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# Regulation of Enzymes of the 3,5-Xylenol-Degradative Pathway in *Pseudomonas putida*: Evidence for a Plasmid

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Constitutive synthesis of enzymes responsible for methyl group oxidation in 3,5-xylenol degradation and an associated *p*-cresol methylhydroxylase in *Pseudomonas putida* NCIB 9869 was shown by their retention at high specific activities in cells transferred from 3,5-xylenol medium to glutamate medium. The specific activities of other enzymes of the 3,5-xylenol pathway declined upon removal of aromatic substrate, consistent with their inducible control. Specific activities of the methyl-oxidizing enzymes showed an eventual decline concomitant with a decrease in the fraction of bacteria capable of growth with 3,5-xylenol; a simultaneous loss of the ability to grow with *m*-hydroxybenzoate was also observed. The property of 3,5-xylenol utilization could be transferred to another strain of *P. putida*. It is proposed that enzymes of the 3,5-xylenol pathway and those for conversion of *p*-cresol to *p*-hydroxybenzoate are plasmid encoded, that the early methyl-oxidizing enzymes are expressed constitutively, and that the later enzymes are inducible.

The catabolism of 3,5-xylenol by *Pseudomonas putida* NCIB 9869 is initiated by a methylhydroxylase and proceeds as shown in Fig. 1 (5, 7). The same enzymes will also oxidize the structurally similar compound, *m*-cresol, as far as D-malate by a parallel pathway whose intermediates, including *m*-hydroxybenzylalcohol, *m*-hydroxybenzaldehyde, *m*-hydroxybenzoate, and gentisate, are more readily available and can be used as substrates in enzyme assays. D-Malate is further metabolized by a different route than citramalate (6). However, a different pathway for *m*-cresol metabolism, involving hydroxylation to 3-methylcatechol as the ring fission substrate, is induced when *m*-cresol rather than 3,5-xylenol is used as growth substrate (8).

Besides the 3,5-xylenol methylhydroxylase, which has the properties of a mixed-function oxygenase, bacteria grown on 3,5-xylenol also contain a *p*-cresol methylhydroxylase, which is a flavocytochrome with no direct oxygen requirement and is specific for *para*-alkylphenols (11). Also present are two distinct $NAD^+$-dependent aromatic alcohol dehydrogenases, each of which is active with both *m*- and *p*-hydroxybenzylalcohols (12), and an $NADP^+$-dependent aromatic aldehyde activity with both *m*- and *p*-hydroxybenzaldehydes (10). There is some indication that two aldehyde dehydrogenases are involved, but this has not been firmly established. Therefore 3,5-xylenol-grown bacteria have enzymes for the oxidation of the methyl group of *p*-cresol (Fig. 1), although the physiological role, if any, of this activity is not known because different enzymes catalyzing these reactions were isolated from *p*-cresol-grown bacteria (11, 12). To understand why enzymes for the oxidation of the methyl group of *p*-cresol are present in 3,5-xylenol-grown cells, a study of the control of these and other activities was undertaken.

## MATERIALS AND METHODS

**Growth of bacteria.** Bacteria were grown in Erlenmeyer flasks at 30°C on an orbital shaker (Gallenkamp & Co. Ltd., Widnes, U.K.) at 150 cycles/min. The medium contained (per liter): 4.33 g of $Na_2HPO_4$; 2.65 g of $KH_2PO_4$; 2.0 g of $NH_4Cl$; 0.1 g of nitrilotriacetic acid; 4 ml of salts solution (15); 0.3 g of aromatic carbon sources or 2.0 g of glutamate or succinate. The pH was adjusted to 7.0, using 0.5 M NaOH. The organism was maintained at 4°C on nutrient agar slants after growth at 30°C for 24 h.

