Simultaneous Degradation of Chloro- and Methylaromatics via Ortho Pathway by Genetically Engineered Bacteria and Natural Soil Isolates

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INTRODUCTION

Industrial sewage frequently contains methylsubstituted and chlorosubstituted aromatics. Methylsubstituted aromatics are degraded by bacteria by a variety of pathways. This class of compounds can in general be considered as biodegradable (1).

Chlorosubstituted aromatics, however, despite many reports on dissimilation by bacteria and fungi (2), are often more or less recalcitrant. In aerobic systems the velocity of substrate disappearance is inversely correlated with the number of chlorine atoms attached to the ring. Accordingly, benzoates substituted with only one chlorine atom are degraded by many soil and water bacteria when supplied as a single substrate. However, serious problems may emerge when mixtures of degradable compounds are fed as carbon sources. This phenomenon can be demonstrated using substituted benzoates as model compounds.

I. METABOLISM OF SUBSTITUTED BENZOATES BY PURE CULTURES

Pseudomonas putida arvilla mt-2, a common soil bacterium, degrades a range of xylenes and methylbenzoates via meta cleavage of methylcatechols (Fig. 1).

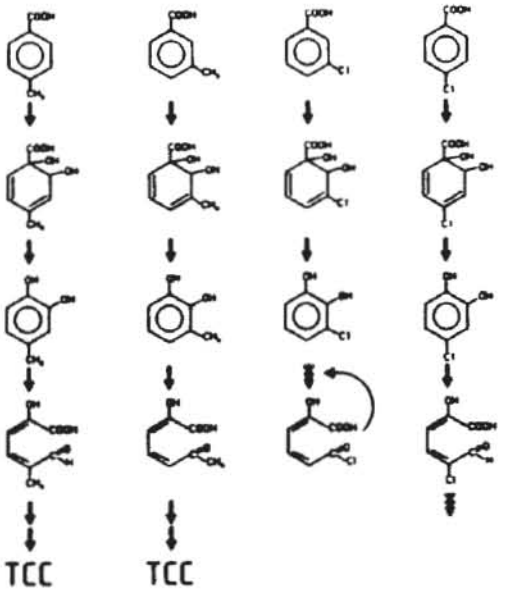

Fig. 1. Productive metabolism of methylbenzoates and cometabolism of chlorobenzoates by Pseudomonas putida mt-2

When resting cells after growth with methylbenzoates are incubated with chlorobenzoates, no growth can be observed with these compounds. Instead, 3-chlorobenzoate is transformed mainly to toxic 3-chlorocatechol which accumulates and causes blackening of the medium by oxidation yielding polymeric materials. A minor part of nonsymmetrically substituted 3CB is transformed to 4-chlorocatechol which can be cleaved by the catechol 2,3-dioxygenase (C230) to 5-chloro-2-hydroxymuconic semialdehyde (5-Chloro-HMS). This compound is turned over very slowly only and its formation is at least unproductive for the cells. Thus the cometabolism of 4-chlorobenzoate via 4-chlorocatechol quantitatively yields 5-chloro-HMS. Alkyl aromatics degrading cultures are therefore very sensitive to shock loads especially of chlorosubstituted aromatics. This can eventually result in a total loss of degradative capacity for alkylaromatics due to destruction of C230 and intoxication of the cells.

On the other hand, *Pseudomonas* sp. B13 degrades 3-chlorobenzoate via 3- and 4-chlorocatechols followed by reactions of a specialized ortho pathway (Fig. 2).

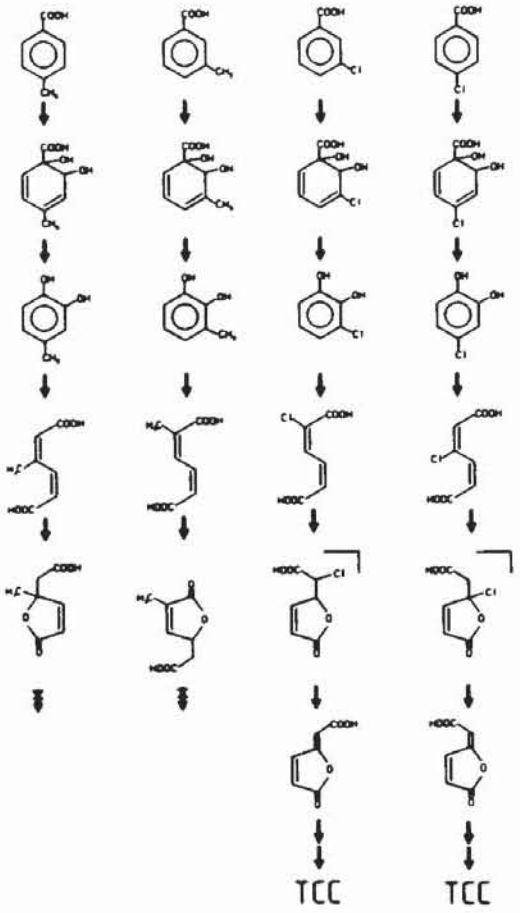

Fig.2. Metabolism of chlorobenzoates and cometabolism of methylbenzoates by Pseudomonas sp. B13FR1

