Extracts of culture cells sampled from the chemostats were prepared by sonication and centrifugation. Subsequently, catechol 2,3-dioxygenase activity was determined according to the method of Sala-Trepat & Evans (1971).

Quantification from population dynamics of the growth-rate advantage of TOL⁻ cells. Consider the competition between two strains A and B, present at population fractions p_A and p_B , and with growth rates μ_A and μ_B . In chemostat culture, the dilution rate D is an externally set parameter and μ_A and μ_B are variables. When it is assumed that the ratio of μ_A and μ_B is constant ($\mu_B/\mu_A = C$), substitution of equation (3) in equation (1) (both equations can be found in the preceding paper: Duetz *et al.*, 1991) yields

$$\left(\frac{dp_A}{dt}\right) = \frac{Dp_A(1-p_A)}{(C/1-C)+p_A} \quad (1)$$

Integration with the boundary condition $p_A(t=0) = p_A^0$ yields

$$\frac{(p_A)^C}{1-p_A} = \frac{(p_A^0)^C}{1-p_A^0} e^{(1-C)Dt} \quad (2)$$

Equation (2) can be used to describe the competition in chemostat culture between a fraction of TOL⁺ cells (p_+) and a fraction of TOL⁻ cells (p_-), with a constant growth-rate ratio $C = \mu_+/\mu_-$ and an initial fraction of the TOL⁻ population of p_-^0 .

$$\frac{(p_-)^C}{1-p_-} = \frac{(p_-^0)^C}{1-p_-^0} e^{(1-C)Dt} \quad (3)$$

Values for C and p_-^0 were generated by non-linear least-square fitting of equation (3) to the experimental data for the change of p_- in time, making use of a subroutine written in FORTRAN (available from the authors). In the fitting procedure the datapoints were weighted equally but datapoints with values for p_- of $< 3\%$ and $> 97\%$ were ignored. The value for p_-^0 is arbitrary since the contribution of segregation in the initial phase is not taken into account.

For persistence experiments in phauxostat culture a value for C was calculated from the value for the growth rates of the TOL⁺ and TOL⁻ cells as determined according to the preceding paper (Duetz *et al.*, 1991).

Estimation of segregation rate. If it is assumed that the first appearing TOL⁻ cells do not have a growth-rate advantage, the segregation rate R can be estimated by

$$\left(\frac{dp_-}{dt}\right) = R \quad (4)$$

Integration with the boundary condition that $p_-(t=0) = 0$ yields

$$R = \frac{p_-(t)}{t} \quad (5)$$

For calculating R in this way we used the first data point of the persistence experiment with a fraction p_- that was significantly over 1%. The assumption of the absence of a growth-rate advantage of the mutant cells and the assumption that $p_-^0 = 0$ both lead to an overestimation of R . Accordingly, we present the resulting values for R as maximal values.

Results

Effects of growth-limiting nutrient on plasmid maintenance

P. putida mt-2 was grown in continuous culture in a mineral medium at a fixed dilution rate of 0.1 h⁻¹. During the first 5 d after inoculation the sole C-source was *m*-toluate. In this period, a steady state of 100%

Fig. 1

Fig. 1. Persistence of pWW0 in *P. putida* mt-2 grown in chemostat culture under succinate-limitation at a dilution rate of 0.1 h^{-1} . ●, ○, Fractions p_+ in two duplicate experiments; —, best-fit curves of equation (3) (see Methods) to the experimental values for p_+ , generating values for the growth-rate ratio C of 0.94 and 0.76 respectively.

Fig. 2

Fig. 2. Persistence of pWW0 in *P. putida* mt-2 grown in chemostat culture under sulphate-limitation at a dilution rate of 0.1 h^{-1} . ●, ○, Fractions p_+ in two duplicate experiments; —, best-fit curve of equation (3) (see Methods) to the experimental values for p_+ , generating a value for the growth-rate ratio C of 0.89. For the duplicate experiment no value for C could be calculated.

TOL⁺ cells was established under phosphate-, sulphate- or carbon- and energy-limitation, but not under ammonium-limitation, where almost immediately a fraction (up to 80%) of TOL⁻ cells appeared. To establish a 100% TOL⁺ population under ammonium-limiting conditions, *m*-methylbenzyl alcohol was used as a C-source instead of *m*-toluate. After this initial stage, the medium containing *m*-toluate or *m*-methylbenzyl alcohol was replaced by a medium with succinate as the sole C-source. The time to reach a TOL⁺ population of 95% or less ($t_{95\%}$) was determined. Subsequently, the ratio (C) of the growth rates of TOL⁺ cells and TOL⁻ cells was calculated by fitting equation (3) to the data points.