**Proportion of 3,5-xylenol positive bacteria.** Samples from cultures were serially diluted in sterile 50 mM $Na_2HPO_4$-$KH_2PO_4$ buffer, pH 7.0, and 0.1-ml portions of the dilutions were spread on nutrient agar plates. After 24 h at 30°C, 100 to 150 colonies were transferred to 3,5-xylenol agar plates, prepared by solidifying liquid medium with 1.5% (wt/vol) agar, and also to nutrient agar plates. Plates were incubated at 30°C, and after 5 days the number of colonies was recorded.

**Preparation of bacterial extracts.** Bacterial suspensions were broken with a Dawe Soniprobe (type 7530A; Dawe Instruments Ltd., London) fitted with a microtip and operated for a total of 4 min in 30-s periods and were centrifuged for 15 min at 25,000 × *g* and 2°C.

**Respirometry.** Respirometric experiments were carried out in a conventional Warburg apparatus with

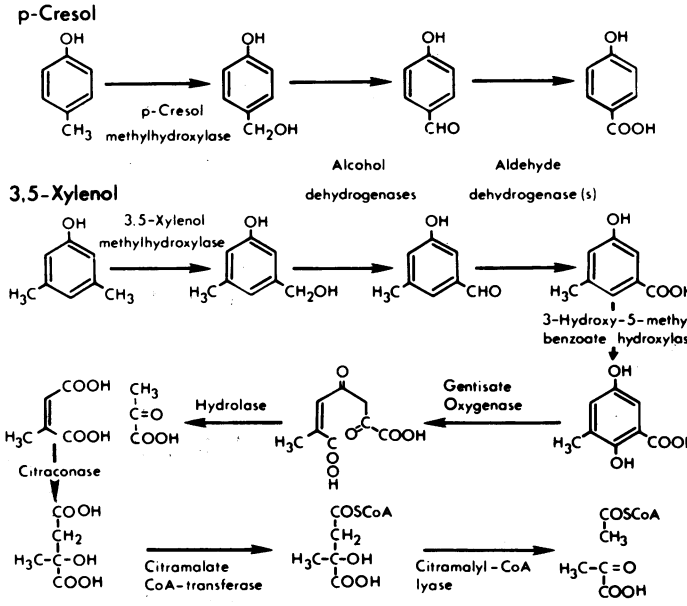


FIG. 1. *Pathway of metabolism of 3,5-xylenol and oxidation of p-cresol by P. putida NCIB 9869.*

flasks containing a suspension of bacteria (5 mg, dry weight) in 2.5 ml of 50 mM $KH_2PO_4$–$Na_2HPO_4$ buffer, pH 7.0, 0.3 ml of 10 mM substrate tipped from the side arm, and 0.2 ml of 20% (wt/vol) KOH in the center well.

**Enzyme assays.** All enzymes were assayed at 30°C. A Gilford 2400S spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio) and 1-cm light path cuvettes were used for spectrophotometric assays.

