Degradation of 2-methylaniline and chlorinated isomers of 2-methylaniline by *Rhodococcus rhodochrous* strain CTM

KLAUS FUCHS,[†] ANGELIKA SCHREINER and FRANZ LINGENS*

Institut für Mikrobiologie, Universität Hohenheim, Garbenstraße 30, D-7000 Stuttgart 70, FRG

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Rhodococcus rhodochrous strain CTM co-metabolized 2-methylaniline and some of its chlorinated isomers in the presence of ethanol as additional carbon source. Degradation of 2-methylaniline proceeded via 3-methylcatechol, which was metabolized mainly by *meta*-cleavage. In the case of 3-chloro-2-methylaniline, however, only a small proportion (about 10%) was subjected to *meta*-cleavage; the chlorinated *meta*-cleavage product was accumulated in the culture fluid as a dead-end metabolite. In contrast, 4-chloro-2-methylaniline was degraded via *ortho*-cleavage exclusively. Enzyme assays showed the presence of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase as inducible enzymes in strain CTM. Extended cultivation of strain CTM with 2-methylaniline and 3-chloro-2-methylaniline yielded mutants, including *R. rhodochrous* strain CTM2, that had lost catechol 2,3-dioxygenase activity; these mutants degraded the aromatic amines exclusively via the *ortho*-cleavage pathway. DNA hybridization experiments using a gene probe revealed the loss of the catechol 2,3-dioxygenase gene from strain CTM2.

Introduction

Because of their high lipophilic character and their ability to interact with DNA (Gorrod & Manson, 1986; Parris, 1980) anilines containing a methyl- and a chlorosubstituent are hazardous compounds. Therefore investigations on their microbial degradation are of special interest. Some bacterial strains that degrade methylanilines completely have been described (Appel *et al.*, 1984; Raabe *et al.*, 1984; McClure & Venables, 1986). The presence of a chlorosubstituent on the aromatic ring, however, often causes problems in the microbial degradation of these compounds (Corke *et al.*, 1979; Iwan *et al.*, 1976; Parris, 1980; Surovtseva & Volnova, 1981). In most of these cases degradation stopped after formation of chlorocatechols or chlorolactones.

In this paper we present data on the metabolism of 2-methylaniline and some of its chlorinated isomers by *Rhodococcus rhodochrous*. To our knowledge this is the first report on the metabolism of chlorinated methylanilines in micro-organisms.

Methods

Chemicals. 2-Methylaniline, 3-chloro- and 4-chloro-2-methylaniline were from Riedel de Haen. Methylcatechols were purchased from EGA Chemie. 5-Chloro-3-methylcatechol was synthesized according to Gaunt & Evans (1971). 3-Chloro- and 4-chlorocatechol, as well as 3,5-dichloro- and 3,6-dichlorocatechol were a generous gift of Professor Dr W. Reineke (Gesamthochschule Wuppertal, FRG).

Isolation and growth of bacteria. *Rhodococcus rhodochrous* strain CTM was isolated from garden soil with 2-methylaniline as sole carbon source. During extended cultivation of strain CTM with 2-methylaniline a stable mutant, strain CTM2, could be isolated. CTM2 no longer coloured the medium greenish-yellow during the degradation of 2-methylaniline. Cells were grown at 28 °C at 130 r.p.m. on a rotary shaker. The medium used was as described by Dorn & Knackmuss (1978b) with the following modification: instead of 10 mg ferric ammonium citrate l⁻¹, 8 mg ferrous ammonium sulphate l⁻¹ was added. The medium was supplemented with 1 ml l⁻¹ of a trace element solution according to Pfennig & Lippert (1966). Aromatic amines were added as a 0.2% (v/v) ethanolic stock solution. For experiments with resting cells, bacteria were grown in minimal medium supplemented with 0.5% (w/v) sorbitol. After 2 d the washed cell pellet was resuspended in fresh medium (1/10th the original culture volume) containing 1 mM of the appropriate aromatic amine. The methods for large-scale growth of induced biomass and preparation of crude extracts are described in the following paper by Schreiner *et al.* (1991).