Under phosphate- or ammonium-limitation the TOL⁺ cells persisted for a period of more than 2000 h (200 vessel volume changes). The fraction of the TOL⁻ population never exceeded 3% of the whole population.

Two replicate experiments done under carbon- and energy-limitation both showed a complete loss of the TOL⁺ population within 1500 h (see Fig. 1). However, the $t_{95\%}$ times (400 and 950 h) and the calculated growth-rate ratios ($C = 0.94$ and 0.76) were dissimilar.

The ingrowth of TOL⁻ cells observed under sulphate-limitation (Fig. 2) did not follow the same distinct pattern as under carbon- and energy-limitation. The $t_{95\%}$ times in the replicates were about the same (830 and 980 h), but in the first experiment the subsequent further decrease in the percentage of TOL⁺ cells ceased at a level of about 10% (until then $C = 0.89$). The other experiment even showed a significant increase in the TOL⁺ population after the initial decrease to 85% TOL⁺ cells.

These results show that the type of nutrient limitation has a pronounced effect on the persistence of the TOL⁺ population. Because carbon- and energy-limitation al-

ways appeared to result in a ready and uncomplicated ingrowth of a TOL⁻ population, we determined the influence of the dilution rate on the persistence of TOL⁺ cells under this limitation.

Effect of growth rate on the persistence of TOL⁺ cells

The persistence of TOL⁺ cells was examined under carbon- and energy-limiting conditions with different dilution rates in the way described above. At the highest dilution rate tested (0.5 h^{-1}) the TOL⁺ population was extremely persistent (see Fig. 3). The percentage of TOL⁻ cells was more than twice the growth rate of the TOL⁺ cells ($C = 0.38$). Table 1 shows that at intermediate dilution rates (0.1 and 0.2 h^{-1}) the values for dilution rate tested (0.05 h^{-1}) the $t_{95\%}$ was reached almost immediately when the medium was switched from *m*-toluate to succinate and the growth rate of the TOL⁻ cells was more than twice the growth rate of the TOL⁺ cells ($C = 0.38$). Table 1 shows that at intermediate dilution rates (0.1 and 0.2 h^{-1}) the values for $t_{95\%}$ and C were intermediate between these two extremes (see also Fig. 3).

In addition to persistence experiments under carbon- and energy-limitation, *P. putida* mt-2 harbouring pWW0 was grown in phauxostat culture, which allows continuous cultivation at non-limited growth rates ($D = 1.04 \pm 0.05 \text{ h}^{-1}$ in this case). The residual concentration of succinate was about 4 mM. The $t_{95\%}$ was 400 h (about 420 vessel volume changes). The ingrowth of TOL⁻ cells ceased at about 15%. The growth-rate ratio C until then was > 0.99 (see Fig. 4 and Table 1).

Fig. 3

Fig. 3. Persistence of pWW0 in *P. putida* mt-2 grown in chemostat culture under succinate-limitation at different dilution rates. Fractions p_+ at dilution rates of 0.5 (●), 0.2 (○), 0.1 (■) and 0.05 (□) are shown. —, Best-fit curves of equation (3) (see Methods) to the experimental values for p_+ , generating values for the growth-rate ratio C of 0.995, 0.965, 0.94 and 0.38 respectively.

Fig. 4

Fig. 4. Persistence of pWW0 in *P. putida* mt-2 grown at a non-limited rate on succinate in phauxostat culture. ●, Fraction p_+ , (error bars indicate 95% reliability intervals); ○, dilution rate (h^{-1}); —, best-fit curve of equation (3) (see preceding paper: Duetz *et al.*, 1991) to the experimental values for p_+ , generating a value for the growth-rate difference v of 0.01 h^{-1} (corresponding to a growth-rate ratio C of 0.99).