*p*-Cresol methylhydroxylase was assayed as described by Hopper and Taylor (9), except that 50 mM glycine–NaOH buffer (pH 9.6) was used. The rate of oxidation of 3,5-xylenol by bacterial suspensions (as an indication of 3,5-xylenol methylhydroxylase activity) was measured with a Clark type $O_2$ electrode in a stirred vessel (oxygen monitor model 53; Yellow Springs Instrument Co., Yellow Springs, Ohio). The reaction mixture contained a suspension of bacteria (5 mg, dry weight) in 3 ml of 50 mM $KH_2PO_4$–$Na_2HPO_4$ buffer, pH 7.0, and 5 μmol of 3,5-xylenol. *m*-Hydroxybenzylalcohol dehydrogenase was assayed as described by Keat and Hopper (12). *m*-Hydroxybenzaldehyde dehydrogenase was assayed by measuring the rate of increase in absorbance at 355 nm of a reaction mixture containing in 1 ml of 0.1 M Tris-hydrochloride buffer, pH 8.0, 0.4 μmol of $NADP^+$, extract, and 0.4 μmol of *m*-hydroxybenzaldehyde; transformation of 1 μmol of substrate gave an increase in absorbance of 4.19. 3-Hydroxy-5-methylbenzoate hydroxylase was assayed by measuring the rate of decrease in absorbance at 340 nm due to oxidation of NADH in a cuvette containing 1.2 μmol of NADH, extract, and 10 μmol of *m*-hydroxybenzoate in 3 ml of 50 mM $KH_2PO_4$–$Na_2HPO_4$ buffer, pH 7.5. Gentisate oxygenase was assayed as described by Hopper and Taylor (8). Maleylpyruvate hydrolase was assayed by following the decrease in absorbance at 330 nm of a cuvette containing 0.15 μmol of maleylpyruvate (molar absorbance coefficient, 13,000 [14]) and extract in 1.0 ml of $KH_2PO_4$–$Na_2HPO_4$ buffer, pH 7.4. Citraconase [(−)-citramalate hydro-lyase] was assayed by measuring the rate of decrease in absorbance at 240 nm of a reaction mixture containing 3 μmol of citraconate and extract in 3 ml of 0.1 M $KH_2PO_4$–$Na_2HPO_4$ buffer, pH 7.4. The conversion of citramalate to pyruvate by the citramalate-coenzyme A transferase, citramalyl-coenzyme A lyase system (Fig. 1) was assayed by coupling to lactate dehydrogenase and following the decrease in absorbance at 340 nm due to NADH oxidation. The reaction mixture (total volume, 1.5 ml), containing 1.5 μmol of $MgCl_2$, 1 μmol of succinate, 5 μmol of ATP, and 0.6 μmol of NADH in 0.1 M Tris-hydrochloride buffer, pH 8.0, was gassed for 1 min with $N_2$, and the cuvette was fitted with a rubber cap. Lactate dehydrogenase (1 μl, 1.5 U) and extract were then injected, followed after 2 min by 5 μmol of (−)-citramalate.

**Estimation of protein.** Protein was assayed by the method of Gornall et al. (4), using bovine serum albumin as standard.

**Mating experiment.** The recipient organism, PaW340, was a streptomycin-resistant, $TOL^-$, tryptophan auxotroph derivative of *P. putida* (*arvilla*) mt-2, provided by P. A. Williams, University College of North Wales, Bangor. Donor and recipient bacteria were grown overnight without shaking in 5 ml of nutrient broth at 30°C. About 3 h before the mating 0.5 ml of each culture was transferred to a further 5 ml of nutrient broth which was incubated at 30°C without shaking. To initiate the conjugation 0.5-ml portions of each culture were mixed; after 1.5 h at 30°C, 0.1 ml of the mixture and of $10^{-1}$, $10^{-2}$, and $10^{-3}$ dilutions in sterile 50 mM $KH_2PO_4$–$Na_2HPO_4$ buffer, pH 7.0, were spread onto 3,5-xylenol agar plates containing 1 mg of streptomycin sulfate per ml and 50 μg

of tryptophan per ml, and colonies were counted after 5 days at 30°C. Numbers of recipients and donors in the mating mix were measured by plate counts of serial dilutions on nutrient agar. No colonies were observed after 10 days at 30°C when 0.1-ml amounts of the separate donor and recipient cultures were spread onto selection plates.

**Chemicals.** Maleylpyruvate was prepared enzymatically as described by Lack (13), except that partially purified gentisate oxygenase from *P. putida* NCIB 9869 was used. 3-Hydroxy-5-methylbenzoic acid was prepared by the method of Berner and Laland (2). (−)-Citramalate was prepared by the enzymatic hydration of citraconic acid (7). Citraconate and 3,5-xylenol were from Aldrich Chemical Co., Gillingham, Dorset, U.K.; *p*-cresol, *m*-hydroxybenzaldehyde and *m*-hydroxybenzoic acid were from BDH Ltd., Poole, Dorset, U.K.; *m*-hydroxybenzylalcohol was from Pfaltz and Bauer, Flushing, N.Y.; $NAD^+$, $NADP^+$, and coenzyme A were from Boehringer (London) Ltd., Lewes, East Sussex, U.K.; and NADH was from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

