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THE METABOLISM OF TOLUENE BY
THERMOTOLERANT BACTERIA

by

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B.Sc. (Hons) London

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DECLARATION

The work in this thesis was the result of original research conducted by myself under the supervision of Professor Howard Dalton. All sources of information have been specifically acknowledged by means of reference.

None of the work in this thesis has been used in any previous application for a degree.

Helen Diana Simpson

SUMMARY

Several thermotolerant organisms capable of growth on toluene as the sole carbon and energy source, were isolated from various soil samples and liquid samples from a waste treatment works. Two isolates were selected for further studies and tentatively characterized as *Bacillus* species. The isolates were designated strain HTB16 and strain AT50 and had optimum growth temperatures of 45°C and 50°C respectively.

An initial aim of the project was to investigate whether thermotolerant organisms could be exploited to produce aromatic *cis*-dihydrodiols. Both strains HTB16 and AT50 oxidized toluene using a dioxygenase-catalyzed reaction, thereby forming *cis*-toluene dihydrodiol as an intermediate. Therefore, a mutant of either strain, which lacked functional *cis*-toluene dihydrodiol dehydrogenase would accumulate *cis*-toluene dihydrodiol when grown in the presence of toluene and a co-substrate.

A mutant of strain AT50 would probably be the most suitable organism for an industrial process since it exhibited a higher optimum growth temperature, which was reflected by a greater thermostability of its enzymes. The enzymes detected in crude extracts of strain AT50 appeared to be more thermostable than those in extracts of HTB16. *cis*-Toluene dihydrodiol dehydrogenase from AT50 was particularly stable. This enzyme was purified and partially characterized and amongst its properties, had a half-life of 100 min at 80°C.

Strains HTB16 and AT50 were subjected to various mutagenic procedures. NTG mutagenesis appeared to be the best method for obtaining a relatively high percentage of mutants amongst the survivors. Unfortunately, all the mutants obtained appeared to lack toluene dioxygenase.

In conclusion, it appears that enzymes from thermotolerant organisms are more thermostable than those from mesophiles. Consequently, it may be advantageous to use strain AT50 to produce *cis*-toluene dihydrodiol, once a suitable mutant is obtained. However, experiments with continuous cultures of strain AT50 suggested that one possible disadvantage of growing toluene-utilizing bacteria at 50°C is the low solubility of toluene in aqueous medium at this temperature.

ABBREVIATIONS

h	Hour
s	Second
min	Minute
approx.	Approximately
rpm	Revolutions per minute
Pa	Pascal
M _r	Molecular ratio
K _m	Michaelis constant
K _i	Inhibition constant
w/v	Concentration, weight by volume
v/v	Concentration, volume by volume
v.v.v. ⁻¹	Volume per volume per minute
pI	Isoelectric point
Em	Erythromycin
Cm	Chloramphenicol
Kn	Kanamycin
PEG buffer	Potassium phosphate buffer containing 10% ethanol and 10% glycerol
TE buffer	10mM Tris-HCl pH8.0 + 1mM EDTA
MS	Mineral salts medium
SDS	Sodium dodecyl sulphate
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
PMS	Phenazine methosulphate
BPB	Bromophenol blue

TCA	Tricarboxylic acid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
A ₅₄₀	Optical density at 540nm
C-2,3-O	Catechol-2,3-oxygenase
C-1,2-O	Catechol-1,2-oxygenase
PCA	Protocatechuate
TCB	<u>cis</u> -Toluene dihydrodiol (<u>cis</u> -1,2-dihydroxy-3-methylcyclohexa-3,5-diene)
3-MC	3-Methylcatechol

Other symbols and units are as detailed in the Biochemical
Journal (1981) 193, 1-21.

CHAPTER 1 : INTRODUCTION

1.1. INTRODUCTORY COMMENTS

1.1.1 Environmental sources of aromatic compounds

Studies on the environmental distribution of aromatic hydrocarbons were initiated in 1947 by Kern who noted the presence of chrysene (1,2-benzphenanthrene) in soil. Subsequently it has become evident that aromatic compounds are very abundant in the biosphere and a single sediment sample can contain thousands of aromatic compounds (Blumer, 1975). As a consequence, the significance of the microbial degradation of aromatic compounds in general, cannot be overestimated.

Some of the aromatic hydrocarbons in the environment have a biosynthetic origin and it is known that certain benzoid terpenes are produced by living organisms (Moshier & Chapman, 1973). Moreover, lignin is an aromatic biopolymer which is synthesised in vast amounts by plants. However, it is currently accepted that most of the compounds found in the environment are produced by the pyrolysis of organic material and do not, in fact, have a biosynthetic origin. The complex mixtures of polycyclic aromatic hydrocarbons found in soils and sediments are a result of the deposition of airborne particles that contain combustion products. This particulate matter may arise naturally from forest fires and similar phenomena (Blumer & Youngblood, 1975), or from the combustion of fossil fuels by man (Hites et

al., 1977). Many pollutants and pesticides are aromatic (Steiert & Crawford, 1985) and in urban areas there are increased concentrations of polycyclic aromatic hydrocarbons (Lee et al., 1976). Chlorinated phenols and their derivatives are used extensively in industry and agriculture as insecticides, fungicides and herbicides. Consequently, this group of compounds may pose serious environmental problems. The susceptibility of halophenols to biodegradation has been related to molecular composition and structure by Alexander (1965), who concluded that multiple halogenation of the ring, particularly with meta substitution, conferred increased resistance to biological attack.