Analytical methods. Degradation products were detected by TLC using toluene/dioxane/acetic acid (72:16:1.6, by vol.) as solvent system. Degradation products were extracted with ethyl acetate from the culture supernatant previously acidified to pH 2 using H₃PO₄. For

[†] Present address: Kronwald Separationstechnik, 6920 Sinsheim, FRG.

Abbreviations: C23O, catechol 2,3-dioxygenase; C12O, catechol 1,2-dioxygenase.

isolation of *meta*-cleavage products, the concentrated ethyl acetate layer was passed through an Adsorbex RP 18 column in order to retain catechol compounds. Compounds separated by preparative TLC were scraped from the silica gel plate and extracted with ethyl acetate or diethyl ether.

The metabolites were identified by coupled gas chromatography/mass spectrometry (GC, Packard model 427 with flame ionization detector and SE30 fused silica capillary column; MS, Varian MAT 311, 70 eV). The oven temperature was raised continuously from 80–200 °C at a rate of 10 °C min⁻¹. Helium was the carrier gas. Hydroxylated and carboxylated compounds were derivatized using diazomethane.

Liberation of Cl⁻ was assayed with a Marius 'Chlor-o-counter', previously calibrated with NaCl. For qualitative identification of halogen substituents on TLC plates AgNO₃/H₂O₂ reagent (Stahl, 1967) and the Beilstein test (Organikum, 1977) were used. The iodoform test was used to detect the presence of the CH₃-CO group (Organikum, 1977). Catechol compounds were assayed as described by Raabe *et al.* (1984). Spraying reagents were prepared as described by Stahl (1967).

Enzyme assays. Catechol 2,3-dioxygenase (C23O) activity was assayed as described by Schreiner *et al.* (1991). Catechol 1,2-dioxygenase (C12O) activity was assayed in 20 mM-potassium phosphate buffer, pH 7.5, by monitoring the formation of muconates. The reaction mixture (final vol. 1 ml) contained an appropriate amount of protein and 300 µmol of substrate. Extinction coefficients for muconates were those described by Dorn & Knackmuss (1978a). When C23O and C12O – which both converted methylcatechols – were present together in an extract, the activity of either enzyme could be determined by taking advantage of the fact that C23O is totally inhibited in the presence of 0.2 mM-Fe³⁺. One unit (U) of enzyme (C12O or C23O) is defined as the amount of enzyme converting 1 µmol substrate min⁻¹.

Protein concentration was measured according to Bradford (1976). The presence of the β -ketoacid pathway was demonstrated qualitatively by the Rothera reaction (Ottow & Zolg, 1974). Phenol hydroxylase was assayed according to Straube (1987).

Gel electrophoresis. This was done as described by Schreiner *et al.* (1991).

Experiments generating the CTM2 phenotype. The influence of aniline derivatives on the loss of C23O activity was tested as follows. Strain CTM was cultivated at 28 °C for 5 d in 25 ml of minimal medium with ethanol (0.2%) as carbon source and one of the following aromatic substrates: aniline, 1.0 mM; 3-chloro- and 4-chloro-2-methylaniline, 0.5 mM; 2-methylaniline, 1.0 mM; 4-chloro-2-methylphenol, 0.25 mM; and benzoate, 2.0 mM. At intervals of 5 d an aliquot (1 ml) from each culture flask was transferred to fresh medium. In this way, strain CTM was cultivated for 10 weeks. Then an aliquot from each flask was plated on minimal agar plates containing sorbitol as carbon source. A total of 500 colonies from every assay were tested on titre plates for *meta*-cleavage activity: 24-well Costar plates were used and each well was filled with 1 ml of minimal medium containing 0.5 mM-3-chloro-2-methylaniline. The formation of the yellow-green dead-end metabolite 2-hydroxy-5-chloro-6-oxoheptadienoic acid indicated *meta*-cleavage activity.

