Mathematical analysis of catabolic function loss in a population of *Pseudomonas putida* mt-2 during non-limited growth on benzoate

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Pseudomonas putida mt-2, harbouring the TOL plasmid PWW0, was grown continuously on benzoate in a phauxostat at a non-limited rate. The gradual decrease in the population carrying the complete TOL plasmid was caused predominantly by a growth-rate advantage of spontaneous mutants carrying a partially deleted plasmid (TOL⁻ cells). The growth-rate difference (v) was quantified both by measuring the increase in the dilution rate (from 0.68 to 0.79 h⁻¹; $v = 0.11$ h⁻¹) and by mathematical analysis of the ingrowth of TOL⁻ cells ($v = 0.12$ h⁻¹). The latter procedure also established that the segregation rate was of the order of magnitude 10⁻⁵ h⁻¹. Similar values for the growth-rate advantage and the segregation rate were found when both benzoate and succinate were present in non-limiting concentrations. It is suggested that the growth-rate disadvantage of the wild-type strain is caused by inhibitory effects of an intermediate in the degradation of benzoate via the plasmid-encoded *meta*-pathway.

Introduction

TOL plasmids encode for the biodegradation of toluene, xylenes and other alkyl substituted benzenes (for a review see Assinder & Williams, 1990). For a number of strains harbouring a TOL plasmid, it has been shown that growth on benzoate results rapidly in a population that has lost the ability to grow on toluene and xylene (Nakazawa & Yokota, 1973; Williams & Murray, 1974; Keshavarz *et al.*, 1985; Stephens & Dalton, 1987). Genetic analysis demonstrated that these mutants contain either a partially deleted plasmid or no plasmid at all (Bayley *et al.*, 1977; Osborne *et al.*, 1988).

Recent publications have disagreed about the mechanism behind this phenomenon. In general the discussion has concentrated on two possibilities. Williams *et al.* (1988) stated that the disappearance of the original strain from a pure culture of *Pseudomonas putida* mt-2 harbouring TOL plasmid pWW0 could be explained adequately by the higher growth rate of spontaneous mutant cells. The relatively high growth-rate advantage of the mutant cells was said to be caused by the use of the chromosomally encoded *ortho*-pathway for the catabo-

lism of benzoate. In contrast, Stephens & Dalton (1987, 1988) proposed that for *P. putida* MT15 carrying TOL plasmid pWW15, benzoate specifically enhances the rate of segregation, and so may cause the gradual disappearance of the wild-type strain even when the wild-type strain is supposed to have no growth-rate disadvantage.

In a number of modelling studies of plasmid instability in other host-vector systems, it has been shown that it is possible to distinguish between the two means of loss of plasmid-encoded functions by analysing the kinetics of disappearance of the organisms bearing the original plasmid (Noack *et al.*, 1984; Cooper *et al.*, 1987; Davidson *et al.*, 1990). By means of a similar model, adapted for growth at maximum rate, we quantified in this study both the growth-rate difference and the segregation rate for *P. putida* mt-2 in phauxostat culture and batch culture, with benzoate or benzoate plus succinate as carbon and energy sources.

Methods

Nomenclature. Table 1 lists the nomenclature used in this paper.

Bacterial strain. *Pseudomonas putida* mt-2 (ATCC 33015) harbouring the TOL plasmid pWW0 was maintained in agar tubes on a mineral

Abbreviation: HMS, 2-hydroxymuconic acid semialdehyde.

Table 1. *Nomenclature*

C-source	Carbon and energy source
<i>D</i>	Dilution rate
OD ₅₄₀	Optical density at 540 nm
<i>p</i> ₊	Fraction of the total population with the complete catabolic TOL function
<i>p</i> ₋	Fraction of the total population lacking the catabolic TOL function
<i>p</i> ₋ ⁰	<i>p</i> ₋ at <i>t</i> = 0
<i>R</i>	Segregation rate (formation rate of cells without the catabolic TOL function from wild-type cells)
TOL ⁺ cells	Cells harbouring the complete catabolic TOL function
TOL ⁻ cells	Cells lacking the catabolic TOL function
TOL ⁺ population	Part of the total population with the complete catabolic TOL function
TOL ⁻ population	Part of the total population lacking the catabolic TOL function
<i>v</i>	Difference in growth rate between the TOL ⁻ population and the TOL ⁺ population