1.1.2. The relevance of aromatic compounds to man.

Interest in the physiological effects of aromatic hydrocarbons can probably be traced to the first recorded case of chemical carcinogenesis in 1761. At that time, Hill published an article entitled "Cautions against the immoderate use of snuff founded on the known qualities of the tobacco plant and the effects it must produce when this way taken into the body, and enforced by cases of persons who perished miserably of diseases occasioned or rendered incurable by its use" (Redmond, 1970). The relationship between skin cancer and skin contact with tars and oils has been established and in 1930 Kennaway & Heiger showed that dibenz(1,2)-anthracene caused skin cancer in mice. The

carcinogenic properties of various aromatic compounds are now well known and it has been suggested that 60-90% of human cancers are caused by exposure to environmental chemicals (Higginson & Muir, 1973). Hence there is current interest in exploiting microorganisms to degrade aromatic compounds and prevent them persisting in the environment where they may be a health hazard and cause ecological problems. Knowledge of the enzymatic breakdown of the benzene nucleus, particularly when bearing halogen substituents, will contribute towards an understanding of the recalcitrance of particular molecules to microbial attack.

Knowledge of the specific pathways of aromatic metabolism is needed when the objective is to alter the structure of an aromatic compound rather than achieve complete mineralization. The abilities of enzymes to act as specific and chiral catalysts have been recognised in the pharmaceutical industry for many years and enzymes are now being used increasingly in organic synthesis. Enzymes can be exploited to effect selective or regiospecific transformation of only one of two or more chemically similar functions in a molecule (Jones, 1986), which can be extremely difficult to achieve by chemical synthesis. There is no single chemical process to date which permits the direct oxidation of benzene or very simple aromatic compounds to synthetically useful substances. However, several intermediates produced by

aromatic-utilizing bacteria have industrial applications. For example, cis-benzene dihydrodiol (an intermediate of benzene oxidation in Pseudomonas putida) can be polymerized to produce high molecular weight polyphenylene, an extremely robust polymer (Ballard et al., 1983). In the future, both by inference from pathways already elucidated and by the discovery of novel reaction sequences, hitherto unknown intermediates may be discovered from which useful bioconversions may be developed. Furthermore, the use of genetic manipulation could vastly increase the scope of potential compounds produced.

1.2. AN OVERVIEW OF AROMATIC-UTILIZING BACTERIA

1.2.1 Occurrence of aromatic-utilizing bacteria

The benzene nucleus is chemically inert since it has a large negative resonance energy due to the conjugation of unsaturated bonds. However, in spite of the stability of the aromatic nucleus, there are many microorganisms which, under the mildest conditions, can completely degrade the benzene ring.

The ability of bacteria to utilize aromatic hydrocarbons for growth was first demonstrated by Stormer (1908) who isolated Bacillus hexacarbovorus which could grow on toluene and xylene. It has since become evident that there is a great diversity of organisms which can degrade aromatic compounds. The ubiquitous distribution of aromatic-utilizing soil bacteria was illustrated by Gray & Thornton, who found that 146 out of 245 soil samples examined contained bacteria capable of metabolizing naphthalene, phenol or cresol (Gibson & Subramanian, 1984).

Since pseudomonads are ubiquitous and possess extraordinary nutritional versatility, it is not surprising that they degrade a wide variety of aromatic compounds. Several catabolic pathways of various degrees of complexity may occur within a single Pseudomonas strain. This is one reason why much of the early work on aromatic-utilizing bacteria was undertaken with Pseudomonas species (Ornston, 1971).

Aerobic, spore-forming, rod-shaped bacteria of the genus Bacillus, are an important component of the microflora of most soil and water environments and thus are potential agents of biological transformation and degradation of aromatic compounds (see Section 1.4). However apart from bacteria belonging to the genus Bacillus, there is a paucity of reports on Gram-positive, aerobic bacteria which degrade aromatic compounds. Nevertheless, the isolation of several aromatic-utilizing coryneform bacteria has been recorded. Coryneform bacteria often grow profusely in soil and are characterized by a marked tendency of the cells to produce branched and coccoid forms. Aromatic utilization by *Nocardia* has been covered in a review by Raymond & Jaisson (1971). In 1928, Gray & Thornton isolated from soil two strains of Mycobacterium agreste (listed as Nocardia corallina in the 7th Edition of Bergey's Manual of Determinative Bacteriology (1957)) that could utilize toluene. They also isolated a Mycobacterium cosmeticum that could grow on phenol. More recently, an Achromobacter sp. and two Nocardia spp. which could grow on benzoate, toluene and ethylbenzene respectively, as the sole carbon and energy source, have been isolated (Horvath & Alexander, 1970; Spokes & Walker, 1974a). It has been well documented that 2,4-dichlorophenol, which is inherently toxic to biological systems, can be metabolized by an Arthrobacter sp. to form tricarboxylic acid cycle intermediates and finally to form carbon dioxide and chloride (Tiedje & Alexander, 1969; Duxbury et al., 1970).

The bacterial catabolism of aromatic compounds in anaerobic environments has been reviewed by Evans (1977) and will not be covered in detail here. However, it is noteworthy that several different bacterial genera can degrade aromatic compounds under anaerobic conditions using inorganic electron acceptors. Several species of the purple non-sulphur bacteria, the Rhodospirillaceae, can grow on simple aromatic compounds as sole carbon source both aerobically in the dark by respiration and anaerobically in the light by photosynthesis. Some bacteria, eg. Pseudomonas and Moraxella, can utilize aromatic compounds under anaerobic conditions through nitrate respiration (Taylor et al., 1970; Williams & Evans, 1975). In fermentation, no external electron acceptor is required and the carbon source is degraded anaerobically by a series of reactions that release energy by substrate-level phosphorylation. Mixed cultures of bacteria, isolated from rumen-liquor or sewage-digester sludge, can ferment aromatic compounds to methane and carbon dioxide (Ferry & Wolfe, 1976; Balba & Evans, 1977). Firstly, the benzene nucleus is reduced and then cleaved to aliphatic acids by facultatively anaerobic, Gram-negative organisms. The aliphatic acids are then converted to suitable substrates for various methanogenic bacteria to complete the process. Two methanogenic strict anaerobes, characterized as Methanobacterium formicicum and Methanospirillum hungatei, were isolated from a mixed culture and were unable to attack benzoate confirming that they served as terminal organisms of a metabolic food chain

composed of several different organisms.