Reversion test. Strain CTM2 was cultivated on minimal medium containing either 2-methylaniline or sorbitol. An aliquot from each culture flask was plated on minimal agar plates containing sorbitol. A total of 2000 colonies from each culture were examined for *meta*-cleavage activity, as described above.

DNA isolation and hybridization. These were done as described by Schreiner *et al.* (1991).

Fig. 1. Proposed pathway for the degradation of 2-methylaniline, 3-chloro- and 4-chloro-2-methylaniline by *R. rhodochrous* strains CTM and CTM2. Compounds are as follows: 1, 2-methylaniline; 2, 3-chloro-2-methylaniline; 3, 4-chloro-2-methylaniline; 4, 3-methylcatechol; 5, 4-chloro-3-methylcatechol; 6, 5-chloro-3-methylcatechol; 7, 2-hydroxy-6-oxoheptadienoic acid; 8, 2-methylmuconic acid; 9, 2-hydroxy-5-chloro-6-oxoheptadienoic acid; 10, 3- or 4-chloro-2-methylmuconic acid. Compounds 4 to 9 were identified as degradation products. —→, pathway used by *R. rhodochrous* strains CTM and CTM2; ---→, degradation steps used by *R. rhodochrous* strain CTM only.

Results

Growth on methylanilines in the presence and absence of ethanol

R. rhodochrous strain CTM was isolated from garden soil with 2-methylaniline (compound 1, Fig. 1) as the elective carbon source. During degradation studies the spontaneous mutant strain CTM2 was detected by its inability to colour the methylaniline growth medium. The mutant strain CTM2 proved stable, no revertants to the phenotype of strain CTM being found. Both strains showed only weak growth on solid and in fluid media containing one of the methylanilines as sole carbon and nitrogen source. Degradation proceeded very slowly and did not exceed 85% within 3 months. However, the presence of ethanol as additional carbon source led to better growth of the bacteria and to total breakdown of the aromatic amines within 6–8 d. Therefore all degradation studies were done in the presence of ethanol. The optimal substrate concentrations of 2-methylaniline were

Table 1. Mass spectrometric data of isolated metabolites

Degradation substrate	Isolated compound/identified as	Major fragment ions (<i>m/z</i> ; percentage relative intensity)
2-Methylaniline	3-Methylcatechol/3-methylveratrole*	M^+ (152; 100%); $M^+ - \text{CH}_3$ (137; 60%); $M^+ - \text{COCH}_3$ (109; 53%); C_6H_5^+ (77; 71%)
2-Methylaniline	2-Methylmuconic acid/2-methylmuconic acid dimethylester*	M^+ (184; 0.7%); $M^+ - \text{CH}_3$ (169; 1.4%); $M^+ - \text{OCH}_3$ (153; 2.9%); $M^+ - \text{COOCH}_3$ (125; 100%)
2-Methylaniline	2-Hydroxy-6-oxohepta-2,4-dienoic acid/respective methylester	M^+ (184; 3%); $M^+ - \text{CH}_3$ (169; 3%); $M^+ - \text{CH}_3\text{CO}$ (141; 18%); $M^+ - \text{COOCH}_3$ (125; 100%); $M^+ - \text{CH}_3\text{CO}, -\text{COOCH}_3$ (82; 13%)
3-Chloro-2-methylaniline	4-Chloro-3-methylcatechol/4-chloro-3-methylveratrole	M^+ (186†; 92%); $M^+ - \text{CH}_3$ (171†; 69%); $M^+ - \text{CH}_3\text{CO}, -\text{Cl}$ (108; 100%); C_6H_5^+ (77; 75%)
3-Chloro-2-methylaniline	2-Hydroxy-5-chloro-6-oxoheptadienoic acid/respective methylester	M^+ (218†; 2%); $M^+ - \text{CH}_3\text{CO}$ (175†; 4%); $M^+ - \text{COOCH}_3$ (159†; 100%); $M^+ - \text{CH}_3\text{CO}, -\text{CH}_3\text{O}$ (144†; 8%); $M^+ - \text{COOCH}_3, -\text{Cl}$ (123; 1%); $M^+ - \text{CH}_3\text{CO}, -\text{COOCH}_3$ (116†; 10%)
4-Chloro-2-methylaniline	4-Chloro-2-methylphenol/4-chloro-2-methylphenol*	M^+ (142†; 30%); $M^+ - \text{H}_2\text{O}$ (124†; 2%); $M^+ - \text{Cl}$ (107; 86%); C_6H_5^+ (77; 53%)
4-Chloro-2-methylaniline	5-Chloro-3-methylcatechol/5-chloro-3-methylveratrole*	M^+ (186†; 94%); $M^+ - \text{CH}_3$ (171†; 68%); $M^+ - \text{OCH}_3, -\text{OCH}_3$ (125†; 26%); $M^+ - \text{COCH}_3, -\text{Cl}$ (108; 100%); C_6H_5^+ (77; 75%)