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. Nitrilotriacetate (NTA) was used as a complexing agent. The medium was supplemented with 5 mM-*m*-toluate (TE-medium), 5 mM- or 15 mM-benzoate (BE-medium), 10 mM-succinate (SE-medium) or both 15 mM-succinate and 10 mM-benzoate (SBE-medium). For batch growth these media were supplemented with 100 mM-K₂HPO₄ buffer and the pH was set at pH 7.0 for TE-medium and BE-medium and at pH 6.7 for SE-medium. For fermenter growth TE-medium was supplemented with 10 mM-K₂HPO₄ and set at pH 6.85, BE-medium with 2.5 mM-K₂HPO₄ (pH 6.4) and SBE-medium with 50 mM-K₂HPO₄ (pH 6.55). For fermenter culture, silicone antifoaming agent (BDH) was added to the growth media at a concentration of 62 µl l⁻¹.

phauxostat experiments. The experiments were done in 100 ml glass fermenters, shaped like Erlenmeyer flasks, with a small headspace volume and stirred magnetically at 900 r.p.m. Medium entered at the bottom of the vessel through a needle with an internal diameter of 1 mm. Air was supplied through the same needle at 300 ml min⁻¹. The fermenter was equipped with two stainless steel baffles and kept at 28 °C.

To ensure identical starting conditions for the persistence experiments, the fermenter was fed with a mineral medium containing *m*-toluate as the sole C-source for 3 d, after inoculation with 30 ml of an overnight batch culture. In this period the culture was carbon- and energy-limited and the dilution rate was increased stepwise to 0.4 h⁻¹. After 3 d the fermenter was fed with SE-, BE- or SBE-medium. The culture was forced to maximal growth rate by running the fermenter as a phauxostat, as described by Martin & Hempfling (1976); when the pH reached 7.02 (due to the conversion of the acid C-source to neutral biomass) the medium pump was switched on automatically (pH controller, 500 series, L & H Engineering). The pH subsequently fell and the pump was switched off automatically at pH 6.98. In this way benzoate or succinate concentrations were stably maintained in excess of 4 mM.

Whenever wall growth was observed, the complete cell suspension was transferred to another sterile fermenter vessel of the same type, without interrupting the addition of medium for more than 1 min.

The dilution rate was calculated by dividing the amount of medium consumed by the length of the time interval (6–24 h). For plotting and mathematical analysis of dilution rate versus time, the time-point was taken as the middle of the time interval during which the dilution rate was so measured.

Sequential batch growth. Batch experiments were done at 30 °C, with 50 ml of medium in Erlenmeyer flasks (250 ml) shaken at 200 r.p.m. *P. putida* mt-2 was grown overnight in TE-medium and then diluted by 10⁻³ in a fresh batch of TE-medium. At the end of the exponential phase (after incubation for about 8 h) 10 µl of this cell culture was used to inoculate a batch culture in BE-medium (5 mM-benzoate). Subsequently, fresh BE-medium cultures were inoculated just before the former batch reached stationary phase (every 8 h) by diluting the former 10⁻³–10⁻⁴, equivalent to about 10⁶–10⁷ cells at the beginning of each batch. The experiment was stopped when no TOL⁺ cells could be detected.

Assay for TOL function loss. At regular intervals, samples were withdrawn from batch or phauxostat cultures, diluted and plated onto peptone agar. After 1 or 2 d growth at 30 °C, between 200 and 2000 colonies were screened for the presence of the plasmid-encoded enzyme catechol 2,3-dioxygenase by spraying the agar plates with a 250 mM-catechol solution. The positive colonies turned yellow almost instantly due to the conversion of catechol to 2-hydroxymuconic semialdehyde (Wigmore *et al.*, 1974). This indirect screening method was checked by testing a number of white and yellow colonies for their growth on agar plates with *m*-toluate or succinate as the sole C-source.