In summary, representatives of several different bacterial genera can degrade aromatic compounds. Soil and water bacteria in general but particularly Pseudomonas species, exhibit great nutritional versatility not found in commensal and parasitic bacteria, such as Escherichia coli.

1.2.2. General pathways of aromatic metabolism

1.2.2.1 Preparation of the aromatic nucleus for aerobic fission

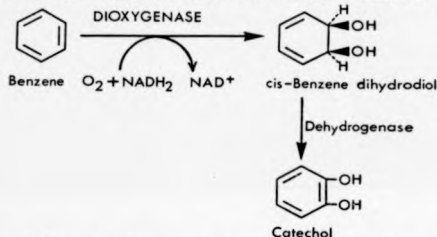
1.2.2.1.1. Activation of the benzene nucleus

There are only a few chemically permissible routes to convert the benzene nucleus into aliphatic products that can enter the tricarboxylic acid cycle. Initially, the microorganism must activate the aromatic nucleus to permit cleavage and further metabolism.

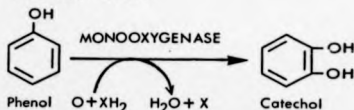
In general, the benzene nucleus must carry two hydroxyl groups situated ortho or para to one another before ring fission can occur (Dagley, 1971). Consequently, although the catabolism of numerous aromatic compounds involves a large number of reactions, the pathways often converge to one of two compounds: catechol or protocatechuate. Exceptions are uncommon but include the reaction catalysed by 1-hydroxy-2-naphthoate 1,2-dioxygenase, where a benzene

ring substitutes for one of the usual two hydroxyls (Kiyohara & Nadao, 1977). Also, the enzyme 5-chlorosalicylate 1,2-dioxygenase from Bacillus brevis cleaves the aromatic ring while it is substituted with only one hydroxyl group (Crawford et al., 1979b). A further example was provided by Que (1979) who showed that 2-aminophenol was slowly attacked by catechol 2,3-oxygenase.

The catechol structure necessary for ring cleavage may be achieved by double hydroxylation using molecular oxygen.



In contrast phenolic aromatics are activated by further hydroxylation by flavoprotein monooxygenases. One of the atoms of molecular oxygen is incorporated into the benzenoid molecule and the other is reduced to water by a hydrogen donor (reduced nicotinamide, flavin nucleotides or pteridines) (Dagley, 1971).



Likewise many singly substituted or 1,2-di-substituted aromatic rings, eg. mandelate, phenylalanine, benzoate and salicylate are degraded to catechol (Figure 1.1a). Conversely, aromatic rings di-substituted in positions 1,3 and 1,4 and multiply substituted rings, eg. 4-hydroxybenzoate, quinate and shikimate, are degraded to protocatechuate (Figure 1.1b).

Polycyclic aromatics are degraded by attack on one aromatic ring at a time. For example, naphthalene is oxidised by a dioxygenase-catalyzed reaction (Gibson & Subramanian, 1984). Meta-cleavage subsequently releases one of the rings leaving ultimately gentisic acid or catechol which are ring-fission substrates.

1.2.2.1.2. Activation of compounds possessing side chains

In compounds possessing side chains on the benzene nucleus, the substituent groups are often, but not always, removed before ring cleavage.

Aliphatic side chains can be modified, shortened or remain intact. For example, some pseudomonads hydroxylate the nucleus of a cresol, leaving the methyl group intact.

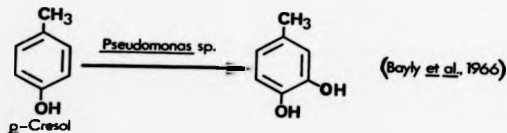
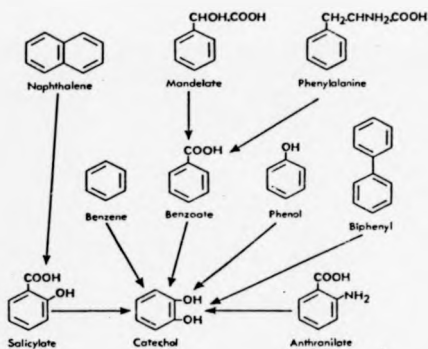
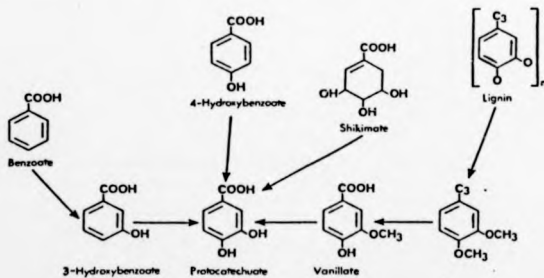


Figure 1.1 Degradative pathways of aromatic compounds leading to (a) catechol and (b) protocatechuete

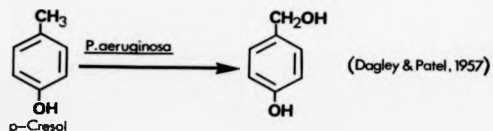
a)



b)

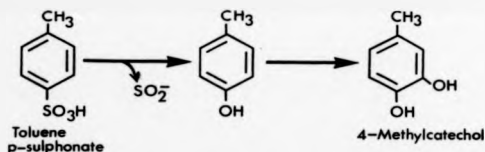


Conversely, others oxidise the methyl group to a hydroxyl group.