## RESULTS

**Oxidations by intact cells.** Succinate-grown cultures of *P. putida* NCIB 9869 do not oxidize 3,5-xylenol or *p*-cresol if the inoculum has previously been grown for many generations on nutrient agar (11). In contrast, this capability was retained longer than expected when bacteria from a 3,5-xylenol-grown culture were grown on a nutrient agar plate for 24 h at 30°C and then a small inoculum was transferred to liquid medium containing either succinate or glutamate as carbon source. After growth for 24 h, the bacteria were harvested and washed, and their ability to oxidize various compounds was measured in a Warburg apparatus. Both 3,5-xylenol and *p*-cresol were oxidized immediately (1.7 μl of $O_2$ $min^{-1}$ $mg^{-1}$, dry weight) with an uptake of about 1.5 μmol of $O_2$ per μmol of substrate, sufficient for oxidation of a methyl group to carboxyl. This was followed by a period of induction and then a further rapid rate of oxygen uptake.

**Enzyme levels in cell extracts.** The activities of the 3,5-xylenol pathway enzymes and the co-occurring *p*-cresol methylhydroxylase were assayed at intervals after bacteria had been transferred from 3,5-xylenol to glutamate medium (Fig. 2). The enzymes clearly fall into two groups, the methyl-oxidizing enzymes for both 3,5-xylenol and *p*-cresol which are retained at high activities for several days (30 to 40 generations in this experiment) and those enzymes for the subsequent degradation of 3-hydroxy 5-methylbenzoate which, as expected, declined during day 1 of growth after transfer to glutamate medium. Although there is an apparent association of the curve for 3,5-xylenol methylhydroxylase with that for the latter group of enzymes, for reasons discussed later, it is not considered to be one of those activities which declines rapidly.

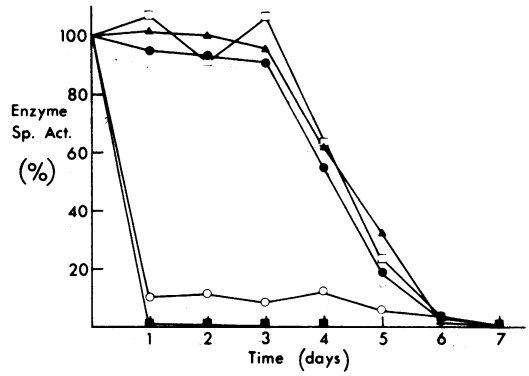


FIG. 2. *Enzyme specific activities in successive glutamate cultures after transfer from 3,5-xylenol medium. After growth in 1 liter of 3,5-xylenol medium, 0.5 ml of culture was inoculated into 1 liter of glutamate medium and the remaining bacteria were harvested for enzyme assays: similar successive transfers were made at 24-h intervals. The specific activities in 3,5-xylenol-grown bacteria represent the zero-time values and are given as 100% (actual activities are given in Table 1). 3,5-Xylenol methylhydroxylase, ○; m-hydroxybenzylalcohol dehydrogenase, ●; m-hydroxybenzaldehyde dehydrogenase, □; 3-hydroxy-5-methylbenzoate hydroxylase, gentisate oxygenase, maleylpyruvate hydrolase, citraconase, citramalate system, ■; p-cresol methylhydroxylase, ▲.*

**Loss of ability to grow on 3,5-xylenol and *m*-hydroxybenzoate.** Along with the decline in activities of the methyl-oxidizing enzymes (Fig. 2), there was a progressive fall in the fraction of bacteria capable of growth on 3,5-xylenol (Fig. 3). A repeat of the experiment, using a culture grown on 3,5-xylenol from a single colony, gave similar results. No colonies appeared when bacteria that had lost their 3,5-xylenol-degrading capacity were plated on 3,5-xylenol agar, and if revertants occurred they did so at a frequency well below $10^{-9}$.

When the organism, grown on 3,5-xylenol, was inoculated as a streak on *m*-hydroxybenzoate agar, isolated colonies appeared, rather than confluent growth, even when a heavy inoculum was used. Despite this, it was possible to use such plates to follow loss of ability to grow on *m*-hydroxybenzoate after transfer from 3,5-xylenol to glutamate medium. In such an experiment, there was simultaneous loss of ability to grow on both 3,5-xylenol and *m*-hydroxybenzoate.