* Authentic compounds available for comparison.

† With chloro pattern.

0.5–1.0 mM and 0.1–0.5 mM for the chloromethylanilines for both strains. Mutant strain CTM2 possessed a slightly higher tolerance to the chloromethylanilines (up to 1.0 mM), but degradation of the aromatic amines took the same time as in strain CTM.

Degradation of 2-methylaniline and chlorinated isomers in wild-type strain CTM

(i) *2-Methylaniline*. The first compound isolated from growth cultures or from cultures of resting cells showed a TLC R_F value of 0.48, identical with the R_F value of authentic 3-methylcatechol. Both spots turned orange-red on spraying the thin-layer plate with nitrite/molybdate reagent. In GC/MS the methylated compound yielded a mass spectral fragmentation pattern identical with authentic 3-methylveratrole (Table 1). When 3-methylcatechol was added to crude extracts of cells grown on 2-methylaniline it was converted to a greenish-yellow product. During degradation of 2-methylaniline the medium also turned greenish-yellow 38 h after inoculation and retained this colour for about 12 h; thereafter it became colourless. The yellow compound isolated from the greenish-yellow culture fluid had an absorption maximum at 387 nm at pH 7 and pH 12. At pH 2 the maximum shifted to 320 nm. These UV data were identical with those described for 2-hydroxy-6-oxoheptadienoic acid (compound 7, Fig. 1) by Catelani *et al.* (1968). The iodoform test was positive, indicating the presence of the $\text{CH}_3\text{—CO}$ group. The methylated substance had a GC retention time of 4.6 min, and mass spectral data could be attributed to 2-hydroxy-6-oxo-

hepta-2,4-dienoic acid methylester (Table 1). From the culture supernatant of resting cells of strain CTM 2-methylmuconic acid (compound 8, Fig. 1) could be isolated. The methylated metabolite showed a mass spectral fragmentation pattern identical with 2-methylmuconic dimethylester (Table 1), prepared by methylation of authentic 2-methylmuconic acid with diazomethane. 2-Methylmuconic acid was metabolized by crude extracts prepared from 2-methylaniline-induced cells of strain CTM, but not by extracts from uninduced cells.

(ii) *3-Chloro-2-methylaniline*. Strain CTM, when grown on a medium containing 1 mM-3-chloro-2-methylaniline, accumulated a brownish compound. Reaction with nitrite/molybdate reagent identified the compound as a catechol derivative and with the Beilstein test the presence of halogen was detected. The chlorocatechol had an R_F value of 0.48 on TLC, and the methylated compound had a GC retention time of 6.1 min. The mass spectrum (Table 1) was nearly identical to that of synthesized 5-chloro-3-methylveratrole, which had a different GC retention time (6.8 min). Therefore, we propose that the compound isolated is identical with the isomer 4-chloro-3-methylcatechol (compound 5, Fig. 1). Crude extracts of cells induced on 3-chloro-2-methylaniline transformed this chloromethylcatechol to a greenish-yellow product. This compound was also found to accumulate in the culture fluid during 3-chloro-2-methylaniline degradation. The isolated compound exhibited a UV spectrum with absorption maxima at 390 nm (pH 7.5) and 325 nm (pH 2.0), shifting back to 390 nm at pH 10 due to keto–enol tautomerism. These characteristics and the mass spectral data (Table 1) led us

to postulate that the isolated substance is identical with 2-hydroxy-5-chloro-6-oxoheptadienoic acid (compound 9, Fig. 1).