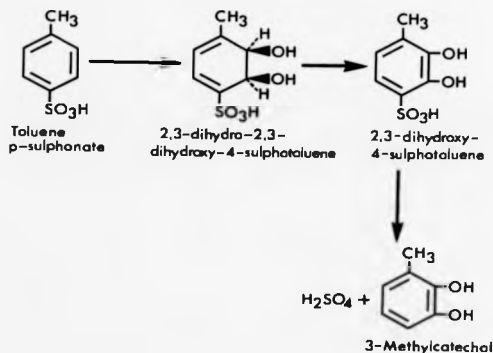
Similarly, toluene can be oxidised either to 3-methyl catechol (via the dihydrodiol) or ultimately to catechol via benzyl alcohol (Gibson & Subramanian, 1984).

Bacteria may elaborate a variety of strategies to deal with inorganic substituents. Cain & Farr (1968) found that toluene p-sulphonate was oxidised by a monooxygenase in a *Pseudomonas* sp. with the release of the sulphonate group as sulphite.

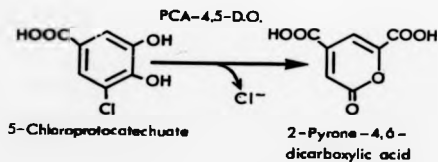


In contrast, another pseudomonad removed the sulphonic acid substituent of toluene p-sulphonate as sulphate (Focht & Williams, 1970). From experiments using radiolabelled

sulphate the following pathway was proposed:



Halogen substituents may be released from the benzene ring fortuitously, due to the mechanism of enzyme action. For example, in non-fluorescent pseudomonads, protocatechuate 4,5-dioxygenase (PCA-4,5-DO) expels methanol from 3-O-methylgallate and can also catalyze the elimination of chloride ions from 5-chloroprotocatechuate (Kersten *et al.*, 1982).



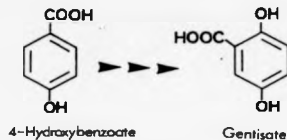
Alternatively, halogen substituents may be retained during ring fission. For example, an Arthrobacter sp., grown on 2,4-dichlorophenoxyacetic acid (2,4-D), retained the chlorine substituents during ring fission (Loos et al., 1967; Tiedje et al., 1969). Initially, monooxygenase attack on the side chain released glyoxylate. Subsequently, the chlorine substituent was removed from position-4 when a maloyl acetic acid was formed. The other chlorine substituent (from the original position-6) was removed when chloromaloylacetic acid was finally metabolized to succinate.

1.2.2.1.3 The "NIH Shift"

Studies with aromatic substrates labelled in specific positions with deuterium have shown that a frequent consequence of hydroxylation is an intramolecular migration or shift of the group displaced by hydroxyl to an adjacent position on the aromatic ring (Buroff et al., 1966; Daly et al., 1972).

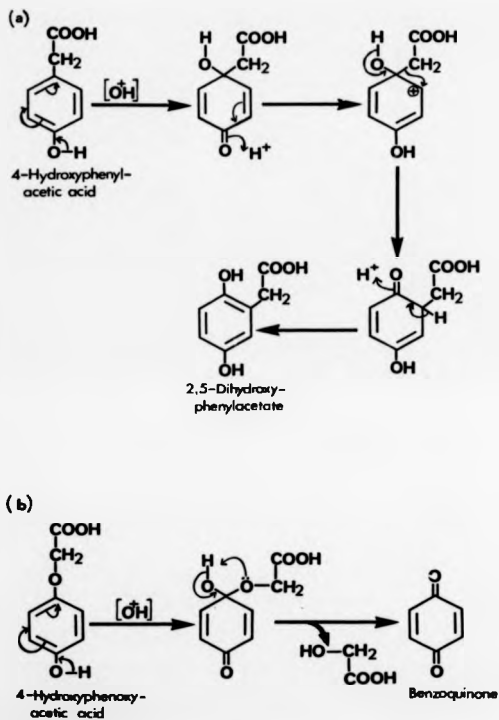
A facultatively thermophilic Bacillus sp. and the mesophilic Bacillus laterosporus were found to convert 4-hydroxybenzoate to gentisate (Buswell & Clarke, 1975; Crawford, 1976) which provides an example of the "NIH shift".

Eg.



The enzymic conversion of 4-hydroxyphenylacetate (4HPA) into homogentisate (2,5-dihydroxyphenylacetate) by the mesophilic Pseudomonas acidovorans provides a similar example (Hareland et al., 1975). Hareland et al. proposed that the side chain does not "migrate" during the reaction but remains bound to the enzyme which then undergoes a conformational change, as a result of carbonium ion formation. This brings C-2 of the aromatic nucleus into close proximity to the bound side-chain. Activity as substrate or inhibitor of 4-HPA 1-hydroxylase was shown only by those analogues of 4-HPA that possessed a hydroxyl group substituent at C-4 of the benzene nucleus. This absolute requirement for the hydroxyl group at C-4 is due to its ability to ionise and thereby supply electrons required for hydroxylation at C-1 (Figure 1.2a). A rearrangement of electrons then leaves C-2 with a positive charge (a carbocation) and results in a shift of the carboxymethyl side-chain to this position. This mechanism accounts for the fact that when the enzyme attacks 4-hydroxyphenoxycetic acid the side-chain does not shift, but is released as glycollate so that benzoquinone is formed (Figure 1.2b). The presence of oxygen in the

Figure 1-2 Reaction mechanisms suggested for the hydroxylation of (a) 4-hydroxyphenylacetic acid and (b) 4-hydroxyphenoxylacetic acid



side-chain would be expected to facilitate fission and so divert the shift of electrons from the formation of a carbonium ion that favours migration.