**Enzyme levels in bacteria grown on various substrates.** Activities of enzymes in extracts of bacteria grown on later intermediates of the pathway or their analogs were measured

and compared with those in glutamate-grown bacteria (Table 1). Since some activities in *P. putida* NCIB 9869 declined only after prolonged growth on glutamate medium (Fig. 2), the bacteria, grown on 3,5-xylenol, were subcultured daily, for five days, in 100 ml and finally 1 liter of glutamate medium. Bacteria from this final culture were used for the assay of enzymes. A similar period of growth was allowed for all other substrates. The organism grew poorly on gentisate agar; however, an inoculum of some of the larger colonies from such a plate grew in gentisate liquid medium. It was not possible to complete similar experiments with later intermediates because methylmaleyl-pyruvate is unstable and no growth was observed on citraconate or citramalate on solid or in liquid medium.

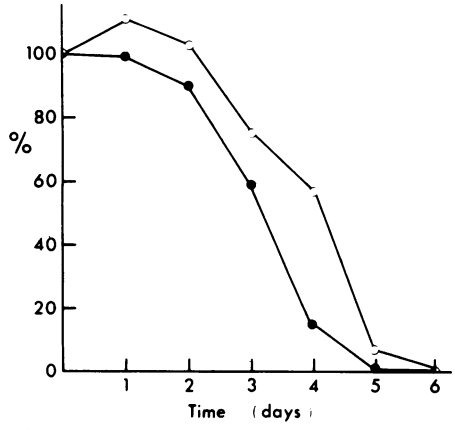


FIG. 3. *The decline in enzyme activity and the percentage of bacteria able to grow on 3,5-xylenol. The specific activity of m-hydroxybenzaldehyde dehydrogenase* (○) *and the percentage of bacteria capable of growth on 3,5-xylenol* (●) *were measured in successive glutamate cultures after transfer from 3,5-xylenol medium. Enzyme levels are shown as a percentage of the specific activity in 3,5-xylenol-grown bacteria (Table 1).*

None of the enzymes could be detected when a strain that had lost the ability to grow on 3,5-xylenol was grown in glutamate medium containing 0.03% (wt/vol) 3,5-xylenol.

**Transfer of 3,5-xylenol-degrading capability.** The ability to grow on 3,5-xylenol was transferred to another strain of *P. putida* in a mating experiment. The recipient strain was PaW340, a streptomycin-resistant, tryptophan auxotroph derived from *P. putida* (*arvilla*) mt-2 which, in contrast to the 3,5-xylenol positive donor, produces fluorescein on King's B agar. Streptomycin-resistant, 3,5-xylenol positive transconjugants were isolated at a frequency of $10^{-6}$. Neither donor nor recipient alone produced colonies on the selection medium. All of the transconjugants tested (42 colonies) were $Trp^-$ and produced fluorescent pigment on King's B agar.

Two of the transconjugants were grown in liquid medium on 3,5-xylenol and the enzyme activities were determined (Table 1). Although PaW340 did not grow on 3,5-xylenol, it did grow

TABLE 1. *3,5-Xylenol pathway enzymes in P. putida NCIB 9869 grown on various substrates and in transconjugant strains grown on 3,5-xylenol*