These enzymes, namely chlorocatechol-1,2-dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase and maleylacetate reductase, well suited for catabolism of chlorocatechols, cometabolize methylcatechols to the corresponding methyllactones (3). These compounds are excreted into the medium and cannot be further transformed by this strain. Thus for either strain one of the two classes of substrates is incompatible and after transformation results in accumulation of more or less toxic intermediates.
This could be demonstrated in continuous culture if *Pseudomonas putida* mt-2 was fed with 3-methylbenzoate (3MB) as a growth substrate. After addition of 3-chlorobenzoate (3CB) the culture due to accumulation of chloro catechols turned black and was washed out. In a control experiment *Pseudomonas* sp. B13 was inoculated prior to addition of 3CB. Now accumulation of methyllactones could be observed accompanied by a marked decrease in C230 activity. This is due to a suicide type inactivation of this meta cleaving enzyme by the action of chlorocatechols (4). As sufficient activity of chlorocatechol 1,2-dioxygenase was present in the mixed culture chlorocatechols were not accumulated in the growth medium.

II. ISOLATION OF TRANSCONJUGANTS BY DIRECTED NATURAL EVOLUTION

Increasing the load of 3CB in the reservoir resulted in transconjugants of the type WR 201/WR 206 which soon took over the culture quantitatively. Transconjugants of the type WR 201 had the background *Pseudomonas putida* mt-2 and had aquired the genes of the chlorocatechol dissimilating ortho pathway from B13 (5). They still had a functional meta pathway operating with methylcatechols and therefore were $3MB^+$, $3CB^+$. Further selection on 3CB or 4CB, however, yielded also strains of the type WR 206 (4) which had lost the C230 activity resulting in a $3MB^-$, $3CB^+$ phenotype. This mutation caused by an insertion of a transposon into the structural gene of the C230 enzyme (xyl E) is of significant advantage for growth with 3CB, as it avoids the futile cycle of production and suicide destruction of enzymes by 3-chlorocatechol. Addition of 3MB resulted, however, in accumulation of methyllactones (5). Transconjugants of the type WR 206 clearly demonstrate that the $3MB^+$ and $3CB^+$ phenotype cannot coexist stably in a single strain as long as there is no productive metabolism of chlorocatechols via meta pathway (B. in Fig. 3) or of methylcatechols via ortho pathway (A. in Fig. 3).

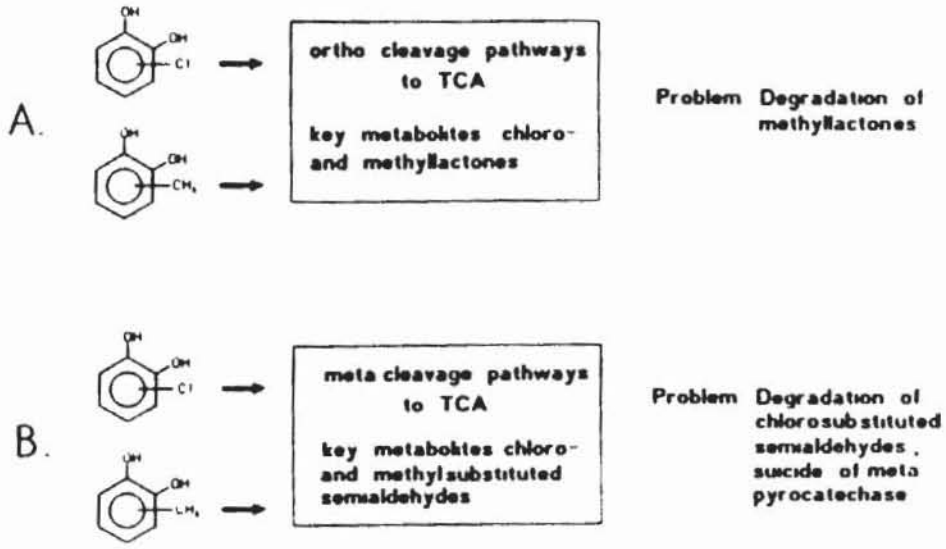

Fig. 3. Strategies for degradation of incompatible substrates

Intensive efforts to break down chlorocatechols via meta cleavage (strategy B) were unsuccessful (unpublished results).