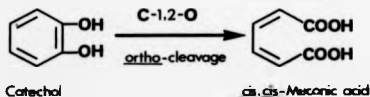
As a consequence of the "NIH shift", hydroxyl group substitution is not limited to positions ortho, meta or para to a ring substituent but frequently occurs on the same carbon atom as the substituent itself.

1.2.2.2. Fission of the benzene nucleus

After conversion of the substrate to a catechol, cleavage of the aromatic ring is carried out by dioxygenases (Figure 1.3) and molecular oxygen is incorporated. The fission occurs either between two neighbouring hydroxyl groups (ortho-cleavage) or between the hydroxylated and the adjacent non-hydroxylated carbon atoms (meta-cleavage).

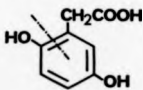
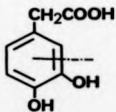
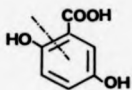
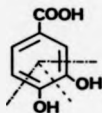
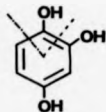
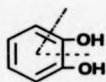
Ortho-fission

Ortho or intradiol cleavage between two neighbouring hydroxylated carbon atoms produces dicarboxylic acids which are further metabolized via the B-ketoadipate pathway. For example, ortho-cleavage of catechol by catechol-1,2-oxygenase (C-1,2-O) produces cis, cis-muconate.



C-1,2-O (pyrocatechase, EC 1.13.11.1) was first isolated

Figure 1.3 Fissions of the benzene nucleus catalyzed by
dioxygenases (Dagley, 1985)

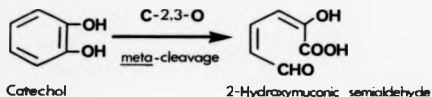


The dotted lines indicate the main points of cleavage
 ----- intra diol cleavage
 ----- extra diol cleavage

and purified from *Pseudomonas fluorescens* (Hayaishi *et al.*, 1957). The C-1,2-O purified from *P. arvilla* C-1 contained 1g atom of iron/ mol of enzyme, based on a M_r of 60 000 and consisted of 2 nonidentical subunits (Nakai *et al.*, 1979). The iron in the enzyme is in the ferric state and is a substrate binding site and hence essential for activity.

Meta-fission

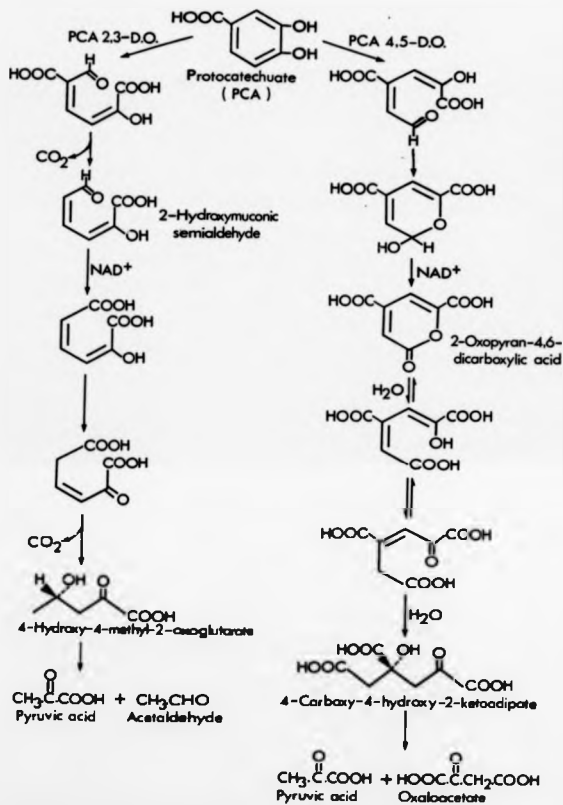
Meta or extradiol cleavage between a hydroxylated and an adjacent non-hydroxylated carbon atom gives rise to 2-hydroxymuconic semialdehydes. For example, meta-cleavage of catechol by catechol-2,3-oxygenase (C-2,3-O) produces 2-hydroxymuconic semialdehyde.



C-2,3-O (metapyrocatechase, EC 1.13.1.2) has been purified from *P. putida* (*arvilla*) mt-2 and contained 4g atoms of iron/mol enzyme, based on a M_r of 140 000, consisting of 4 identical subunits. The iron is present in the high spin ferrous state and is an integral part of the active site (Nakai *et al.*, 1983).

There are two meta-cleavage pathways for protocatechuate (PCA) (as well as an ortho-cleavage pathway) (Figure 1.4).