| Enzyme | sp act of:[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | *P. putida* NCIB 9869 grown on: | | | | | Transconjugant 1 grown on 3,5-xylenol | Transconjugant 2 grown on 3,5-xylenol |
| | 3,5-Xylenol | 3-Hydroxy-5-methylbenzoate | *m*-Hydroxybenzoate | Gentisate | Glutamate | | |
| 3,5-Xylenol methylhydroxylase | 0.11 | 0.12 | 0.12 | 0.09 | <0.002 | 0.18 | 0.13 |
| *p*-Cresol methylhydroxylase | 0.17 | 0.25 | 0.13 | 0.09 | 0.03 | 0.28 | 0.08 |
| *m*-Hydroxybenzylalcohol dehydrogenase | 0.57 | 0.65 | 0.78 | 0.70 | 0.04 | 1.28 | 1.17 |
| *m*-Hydroxybenzaldehyde dehydrogenase | 0.59 | 0.70 | 0.68 | 0.40 | 0.04 | 0.44 | 0.12 |
| 3-Hydroxy-5-methylbenzoate hydroxylase | 0.15 | 0.14 | 0.15 | 0.02 | <0.002 | 0.08 | 0.05 |
| Gentisate oxygenase | 1.74 | 1.08 | 1.01 | 0.25 | <0.005 | 0.51 | 0.66 |
| Maleylpyruvate hydrolase | 0.07 | NA[b] | NA | 0.03 | 0.0008 | 0.05 | 0.04 |
| Citraconase | 0.58 | NA | 0.50 | 0.21 | 0.01 | 0.23 | 0.22 |
| Citramalate system | 0.10 | NA | 0.02 | NA | <0.001 | 0.05 | 0.09 |

[a] The activity of 3,5-xylenol methylhydroxylase, measured with a bacterial suspension, is expressed as μmol of $O_2$ utilized $min^{-1}$ $mg^{-1}$ (dry weight). All other activities are expressed as micromoles of substrate transformed $min^{-1}$ mg of $protein^{-1}$.

[b] NA, Not assayed.

at a very slow rate on citramalate.

**Agarose gel electrophoresis of DNA.** The presence of a plasmid in *P. putida* NCIB 9869 was demonstrated by electrophoresis of its DNA on agarose gel followed by staining with ethidium bromide. The plasmid (Fig. 4c) appears to be considerably larger than the TOL plasmid (Fig. 4G; $78 \times 10^6$ daltons) from *P. putida* mt-2, run as a standard. Three 3,5-xylenol negative strains derived from *P. putida* NCIB 9869 were also examined in this way and all contained plasmids. In two cases (Fig. 4d and e) the plasmids were markedly smaller than that of the parent strain. In the third 3,5-xylenol negative strain (Fig. 4g), the plasmid appeared only slightly smaller than that from the parent strain but, considering the large size of the plasmid and the logarithmic relationship between size and distance moved, this could still represent deletion of sufficient DNA to code for the 3,5-xylenol pathway.

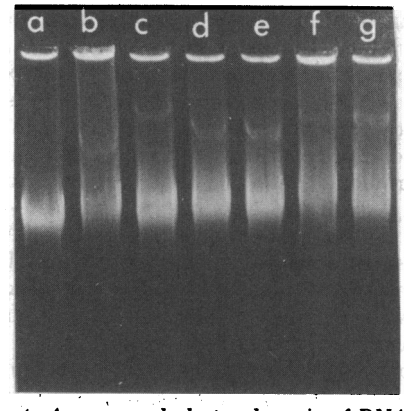


FIG. 4. *Agarose gel electrophoresis of DNA from P. putida NCIB 9869 and from 3,5-xylenol negative strains. (a) P. putida PR52000, a plasmidless standard; (b) P. putida mt-2 (PaW1) containing TOL plasmid; 3,5-xylenol positive P. putida NCIB 9869 grown on nutrient broth (c) and grown on 3,5-xylenol (f); 3,5-xylenol negative strains derived from P. putida NCIB 9869 (d, e, and g). Chromosomal fragments are seen as the heavy, common band.*

## DISCUSSION

The results suggest that enzymes for the oxidation of methyl groups of 3,5-xylenol and *p*-cresol are synthesized constitutively, but that the genes which encode them are located on a plasmid and may be lost spontaneously on nonselective media. Several degradative plasmids for pathways of metabolism of less common organic compounds have now been found in pseudomonads, and these can be cured spontaneously although usually at a low frequency (3, 16). From the pattern of extracted DNA after electrophoresis (Fig. 4), the plasmid present in *P. putida* NCIB 9869 is considerably larger than $78 \times 10^6$ daltons, the size of the TOL plasmid from *P. putida* mt-2 (16). Although plasmids are also present in the 3,5-xylenol negative strains derived from *P. putida* NCIB 9869, they are smaller than that in the parent strain. Thus, there appears to have been deletion of part of the plasmid bearing the 3,5-xylenol pathway genes rather than total loss of plasmid. Excision of a specific region of a plasmid has been suggested as one of the modes of loss of the TOL function in *P. putida* mt-2 (1).