Table 1. Effect of dilution rate on catechol 2,3-dioxygenase activity, the moment at which the fraction of TOL⁺ cells drops below 95% ($t_{95\%}$) and the ratio (C) of the growth rate of TOL⁺ and TOL⁻ cells as measured during persistence or competition experiments

Dilution rate (h^{-1})	Type of experiment	Catechol 2,3-dioxygenase activity [$\text{mU (mg protein)}^{-1}$]	$t_{95\%}$		$C (= \mu_+/\mu_-)$
			h	Vessel vol. changes	
1.04 ($\mu_{\max}$)	Persistence	ND	390	400	> 0.99
	Competition	ND			0.99†
0.5	Persistence, exp. I	116 ± 15	1240	620	0.995
	Persistence, exp. II	ND	> 1150	> 575	
	Competition	ND			> 0.94
0.2	Persistence, exp. I	336 ± 38	820	164	0.965
	Persistence, exp. II	ND	480	96	0.982
	Competition	ND			> 0.96
0.1	Persistence, exp. I	492 ± 2	950	95	0.76
	Persistence, exp. II	ND	400	40	0.94
	Competition	ND			0.82
0.05	Persistence	1131*	0	0	0.38
	Competition	ND			0.60

ND, Not determined.

* Single measurement.

† Result from a competition experiment in sequential batch culture.

Competition experiments

Persistence experiments showed that the value of the growth-rate ratio C was strongly dependent on the dilution rate. We wanted to establish if this effect of the dilution rate is not due to the selection of different TOL⁻ genotypes. We therefore isolated a TOL⁻ strain (carrying a partially deleted plasmid) and a TOL⁺ strain simultaneously from the same persistence experiment ($D = 0.2 \text{ h}^{-1}$). Subsequently, we did competition experiments with this set of strains at different dilution rates.

The ingrowth of an initial population of 10% TOL⁻ cells was monitored and analysed mathematically. The influence of the dilution rate on the growth-rate ratio C was similar to that found during the persistence experiments (see Table 1). Fig. 5 shows the result of the competition experiment at a dilution rate of 0.05 h^{-1} .

Catechol 2,3-dioxygenase activities

The activity of catechol 2,3-dioxygenase was determined during the stage in persistence experiments when the

Fig. 5. Competition between TOL⁻ and TOL⁺ cells in chemostat culture when grown on succinate under succinate-limitation at a dilution rate of 0.05 h⁻¹. ●, Fraction p_+ ; —, best-fit curve of equation (3) (see Methods) to the experimental values for p_+ , generating a value for the growth-rate ratio C of 0.38.

culture was already adapted to succinate as the sole C-source, but did not yet contain a significant fraction of TOL⁻ cells (see Table 1). At the lowest dilution rate tested ($D = 0.05$ h⁻¹) the catechol 2,3-dioxygenase activity was about 10 times higher than at $D = 0.5$ h⁻¹. In separate experiments the activity remained constant but decreased parallel to the percentage of TOL⁺ cells.

Segregation rates

We calculated (maximal) segregation rates R from the results from the persistence experiments. Values for R ranged from 6.2×10^{-5} to 1.0×10^{-4} h⁻¹. No correlation with the type of limitation or dilution rate could be found. These values for R are too low to influence the competition pattern significantly when the fraction of TOL⁻ cells is more than 2% and the value for C is less than about 0.95.

Genetic characterization of TOL⁻ mutants

From all persistence experiments one or more TOL⁻ mutants were isolated. All TOL⁻ mutants appeared to harbour plasmids of 78 kb. *Hind*III restriction patterns were identical to that of plasmid pWW0-8, a derivative of pWW0 lacking all TOL catabolic genes and described previously by Meulien *et al.* (1981).

Discussion

The purpose of this study was to examine the behaviour of *P. putida* mt-2 harbouring TOL plasmid pWW0 under conditions where the plasmid-encoded catabolic capacity was unnecessary. The influence of the limiting nutrient was determined at a dilution rate of 0.1 h⁻¹ with succinate as the sole C-source.

High persistence of the wild-type strain was found under phosphate- or ammonium-limitation with succin-

ate as a C-source. The observation that small populations of TOL⁻ cells (up to 3% of the whole population) evolved without further ingrowth indicates that the growth-rate advantage of TOL⁻ cells is very small under these conditions. The finding that TOL⁺ plasmid-containing cells are very persistent under phosphate-limiting conditions is the most pronounced difference with similar studies undertaken with other plasmids. For plasmids encoding antibiotic resistance (R-plasmids) like TP120 (Godwin & Slater, 1979) and pBR322 (Wouters *et al.*, 1980), phosphate-limiting conditions appear to select strongly for cells lacking the resistance genes. In these studies counterselection for the wild-type strain was less strong under carbon- and energy-limitation. This is in contrast to the poor persistence of our catabolic plasmid under carbon- and energy-limitation.