Figure 1.4 Meta-fission pathways for degrading
protocatechuete



Certain bacilli, eg. B.circulans, degrade PCA via PCA 2,3-dioxygenase forming 2-hydroxy-5-carboxy-cis, cis-muconic semialdehyde. Decarboxylation of this product produces 2-hydroxymuconic semialdehyde which is degraded by the catechol meta-fission enzymes (Crawford, 1975a). Conversely, when non-fluorescent pseudomonads, namely P.acidovorans and P.testosteroni, are grown with 4-hydroxybenzoate the meta-fission PCA 4,5-dioxygenase is induced. The 4-carboxy-2-hydroxymuconic semialdehyde then undergoes oxidation in its hemiacetal form and the resulting pyrone is hydrolyzed to give an open tricarboxylic acid. This is then hydrolyzed to form 4-carboxy-4-hydroxy-2-ketoadipate which undergoes aldol fission yielding pyruvate and oxaloacetate. The occurrence of the three different modes of enzymic ring-fission for PCA illustrates the fact that the point of cleavage of the benzene nucleus is not determined solely by the position and chemical nature of the substituents.

Ortho- or meta-fission ?

Many bacteria have the potential to use either fission pathway. For example, Pseudomonas fluorescens utilises the meta pathway for aromatic compounds catabolized via catechol, whereas those catabolized via PCA undergo ortho-cleavage (Seidman et al., 1969). Whether ortho or meta fission occurs depends on a combination of 2 factors, namely the mechanisms available for induction of the enzymes required and secondly, their substrate

specificities. Enzymes for meta-cleavage pathways are relatively tolerant of substituents on the benzene nucleus. Indeed, in bacteria, methyl-substituted aromatic compounds can usually only be degraded by meta-cleavage since catechol 1,2-oxygenase has a very low affinity for methylcatechols or produces 'dead end' lactone intermediates with the methyl group blocking delactonization (Catalani et al., 1971). However, two exceptions include the purified C-1,2-O from Bravibacterium fuscum which oxidised both 3- and 4-methylcatechol to give corresponding methylsuccinic acids (Nakagawa et al., 1963). A further example of the ortho-fission of the methylcatechols was illustrated when a C-1,2-O, purified from a Pseudomonas sp., oxidised 4-methylcatechol at approximately the same rate as catechol. However, 3-methylcatechol was oxidised much more slowly (Kojima et al., 1967). Since enzymes for the ortho-cleavage pathway are highly specific, bacteria which degrade catechol and PCA by the ortho route probably exercise their biochemical versatility by modifying side chains with non-specific enzymes before opening the benzene nucleus (Kennedy & Fewson, 1968). The subsequent enzymes (the ortho-fission enzymes) are then very specific.

Halogenated aromatics are usually degraded by ortho-fission since, although methyl-substituted aromatic compounds are often degraded by meta-fission, this mode of attack probably converts chlorinated catechols into acylchlorides. The

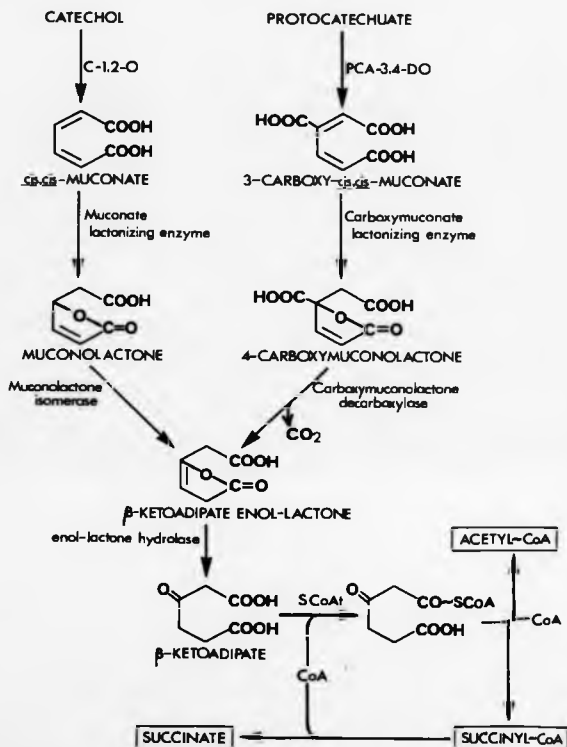
acylchlorides so formed would be open to nucleophilic attack by certain amino acid residues of the enzyme which would consequently be degraded (Dagley, 1985). This is avoided in the ortho-fission route since acylation does not result.

1.2.2.3 Further metabolism to tricarboxylic acid cycle intermediates

1.2.2.3.1 Reactions which follow ortho-fission

Ortho-cleavage of catechol and protocatechuate converges on β -ketoadipate (3-oxoadipate). This compound is activated by a CoA-transferase and cleaved to form succinyl CoA and acetyl CoA, which are metabolized via pathways of intermediary metabolism (Figure 1.5). Although the pathways for catechol and protocatechuate metabolism are analogous chemically, the enzymes responsible are very different and highly specific for their substrates. For example, muconate-lactonizing enzyme exhibits no detectable activity towards 3-carboxy-cis, cis-muconate, and the carboxymuconate lactonizing enzyme does not catalyze the lactonization of cis, cis-muconate. The ortho pathways for degradation of catechol and protocatechuate converge upon β -ketoadipate enol-lactone, which is hydrolyzed enzymically to β -ketoadipate. After esterification with coenzyme A, thiolytic fission can then occur producing succinate and acetyl-CoA.