Our results imply either a high frequency of spontaneous deletion of the 3,5-xylenol genes from the plasmid or a faster growth rate for the negative strains on nonselective medium. The strains lacking the 3,5-xylenol pathway genes are, of course, no longer carrying out the synthesis of the constitutive enzymes of the pathway and could, therefore, be more competitive.

The fraction of 3,5-xylenol positive bacteria appears to fall slightly earlier than the enzyme level in Fig. 3, but this may be due to further deletion from the plasmid during the estimation procedure when the bacteria were grown on nutrient agar. The similar results obtained when this experiment was repeated using a culture grown from a single colony shows that decline in growth capability was due to loss during the course of the experiment rather than the presence in the original culture of 3,5-xylenol negative bacteria which eventually outgrew the positive organisms under nonselective conditions.

Although most of these methyl-oxidizing enzymes are constitutive, the status of the 3,5-xylenol methylhydroxylase is uncertain. Because of difficulties in assaying this enzyme in extracts, the rate of whole-cell oxidation of 3,5-xylenol was taken as a crude measure of activity. The rate measured could be affected by changes in other oxygen-utilizing systems, including activities of oxygenases further along the pathway, in intracellular levels of available reduced pyridine nucleotides and also in the rate of entry of substrate into bacteria. Thus, the lower rate given by the first sample (day 1, Fig. 2) is not necessarily due to a decrease in 3,5-xylenol methylhydroxylase activity itself. This lower rate was maintained for several days and then, like the other constitutive activities, it fell to zero.

The rest of the enzymes of the 3,5-xylenol pathway behave in the way expected for inducible enzymes (Fig. 2). However, the simultaneous loss of ability to grow on 3,5-xylenol and *m*-hydroxybenzoate suggests that the gene for 3-hydroxy-5-methylbenzoate hydroxylase, which

belongs to the inducible group of enzymes, is linked with those for the earlier constitutive group. Cells grown for 5 days on *m*-hydroxybenzoate or 3-hydroxy 5-methylbenzoate should maintain the plasmid and, therefore, high activities of the constitutive enzymes and this was confirmed (Table 1). The presence of the methyl-oxidizing enzymes in gentisate-grown cells (Table 1) also suggests a linkage between gentisate oxygenase and earlier enzymes. Indeed it is possible that genes for all of the 3,5-xylenol pathway enzymes are located on the plasmid, because all the enzymes were present in transconjugants (Table 1). However, this is uncertain because PaW340 is able to grow on citramalate, although at a very slow rate.

These results show how metabolic capabilities may be lost from bacteria when maintained in the laboratory by periodic subculture on nutrient agar. Attention should also be drawn to the potential difficulties that can arise from this type of situation when studying metabolic pathways. Bacteria grown on *m*-cresol while still retaining the 3,5-xylenol pathway genes will contain the constitutive methyl-oxidizing enzymes. Although the initial methylhydroxylase is much less active with *m*-cresol than 3,5-xylenol (11), oxidation does occur (5). This could lead to accumulation of intermediates and, hence, to induction of later enzymes, indicative of the gentisate pathway for *m*-cresol catabolism. However, this probably represents only a minor route, as judged from the limited oxidation of 3,5-xylenol by *m*-cresol-grown bacteria (5). A different pathway involving hydroxylation to 3-methylcatechol, which is then cleaved by an oxygenase, is induced by growth on *m*-cresol, and this becomes more apparent only after loss of the 3,5-xylenol pathway genes from the plasmid.

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