III. DEGRADATION OF METHYLCATECHOLS VIA ORTHO PATHWAY IN NATURAL SOIL ISOLATES

The second strategy A, metabolism of methylcatechols via ortho pathway, seemed to be of greater promise.
A continuous culture degrading 3MB was established with soil as an inoculum. Then the 3CB degrading *Pseudomonas* sp. B13 together with 3CB was added to the culture. A transient accumulation of methyllactones (ML) was observed and ML degrading bacteria became stable members of the mixed culture. They could be isolated as pure cultures and thereby showed methyllactones to be biodegradable substrates. In addition methyllactones were used as a sole source of carbon and energy to select more bacteria which eventually could degrade methylcatechol via reactions of modified classical or chlorocatechol ortho pathways. A number of biochemically different strains could be isolated which besides 2ML utilized 3MB via ortho cleavage. No C230 activities could be detected in some of these isolates after growth with 3MB . There were, however, also other solutions to the problem of simultaneous utilization of incompatible substrates. In one group of strains both meta- and ortho pathways were detected resembling the WR 201 type, i.e. resulting in the wasteful mechanism of suicide destruction of C230 enzyme.
From a coenrichment experiment with cresols plus chlorophenols as substrates supplemented with the 3CB degrading *Pseudomonas* sp. B13, strains were isolated which represent the most evolved type with respect to simultaneous utilization of 3MB and 3CB (for example strain D7-4). These organisms exhibited no C230-activity and did not break down chlorocatechols unproductively. Strains of this type, however, grew slowly with 3MB. Since we could not improve the strains by subcultivation on mixtures of 3MB/3CB we decided to evolve such an organism by the methods of genetic engineering.

IV. CONSTRUCTION OF A HYBRID PATHWAY BY GENETIC ENGINEERING

During the selection for ML degrading organisms *Alcaligenes eutrophus* JMP 134 and *Pseudomonas* sp. B13 were found to utilize 4ML and 3ML respectively. As degradation of 4ML proceeded via 3ML (ML isomerase) (7) a strategy for combining the 4ML isomerase of the *Alcaligenes* strain with the 3ML phenotype of *Pseudomonas* sp. B13 was developed. Total DNA of *Alcaligenes* strain was partially digested and cloned into a cosmid vector pLAFR3. This bank of hybrid plasmids was transferred to a derivative strain of *Pseudomonas* sp. B13 which carried already the genes of the upper part of toluate metabolism of *Pseudomonas putida* (arvilla) mt-2. These genes were introduced to enable the strain to transform also 4-substituted benzoates, a property not present in the parent strain B13. Successful cloning of ML isomerase should result in strains degrading 4MB as well as 3CB and 4CB by reactions of ortho pathways. Such clones, for example B13 FR1 (pFRC20P) could indeed be detected (8). Growth experiments showed that after growth with 4MB both 4CB and 4MB alone or in combination were transformed without any misrouting meta cleaving activity. It therefore resembles the strain D7-4 isolated after naturally occuring gene transfer. However, both strains are still restricted with respect to their substrate range: whereas strain D7-4 cannot utilize 4MB due to its inability to transform 4ML to 3ML, FR1(pFRC20P) is unable to utilize 3MB. Most of this substrate is transformed to 2ML which is not a carbon source for this strain (Fig. 4). As *Alcaligenes eutrophus* JMP134 can be mutated for growth with 2ML, studies are currently undertaken to clone the 2ML genes into the constructed strain FR1 (pFRC20P) in order to create a strain degrading 3MB, 4MB, 3CB and 4CB exclusively via ortho pathways.

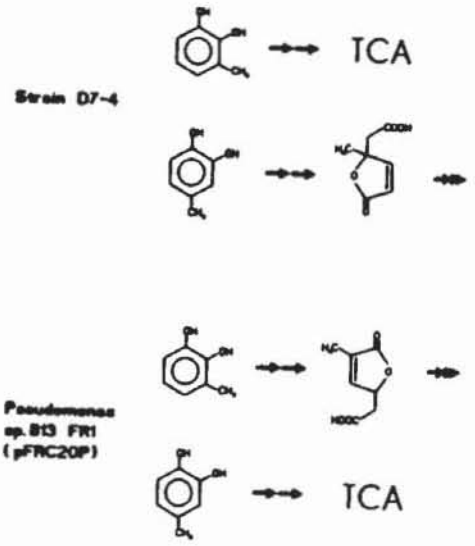

Fig.4. Incapability of two bacterial strains to degrade 3-methyl- and 4-methylcatechol via ortho cleavage pathway

The assembly of complementary catabolic properties for 4MB plus 4CB degradation in a single organism is clearly advantageous over a two species system.

The above mentioned experiments demonstrate that bacterial populations of activated sludge can be augmented and manipulated with special multifunctional strains in order to achieve total degradation of otherwise incompatible substrates in sewage model systems.

SUMMARY

The simultaneous bacterial metabolism of chloro- and methylaromatics via ortho- or metapathways normally results in incomplete degradation and death of the organisms. This is caused by misrouting of central intermediates, i.e. substituted catechols into unproductive pathways and suicide inactivation of the key enzyme of

meta pathway, (catechol 2,3-dioxygenase). The meta pathway proved to be definitely unsuited for productive metabolism of chloroaromatics. Therefore two strategies were used for simultaneous degradation of mixtures of chloro- and methylaromatics via ortho pathways: Methyllactons or certain mixtures of chloro- and methylaromatics were used as enrichment substrates, yielding strains which metabolized these compounds almost exclusively via the desired pathway. Alternatively, relevant enzymes from five different catabolic pathways of three distinct soil bacteria were combined in a patchwork fashion generating a functional ortho cleavage route for methylaromatics coexisting with the ortho cleavage pathway of chloroaromatics.

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