Figure 1.5 The β -ketoadipate pathway



Abbreviations:

C-1,2-O, Catechol-1,2-oxygenase; PCA-3,4-DO, Protocatechuate-3,4-dioxygenase;

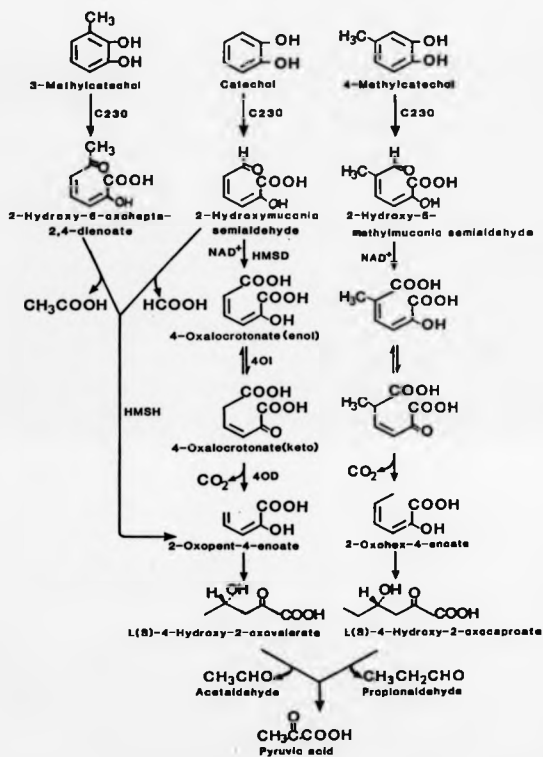
S-CoA: β -ketoadipate succinyl-CoA transferase

1.2.2.3.2 Reactions which follow meta-fission

The products of meta-cleavage are 2-hydroxybenzoic semialdehydes, which can subsequently be metabolized either hydrolytically or oxidatively (Figure 1.6) (Dagley, 1985). The hydrolase enzyme is cofactor-independent and catalyzes the release of formate to give 2-oxopent-4-enoate or its derivatives. The oxalocrotonate branch (oxidative route) is NAD⁺-dependent and involves three enzymatic steps effected by a dehydrogenase, an isomerase, and a decarboxylase. When 4-methylcatechol is degraded by the meta-cleavage pathway (and the oxidative route) then the cis isomer of 2-oxohexa-4-enoate is an intermediate (Collinsworth *et al.*, 1973), indicating that isomerization must occur to remove the double bond between C-4 and C-5, thereby permitting rotation of the methyl group at C-5 to a cis position.

The presence of both pathways for 2-hydroxybenzoic semialdehyde (2-HMS) metabolism have been reported in Pseudomonas spp. Nishizuka *et al.* (1962) demonstrated a NAD⁺-dependent dehydrogenation of 2-HMS, whereas Dagley & Gibson (1965) showed that with their strain hydrolysis occurred yielding formate and 2-oxopent-4-enoate. Subsequent experiments with Pseudomonas putida NCIB 10015 (the strain used by Dagley & Gibson in 1965) revealed the existence of both oxidative and hydrolytic meta-cleavage pathways for degradation of phenol and cresols (Sala-Trepat

Figure 1.6 Meta-fission pathways for degrading catechol,
3-methylcatechol and 4-methylcatechol



et al., 1972). Both 2-HMS hydrolase and 2-HMS dehydrogenase activities have been detected in benzoate-grown Azotobacter cells (Sala-Trepat & Evans, 1971 a & b) and in a naphthalene-grown Pseudomonas (Catterall et al., 1971). However, in both these cases the hydrolase activity was very low and was deemed not to have any physiological significance for the metabolism of 2-HMS.

By isolating mutants defective in one or the other pathway and analysing their phenotypes, Harayama et al. (1987) have elucidated the roles of the divergent branches of the TOL-plasmid encoded meta-cleavage pathway. 3-Methylcatechol is degraded exclusively by hydrolysis liberating acetate. Oxidation of 3-methylcatechol is prevented because it does not possess an oxidizable aldehyde, due to the presence of a methyl group at C-6 instead of a hydrogen atom so that it cannot be attacked by 2-HMS dehydrogenase. Consequently, mutants which lacked 2-HMS hydrolase could not metabolize p-toluate since this compound is degraded via 3-methylcatechol. In contrast, the meta-cleavage products of catechol and 4-methylcatechol, can be degraded by either the hydrolytic or the oxalocrotonate pathway. However, 2-HMS dehydrogenase activity was found to be several times higher than that of 2-HMS hydrolase in TOL plasmid-carrying cells of Pseudomonas putida at-2 previously grown on benzoate or p-toluate (Murray et al., 1972), indicating that catechol and 4-methylcatechol are metabolized preferentially via the oxalocrotonate route. The much higher activity of

HMS dehydrogenase at lower substrate concentrations was due to the lower K_m of HMS dehydrogenase ($2 \mu\text{M}$) compared with that of HMS hydrolase ($> 50 \mu\text{M}$) (Harayama *et al.*, 1987).

The coexistence of the two branches for the catabolism of catechol and 4-methylcatechol can be explained in terms of the non-specificity of the hydrolase activity, which is functional in the degradation of 3-methylcatechol. Also, the structural genes for the production of HMS hydrolase and HMS dehydrogenase are present on an operon and so both enzymes are coordinately induced (Sala-Trepat *et al.*, 1972). Since some bacteria, eg. *Azotobacter*, utilize only the 4-oxalocrotonate pathway for 2-HMS metabolism, it is possible that, originally, only this pathway existed (Sala-Trepat & Evans, 1971b). Bacteria may then have evolved and developed the hydrolytic pathway to increase their substrate range. Modification of a hydrolase already present in the cell to give a product (eg. 2-oxopent-4-enoate) which was also already present (and so could be metabolized further by existing enzymes) would be an economical way of increasing substrate range with minimal genetic alteration. However, a high-affinity hydrolase has not evolved, probably reflecting the fact that the 4-oxalocrotonate branch is more energetically favourable. Metabolism via the 4-oxalocrotonate branch produces NADH, whereas metabolism by hydrolysis produces formate but no NADH. The further catabolism of formate by formate dehydrogenase produces NADH but to obtain the same

energetic gain as is obtained from the 4-oxalocrotonate branch, coordinate synthesis of formate dehydrogenase is required.