The amount of chlorinated heptadienoic acid formed was estimated with the aid of the molar absorption coefficient of the structural analogue 2-hydroxy-6-oxoheptadienoic acid (compound 7, Fig. 1). During degradation of 3-chloro-2-methylaniline samples were taken from the culture fluid and the amount of *meta*-cleavage product was estimated. The *meta*-cleavage product increased continuously up to 0.05 mM during breakdown of 0.5 mM-3-chloro-2-methylaniline. This amount remained constant during the stationary growth phase, indicating the transformation of about 10% of the original substrate to a dead-end metabolite, whereas 90% of the original substrate must have been channelled into the *ortho*-cleavage pathway. This was confirmed by a Cl^- liberation of about 90% and a positive Rothera reaction in crude extracts.

(iii) *4-Chloro-2-methylaniline*. During degradation of this compound (3, Fig. 1) no *meta*-cleavage product was detectable in culture fluids; instead two compounds could be isolated from ethyl acetate extracts of culture supernatant. The first compound (GC retention time 5.6 min) showed a mass spectrum identical with authentic 4-chloro-2-methylphenol (Table 1); the second (R_F value 0.56 on TLC; UV maxima at 283 nm and 213 nm) was identified as 5-chloro-3-methylcatechol (compound 6, Fig. 1). This latter compound (GC retention time 6.6 min) generated a mass spectrum identical with authentic 5-chloro-3-methylveratrole (Table 1). Crude extracts prepared from cells grown on 4-chloro-2-methylaniline converted 5-chloro-3-methylcatechol to colourless products. About 96% of the theoretical amount of Cl^- was released. This fact, together with a positive Rothera reaction, suggests degradation of the catechol via the *ortho*-cleavage pathway. Phenol hydroxylase activity, converting 4-chloro-2-methylphenol to 5-chloro-3-methylcatechol, could not be demonstrated. Therefore we assume that 4-chloro-2-methylphenol is not a degradation product of 4-chloro-2-methylaniline but rather an artefactual product formed by dehydration of the corresponding dihydrodiol during the acid-extraction procedure.

Degradation of 2-methylaniline and chlorinated isomers by mutant strain CTM2

During degradation of 2-methylaniline, 3-chloro- and 4-chloro-2-methylaniline the culture fluid did not become greenish-yellow. Crude extracts obtained from each culture converted the corresponding catechols to colourless products which gave a positive Rothera reaction. In the case of 3-chloro-2-methylaniline 96.5% and in the

case of 4-chloro-2-methylaniline 96% of the theoretical amount of Cl^- was released during degradation, suggesting breakdown exclusively via *ortho*-cleavage. From cultures of resting cells, induced with 2-methylaniline, 2-methylmuconic acid (compound 8, Fig. 1) could be isolated and identified. From culture fluids of cells grown on 4-chloro-2-methylaniline, 5-chloro-3-methylcatechol (compound 6, Fig. 1) was isolated and identified.

Induction of catechol dioxygenases in strains CTM and CTM2

Crude extracts of cells grown in the presence and absence of aromatic amines were assayed for *meta*- and *ortho*-cleaving dioxygenases (Table 2). Dioxygenase activity was only detectable in crude extracts of cells grown in the presence of aromatic amines. C23O, the *meta*-cleaving enzyme was not detectable in strain CTM2.