Quantification from population dynamics of the growth-rate advantage of TOL⁻ cells. The basic equations for competition in continuous culture at a dilution rate *D* between two strains A and B present at population fractions *p*_A and *p*_B, and with growth rates *μ*_A and *μ*_B were formulated by Cooper *et al.* (1987).

$$\left(\frac{dp_A}{dt}\right) = (\mu_A - D)p_A \quad (1)$$

$$\left(\frac{dp_B}{dt}\right) = (\mu_B - D)p_B \quad (2)$$

$$\mu_A p_A + \mu_B (1 - p_A) = D \quad (3)$$

In phauxostat culture *μ*_A and *μ*_B are considered constant and *D* is variable. Substitution of equation (3) in equation (1) yields

$$\left(\frac{dp_A}{dt}\right) = v(1 - p_A)p_A \text{ with } v = \mu_A - \mu_B \quad (4)$$

Integration with the boundary condition that *p*_A(*t* = 0) = *p*_A⁰ yields

$$p_A(t) = \frac{p_A^0}{p_A^0 + (1 - p_A^0)e^{-vt}} \quad (5)$$

Equation (5) can be used to describe the competition at non-limited rates between a TOL⁺ cell fraction *p*₊ and a TOL⁻ cell fraction *p*₋, with a growth-rate difference *v* = *μ*₋ - *μ*₊ and an initial fraction of the TOL⁻ population *p*₋⁰.

$$p_-(t) = \frac{p_-^0}{p_-^0 + (1 - p_-^0)e^{-vt}} \quad (6)$$

It can be proven that equation (6) is also valid for sequential batch experiments with the condition that the cells remain in the exponential phase throughout the experiment.

Values for *v* and *p*₋⁰ were generated by non-linear least-square fitting of equation (6) to the experimental data for the change of *p*₋ in time, making use of the computer program ENZFITTER (Elsevier Biosoft). In the fitting procedure the datapoints were weighted equally but datapoints with values for *p*₋ of less than 3% and more than 97% were ignored. The resulting value for *p*₋⁰ is arbitrary as the contribution of segregation in the initial phase is not taken into account.

Estimation of segregation rate. When R is defined as the fraction of the strain B population that is transformed to strain A cells per hour, the rate of formation of strain A cells from strain B cells is given by

$$\left(\frac{dp_A}{dt}\right) = Rp_B \quad (7)$$

The total rate of increase of p_A is obtained by summation of equations (1) and (7).

$$\left(\frac{dp_A}{dt}\right) = Rp_B + (\mu_A - D)p_A \quad (8)$$

When $p_A \ll 1$, p_B can be approximated by 1, and in the case of a phauxostat culture, $(\mu_A - D)$ can be approximated by v ($= \mu_A - \mu_B$). This yields

$$\left(\frac{dp_A}{dt}\right) = R + vp_A \quad (9)$$

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

$$p_A = \frac{R(e^{vt} - 1)}{v} \quad (10)$$

or

$$R = \frac{vp_A}{e^{vt} - 1} \quad (11)$$

In the present case of formation of TOL⁻ cells from TOL⁺ cells the segregation rate R can be approximated from the first data point with a reliable value for p_- by

$$R = \frac{vp_-}{e^{vt} - 1} \quad (12)$$

Analyses. The optical density of cell cultures at 540 nm (OD_{540}) was measured using a Beckman 40 spectrophotometer. The dry weight was determined according to the method of Herbert *et al.* (1971). Succinate concentrations were determined using an HPLC apparatus (LKB) fitted with a Chromspher C18 reversed phase column (Chrompack), a UV-detector (LKB) set at 210 nm and an C-R3A integrator (Shimadzu). The column temperature was ambient (18–22 °C) and the eluent was 5 mM-H₂SO₄. The concentration of benzoate was determined spectrophotometrically at 225 nm.

Genetic characterization of phenotypical TOL⁻ mutants

TOL⁻ mutants were assayed for the presence of plasmid DNA by one or both of the following methods.

(1) *Plasmid extraction, purification and detection on an agarose gel.* This was done according to the method of Kado & Liu (1981) with the following changes. Cell cultures (1.5 ml) were grown overnight in SE-medium in Eppendorf tubes at 30 °C and subsequently centrifuged and resuspended in 30 µl of water. Cells were lysed by the addition of 30 µl of a solution of 125 mM-Tris, 80 mM-NaOH and 2.5% (w/v) SDS, followed by incubation at 65 °C for 30 min. HindIII restriction analysis was done as described by Wheatcroft & Williams (1981).