The low persistence of the wild-type strain under sulphate limitation compared to phosphate- and ammonium-limitation is also different from results found with R-plasmids. Chew *et al.* (1988) reported a relatively high persistence of plasmid pAT153 (a derivative of pBR322) under sulphate-limitation.

The fast ingrowth of TOL⁻ cells under ammonium-limitation with *m*-toluate as the sole C-source is difficult to explain since TOL⁻ cells cannot metabolize *m*-toluate. Cell debris and excretion products of TOL⁺ cells probably support growth of the TOL⁻ population. This phenomenon may possibly be related to the inhibitory effect of benzoate on TOL⁺ cells described in the preceding paper (Duetz *et al.*, 1991).

A strong influence of dilution rate on persistence was found under carbon- and energy-limitation. At the lowest dilution rate tested ($D = 0.05$ h⁻¹) TOL⁻ cells grew about twice as fast as TOL⁺ cells. At a dilution rate of 0.5 h⁻¹, or at a non-limited rate in phauxostat culture, TOL⁻ cells exhibited almost no growth-rate advantage (< 1%). A similar dependence on dilution rate was found during competition experiments (see Table 1) when the same isolated TOL⁻ strain and TOL⁺ strain were used for each dilution rate. This rules out the possibility that the influence of the dilution rate found during the persistence experiments was caused solely by different TOL⁻ genotypes evolving. The higher growth-rate advantage of segregants at low dilution rate is in agreement with the studies on R-plasmids mentioned above.

Under carbon- and energy-limitation a relationship seems to exist between the high growth-rate advantage of TOL⁻ cells and a high activity of catechol 2,3-dioxygenase in TOL⁺ cells. A high level of expression probably also holds for other plasmid-encoded catabolic enzymes because all enzymes of the *meta*-pathway are expressed as part of a single regulon (see Assinder & Williams, 1990). With high-copy-number plasmids in *Escherichia coli* (e.g. Seo & Bailey, 1985; Bentley *et al.*,

1990) a similar relationship was found between the growth rate, the induction level of plasmid-encoded enzymes and the growth-rate advantage of segregants.

Different views exist on the dominant factors behind the burden that a plasmid imposes on its host. Lee & Bailey (1984) emphasized the importance of the growth inhibitory effect as a consequence of competition for RNA polymerase between chromosomal and plasmid DNA. Bentley *et al.* (1990) concluded from the results of their experiments with pBR329 in *E. coli* RR1 that the level of synthesis of plasmid-encoded protein itself determines the extent of growth retardation. They also demonstrated that the effect of plasmid replication on cell growth is negligible.

It is difficult to identify the dominant factors in the growth-rate-retarding effect of the presence of TOL plasmid pWW0. During the chemostat experiments described in this study we never found any difference in growth yield on succinate between TOL⁺ cells and TOL⁻ cells (results not shown). Furthermore, not enough data are available to estimate the contribution of plasmid-encoded proteins to the total amount of proteins and the energy costs related therewith. Other factors, however, may be more decisive for the outcome of competition between TOL⁺ cells and TOL⁻ cells. For instance, a difference in affinity for the limiting nutrient might be caused by a difference in cell size. Electron microscopy studies indicate that wild-type cells of *P. putida* MT84 harbouring TOL plasmid pWW84 are significantly bigger than cells from a plasmid-free derivative strain (L. K. Chatfield, personal communication). A small cell size, and so a relatively high surface-to-volume ratio, may give a growth-rate advantage that is particularly high at very low concentrations of limiting substrates (Kuenen *et al.*, 1977).

The discovery of mutants harbouring partially deleted plasmids instead of plasmid-free mutants gives rise to speculation about additional plasmid-encoded functions that could be advantageous under the given conditions. For instance, Bradley & Williams (1982) found that the TOL plasmid also encodes for cell-surface-linked functions, such as pili. The structure of the cell surface may also be of importance for the uptake of nutrients at limiting concentration.

Our results may have some significance in explaining the slow biodegradation of certain xenobiotics in natural environments. The experiments indicate that the presence of a complex catabolic pathway, such as is encoded by TOL plasmids, may be a severe burden for the host under conditions that are strongly growth-limiting. The proliferation of strains harbouring catabolic plasmids may be limited if the benefits of the extra carbon and energy gain are not sufficient to compensate for this burden. Further quantitative research is required on this subject.

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