Substrate range of the C12O of mutant strain CTM2

Cells of strain CTM2 grown on 4-chloro-2-methylaniline yielded crude extracts which converted the following catechols (relative activity in %): catechol (100%); 3-chlorocatechol (155%); 4-chlorocatechol (32%); 3-methylcatechol (36%); 4-methylcatechol (18%); 5-chloro-3-methylcatechol (159%); 3,5-dichlorocatechol (86%); 3,6-dichlorocatechol (150%); tetrachlorocatechol (0%). [100% was 0.7 mU (mg crude extract)⁻¹.] A positive Rothera reaction, evidence for the presence of end products of the *ortho*-cleavage pathway, indicated an intact degradative sequence after muconic acid derivatives.

Experiments generating the CTM2 phenotype

Repeated transfers of cells of strain CTM grown on aromatic amines gave rise to cells of the CTM2 phenotype. Especially with 2-methylaniline and with 3-chloro-2-methylaniline as growth substrates, a nearly complete loss of the *meta*-cleaving enzyme, as shown by a colourless test medium, was observed after ten transfers (Table 3).

Genetics of spontaneous C23O⁻ mutants

From the culture with 2-methylaniline, we selected one mutant (strain CTM2) to investigate the genetic background of the C23O⁻ phenotype.

R. rhodochrous was found to harbour two plasmids, pTC1 and pTC2 (see following paper; Schreiner *et al.*, 1991). Plasmid DNA and total DNA from the wild-type

Table 2. Induction of catechol dioxygenases tested with 3-methylcatechol as substrate in *R. rhodochrous* strains CTM and CTM2

Specific activities of the *meta*-cleaving enzyme C23O and the *ortho*-cleaving enzyme C12O in crude extracts of strains CTM and CTM2 grown on different media are shown. Enzymes are assayed as described in Methods; activities are given in mU (mg crude extract protein)⁻¹.

Growth medium	Strain CTM		Strain CTM2	
	C23O	C12O	C23O	C12O
2-Methylaniline + ethanol	12.5	0.55	0	1.7
3-Chloro-2-methylaniline + ethanol	6.07	0.77	0	2.1
4-Chloro-2-methylaniline + ethanol	6.4	0.6	0	1.0
Ethanol	0	0	0	0
Nutrient broth	0	0	0	0

Table 3. Effect of different aromatic amines on the loss of *meta*-cleavage activity

From each transfer 500 colonies (= 100%) were tested as described in Methods. UV spectra of the yellow media showed maxima at 390 nm (pH 7.5) and 325 nm (pH 2.0), identical with the UV data of 2-hydroxy-5-chloro-6-oxoheptadienoic acid.

Growth medium	1st transfer		10th transfer		
	% Yellow-green	% Colourless (C23O ⁻)	% Yellow-green	% Colourless	% Brownish*
Aniline	100	0	29	11	60
2-Methylaniline	99.6	0.4	4.2	95.8	0
3-Chloro-2-methylaniline	100	0	0	100	0
4-Chloro-2-methylaniline	100	0	93.8	6.2	0
5-Chloro-2-methylaniline	100	0	94.2	5.8	0
4-Chloro-2-methylphenol	100	0	99.2	0.8	0
Benzoate	99.6	0.4	99.8	0.2	0
Ethanol	100	0	99.8	0.2	0

* Brownish colourization of the medium occurred due to the autoxidation of accumulated 4-chloro-3-methylcatechol.

strain CTM and the mutant strain CTM2 were isolated. Restriction enzyme analysis revealed the deletion of a continuous DNA region of about 27 kb in plasmid pTC1. After digestion of plasmid and total DNA with *Bgl*II and size fractionation by gel electrophoresis, the blotted DNA was probed with a radiolabelled oligonucleotide obtained from the sequence of C23OI. A strong signal was obtained from a 3.5 kb fragment of the plasmid DNA and total DNA of strain CTM, but plasmid DNA and total DNA from the mutant strain CTM2 gave no signal on the autoradiogram. Restriction enzyme analysis showed that the same 3.5 kb *Bgl*II restriction fragment was absent. Therefore, we conclude that the C23OI gene is completely lost in mutant strain CTM2. On the same restriction fragment a second catechol 2,3-dioxygenase (C23OII) was found during cloning experiments (Schreiner *et al.*, 1991). Obviously, the C23OII gene was lost in mutant strain CTM2, too. To find out whether mutants selected by repeated transfer on various methylanilines (see above) had lost only one specific part of the plasmid, we isolated plasmid DNA from a further