(2) *Colony hybridization.* A sample of TOL⁻ colonies (detected by spraying with catechol) were maintained in 8 × 6 arrays on peptone agar plates. After incubation for 18 h at 30 °C the colonies were transferred to a Biotyne nylon filter membrane (Pall Ultrafine Filtration Corp.) using a replicating device. The membrane was laid onto filter paper (Whatman 3MM) soaked in 10% (w/v) SDS for 5 min to lyse the cells, followed by 0.5 M-NaOH/1.5 M-NaCl (5 min) and 3 M-Tris/1.5 M-NaCl (5 min). The membrane was air-dried and then heated at 80 °C for 2 h. Nick-translation and hybridization were done as described by Keil & Williams (1985), using the HindIII fragment HI from pWW0 cloned into pKT230 as the radiolabelled probe. The HindIII fragment was separated from the vector DNA by agarose gel electrophoresis and extracted according to Girvitz *et al.* (1980).

Statistics. For the average values of the optical density and for the calculated values of the growth-rate difference v , the double standard deviations (2σ) of the individual data are given. For the average dilution rates at the beginning and end of the phauxostat experiments, the double standard error of the mean was calculated [this results in a 95% reliability interval for the mean values of $\bar{x}$ of $(\bar{x} - 1.96\sigma, \bar{x} + 1.96\sigma)$]. For the individual determinations of the fractions of TOL⁺ cells, 95% reliability intervals were calculated and are presented in the graphs. We assumed a binomial distribution, approximated by the normal distribution.

Results

Growth of *P. putida* mt-2 on benzoate in a phauxostat

Non-limited growth of *P. putida* mt-2 on benzoate was achieved through control of the fermenter medium pump by the pH of the culture. In this system (phauxostat) a residual concentration of 4 mM-benzoate (out of 15 mM in the ingoing medium) could be maintained stably throughout the experiment. The biomass remained fairly constant ($OD_{540} = 1.02 \pm 0.05$, corresponding to a dry weight of about 550 mg l⁻¹) indicating a constant growth yield.

During the course of the experiment (120 h) the percentage of the population harbouring the complete TOL plasmid (TOL⁺ population) dropped from 100.0% to below 0.5% (see Fig. 1a). In the period when the percentage of the TOL⁺ population was over 99% (50 h, 33 vessel volume changes) the dilution rate remained constant at 0.68 ± 0.01 h⁻¹. During the stage that the percentage of the TOL population dropped to below 1%, the dilution rate increased and eventually stabilized at 0.79 ± 0.01 h. The cell density remained constant so it may be assumed that this increase in the dilution rate of 0.11 ± 0.02 h⁻¹ is equal to the growth-rate advantage v of the TOL⁻ population.

Fitting equation (6) to the data points for the percentage of the TOL⁺ population in the course of the experiment generates a value for v of 0.12 ± 0.02 h⁻¹.

It is not possible to determine low percentages (below 1%) of the TOL⁻ population accurately. Therefore, in the initial stage of the experiment we could not distinguish segregation from the faster growth of already existing TOL⁻ cells by mathematical analysis. If, at the start of the experiment, the fraction of TOL⁻ cells was 0.008%, their faster growth could be solely responsible for the results presented in Fig. 1(a). Assuming, however, that at time $t = 0$ the population consisted solely of the original strain ($p_-^0 = 0$), a maximal value for the segregation rate can be calculated. For this purpose we applied equation (12) to the first data point in which p_- could be determined with reasonable accuracy ($p_- = 0.011$ at $t = 46.5$ h) assuming a constant value for v of 0.11 h⁻¹. This yielded a value for the segregation rate R of 7×10^{-6} h⁻¹.

Fig. 1. Persistence of plasmid PW0 in *P. putida* mt-2 grown at non-limited rate on (a) benzoate in phauxostat culture, (b) benzoate in sequential batch culture and (c) both benzoate and succinate in phauxostat culture. $\circ$, Dilution rate or growth rate (h^{-1}); $\bullet$, fraction p_+ , (error bars indicate 95% reliability intervals); —, best-fit curve of equation (6) (see Methods) to the experimental values of p_+ , generating values for the growth-rate difference v of (a) 0.12 h^{-1} , (b) 0.22 h^{-1} and (c) 0.095 h^{-1} , and for the initial fraction p_0^- of (a) 8.4×10^{-5} , (b) 2.0×10^{-5} and (c) 1.1×10^{-5} .