30 randomly chosen colonies. Restriction enzyme analysis and probing of *Bgl*II-restricted DNA with the radio-labelled oligonucleotide showed that the genetic material of colonies devoid of C23OI activity possessed deletions in the 3.5 kb *Bgl*II restriction fragment and additionally in other plasmid DNA regions. Unspecific deletions in the plasmid DNA, however, were also observed in colonies which still expressed C23OI activity. Obviously, cultivation on methylanilines caused an unspecific loss of DNA regions.

Discussion

The wild-type strain *R. rhodochrous* CTM, as well as its mutant strain CTM2, was able to grow with 2-methylaniline, 3-chloro- and 4-chloro-2-methylaniline as sole sources of carbon and nitrogen.

Weak growth on solid and in liquid media, however, suggested difficulties in the metabolism of these compounds. This was probably due to an insufficient amount

of energy or reduced cofactors for the initial dioxygenase reaction, as stated by D. Janke and co-workers (Janke & Fritsche, 1985; Janke *et al.*, 1988) when discussing chloroaniline co-metabolism by *Rhodococcus* AN117.

The degradation of the methylanilines proceeded via the corresponding catechols in both *R. rhodochrous* strains, suggesting a common degradative base branch, including oxidative deamination as described for aniline metabolism by *Nocardia* (Bachofer & Lingens, 1983). 2-Methylaniline was metabolized mainly by *meta*-cleavage, whereas a positive Rothera reaction together with a nearly quantitative release of Cl^- during 3-chloro- and 4-chloro-2-methylaniline degradation indicated the presence of an active *ortho*-cleavage pathway in strain CTM. Therefore we propose a degradation pathway for the chloromethylanilines similar to that described for the metabolism of 4-chloro-2-methylphenoxyacetate and *p*-chlorotoluene (Gaunt & Evans, 1971; Haigler & Spain, 1989).

The difference between the wild-type strain CTM and the mutant CTM2, as described in this paper, lies in the different conversion of 3-methylcatechol. The enzyme C23O catalyses an efficient transformation of 3-methylcatechol in strain CTM, whereas the mutant CTM2 does not possess C23O activity. The loss of the *meta*-cleaving enzyme leads to a phenotype with lower degradation capacity towards 2-methylaniline, but – due to the elimination of a misrouting *meta*-cleavage pathway – to a more efficient degradation of chloromethylcatechols via *ortho*-cleavage.

Long-duration exposure of *R. rhodochrous* wild-type strain CTM to different aniline derivatives generated cells of the CTM2 phenotype with a high frequency. Two aspects may contribute to this phenomenon. (i) The most effective 'curing agents' were 3-chloro-2-methylaniline, 2-methylaniline and aniline. The spontaneous mutation rate of 0.2% indicated that all aromatic amines had a high 'curing' potential (Table 3). These compounds may affect the stability of plasmid pTC1 perhaps by causing unspecific deletions of DNA regions, resulting in the C23O⁻ phenotype. From studies with eukaryotic DNA it is known that *N*-hydroxylation of aromatic amines leads to reactive compounds which can interact with DNA (Gorrod & Manson, 1986; Parris, 1980). A direct influence of a growth substrate on 'curing' of catabolic genes was proposed by Stephens & Dalton (1987, 1988) in the case of 'benzoate-curing' of catabolic genes from TOL plasmids. The authors assumed that benzoate directly influenced the stability of TOL plasmids. Perhaps aromatic amines or their reactive derivatives also directly influence the DNA in *R. rhodochrous*. (ii) Cells that have lost the C23O gene(s) may acquire a growth advantage, as described by Williams *et al.* (1988) for 'benzoate-cured' segregants.

Perhaps both aspects play a role in the 'curing' event exerted by the growth substrate.

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