Growth of *P. putida* mt-2 on benzoate in sequential batch culture

In order to facilitate comparison of our results with previous studies, *P. putida* strain mt-2 was also grown on benzoate in sequential batches. The results, although inherently less accurate, are similar to those of the phauxostat system with respect to the offset of significant ingrowth of a TOL⁻ population and the growth-rate difference derived from equation (6) ($v = 0.22 \pm 0.08 \text{ h}^{-1}$). This value for v is in agreement with the growth rate increase of 0.2 h^{-1} found in the subsequent batches upon ingrowth of the TOL⁻ population (from

0.75 to 0.96 h^{-1} ; see Fig. 1b). The value for the segregation rate R derived from the batch experiments ($4 \times 10^{-6} \text{ h}^{-1}$) is of the same order of magnitude as the value for R derived from the phauxostat experiment.

Growth of *P. putida* mt-2 on a mixture of benzoate and succinate in a phauxostat

Experiments in the phauxostat similar to those described above were done, but with a growth medium containing both succinate and benzoate as C-sources (SBE-medium). The culture consumed approximately 4 mM -benzoate and 6 mM -succinate simultaneously while the residual concentrations were 6 mM and 9 mM respectively. The dilution rate was initially $0.79 \pm 0.02 \text{ h}^{-1}$ and increased gradually to $0.98 \pm 0.02 \text{ h}^{-1}$ after the TOL⁺ population was replaced almost completely by a TOL⁻ population (see Fig. 1c). Thus the value for v determined from the dilution rates was 0.19 h^{-1} , while the value for v derived by fitting equation (6) to the data points was 0.10 h^{-1} . The latter value, however, is not very reliable due to a smaller data set. The (maximal) segregation rate was calculated to be $5 \times 10^{-8} \text{ h}^{-1}$. The OD_{540} in this experiment was 0.80 .

Reproducibility of phauxostat results

The experiments with benzoate as sole C-source were reproducible. Two duplicate experiments (data not given) gave values for v and R that were very similar to the experiment presented in this paper. These data were less accurate mainly due to a lower frequency of sampling and therefore they are not presented.

The phauxostat experiments with a mixture of benzoate and succinate, however, showed less consistency. In particular, the time at which the TOL⁻ population became predominant varied in repeat experiments. In all repeat experiments, however, the TOL⁻ population eventually took over at a rate that was comparable to the experiment presented here in detail.

Genetic characterization of TOL⁻ mutants

All TOL⁻ mutants appeared to contain 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 (Meulien *et al.*, 1981). Neither during the pHauxostat experiment (48 colonies tested) nor during the two sequential batch experiments (more than 100 colonies tested) were plasmid-free mutants found.

This is in agreement with previous benzoate curing experiments in batch culture where a vast majority of the TOL⁻ cells contained a partially deleted plasmid (Williams *et al.*, 1988).

Discussion

The use of a phauxostat was shown to be a reliable way to grow *P. putida* mt-2 on benzoate at a non-limited growth rate for a prolonged period of time. Starting with a TOL⁺ population, the dilution rate increased during the course of the experiment concomitantly with the gradual takeover by a TOL⁻ population. The growth-rate difference derived from the difference in the initial and final average dilution rates (0.11 h⁻¹) is in good agreement with the growth-rate difference derived from the kinetics of ingrowth of the TOL⁻ population (0.12 h⁻¹). This represents a 15% faster growth rate of the TOL⁻ population compared to the TOL⁺ population.

From the stage in the phauxostat experiment in which the culture contained maximally 2% of TOL⁻ cells the segregation rate was estimated to be 7×10^{-6} h⁻¹. This value may be too low in the case that segregants may also appear that do not have a growth-rate advantage. More probable, however, is that this value is an overestimation because of the assumption in the calculations that there were no TOL⁻ cells at the start of the experiment. In any case it indicates that the segregation rate with benzoate as a C-source is not abnormally high when compared to the segregation rate observed for the same organism with succinate as C-source (see accompanying paper: Duetz & van An del, 1991), or compared to other values for segregation rates of relatively stable plasmids (e.g. Cooper *et al.*, 1987).

We calculated the relative contribution of the growth-rate difference and the rate of segregation to the loss of the TOL catabolic function in phauxostat culture for different fractions (p_-) of the TOL⁻ population (see Table 2). The table shows that after p_- surpasses 0.01%, the growth-rate difference is the predominant factor in the gradual takeover of the culture by the TOL⁻ population. Furthermore, these calculations demonstrate that it is not necessary to include the contribution of segregation in the applied method for deriving a value for v from the kinetics of increase of the TOL⁻ population from 3% to 97%. The contribution of segregation to the increase in the TOL⁻ population is less than 1% during this stage. The values found for the growth-rate difference and the segregation rate are not significantly different when determined in sequential batch cultures. The higher growth rates found in sequential batch culture may be caused partially by a lower average cell density and a higher temperature (30 °C versus 28 °C).

The rationale behind the growth-rate disadvantage of the TOL⁺ cells remains unclear. A similar growth-rate difference was observed when succinate and benzoate were present simultaneously (Fig. 1c), while almost no growth-rate difference was observed with succinate

Table 2. Relative contribution of segregation and growth-rate difference to the disappearance of the TOL⁺ population of *P. putida* mt-2 during non-limited growth on benzoate, for different values of p_-

For the calculations we used equations (4) and (7) (see Methods) and the values for R and v derived from the pHauxostat experiment shown in Fig. 1(a).

p_-	Dp_-/dt (h ⁻¹)		Relative contribution (%)	
	Segregation rate	Growth-rate difference	Segregation rate	Growth-rate difference
10 ⁻⁶	7.00×10^{-6}	1.07×10^{-7}	98.49	1.51
10 ⁻⁴	7.00×10^{-6}	1.07×10^{-5}	39.55	60.45
10 ⁻²	6.93×10^{-6}	1.06×10^{-3}	0.65	99.35
0.1	6.30×10^{-6}	9.63×10^{-3}	0.07	99.93
0.5	3.50×10^{-6}	2.67×10^{-2}	0.01	99.99

alone (for the latter results see the accompanying paper: Duetz & van An del, 1991). As benzoate was not necessary to sustain growth, this suggests that the previously proposed greater kinetic efficiency of the *ortho*-pathway (e.g. Williams *et al.*, 1988) cannot be solely responsible for the lower growth rate of the TOL⁺ population.

The growth rate of TOL⁺ cells when both benzoate and succinate are present simultaneously is lower than when succinate is the sole C-source. The growth rate of TOL⁻ cells on succinate, however, is unchanged by the additional presence of benzoate. These results indicate that benzoate is causing an inhibitory effect specific to TOL⁺ cells. This has been proposed previously by Stephens & Dalton (1987) who found that benzoate had a strong inhibitory effect when *P. putida* MT15 was grown on gluconate. However, Stephens & Dalton (1987) stated that the subsequent increase in the fraction of TOL⁻ cells was too rapid to be explained simply by the growth-rate difference. An additional explanation, they assumed, was that benzoate induced segregational instability of the plasmid. According to our calculations, however, the reported lag phase of about 20 generations before a significant TOL⁻ population is detectable rules out the possibility that segregation makes an important contribution to the disappearance of TOL⁺ cells. If – under some conditions – the decrease in the fraction of TOL⁺ cells is indeed too fast to be explained by the growth-rate difference (also observed by us at low percentages of TOL⁺ cells, see Fig. 1a and 1c), the cause may be a difference in viability of the two populations on the agar test plates.

It is improbable that the inhibitory effect of benzoate on TOL⁺ cells is caused only by the extra energy expenditure on the expression of enzymes of the *meta*-pathway. An alternative possibility would be that an

accumulating *meta*-pathway intermediate, such as 2-hydroxymuconic semialdehyde (HMS), is inhibitory. It was found (data not shown) that during growth on benzoate extracellular concentrations of 0.2 mM-HMS may occur. It is possible that the intracellular concentrations are even higher. The accumulation of HMS is caused by the very high specific activities of benzoate oxidase and catechol 2,3-dioxygenase for benzoate and catechol respectively (Murray *et al.*, 1972).

Further research needs to be done in order to quantify the energetic burden that TOL plasmids put on their host and to identify possible specific inhibitory effects of pathway intermediates.

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