1.2.3 Taxonomic and evolutionary relationships

It has been suggested that evolutionary relationships between microorganisms may be revealed by comparing the catabolic routes they use to degrade aromatic compounds, assuming that one particular pathway has a single evolutionary origin (Dagley, 1971; Hagean & Rosenberg, 1970; Ornston & Stanier, 1966). Thus, pseudomonads can be divided into two categories: those which cleave protocatechuate by ortho-cleavage (forming 2-carboxy-cis, cis-succinic acid) and those which use meta-cleavage (forming 4-carboxy-2-hydroxy-cis, cis-succinic semialdehyde) (Stanier et al., 1966). However, this classification only holds under strictly specified conditions, namely when the pseudomonads are grown with *p*-hydroxybenzoate. It was found that the meta-cleavage mechanism was relatively rare, being confined exclusively to two species of non-fluorescent pseudomonads, namely *P. acidovorans* and *P. testosteroni*. Conversely, ortho-cleavage of protocatechuate was characteristic of the entire fluorescent group, *P. pseudomallei*, *P. multivorans* and *P. stutzeri*. Since the metabolic fates of the two different ring-cleavage products are wholly distinct and require

different specific enzymes for subsequent degradation, the pathways must have evolved independently.

Similarly, the observation that three different Bacillus spp. degrade 4-hydroxybenzoate by three different mechanisms suggests that bacteria of the genus Bacillus may be derived from a diverse evolutionary background. Crawford (1976) investigated pathways of 4-hydroxybenzoate (4-HBA) degradation among three bacilli, namely B.brevis, B.circulans and B.laterosporus (Figure 1.7). B.circulans degraded 4-HBA via protocatechuate 3,4-dioxygenase, the ortho-cleavage route through the protocatechuate branch of the β -ketoadipate pathway (Ornston & Stanier, 1966). Further divergence was observed in B.laterosporus which converted 4-HBA to gentisate (2,5-dihydroxybenzoate) which was further catabolized by the gentisate pathway via maleyl pyruvate forming D-salate (Hopper et al., 1968).

Alternatively, a comparison of the control mechanisms of a particular pathway in representatives of several different bacterial groups may reveal evolutionary relationships. Studies on the regulation of the β -ketoadipate pathway in two Gram-negative organisms, Moraxella calcoacetica and Pseudomonas putida, have illustrated that the control mechanism in the two organisms is entirely different (Dagley, 1971) (see Figure 1.8). The regulation of the

Figure 1.7 Metabolism of 4-hydroxybenzoate by three different *Bacillus* species

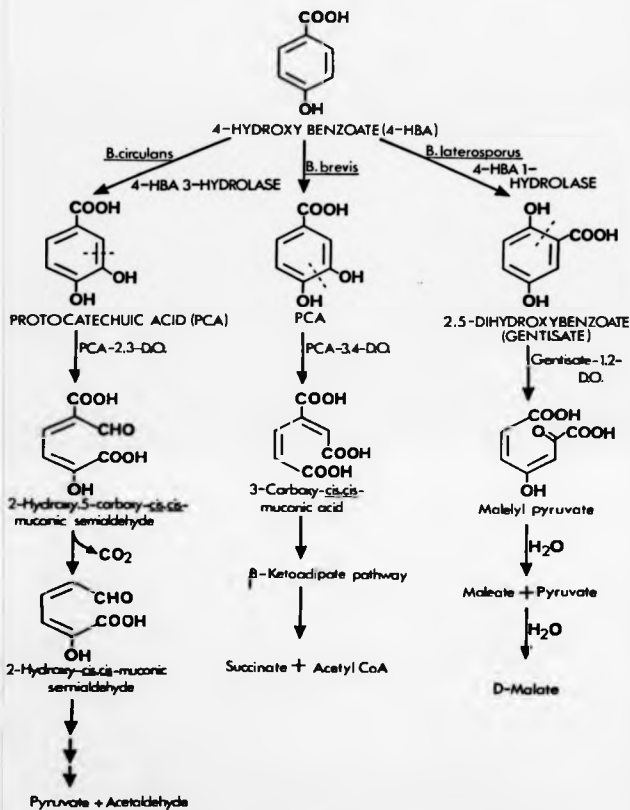
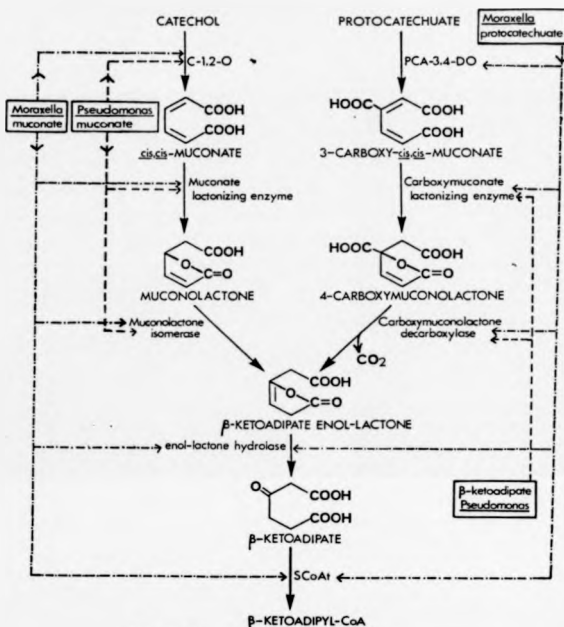


Figure 1.8 Regulation of the synthesis of enzymes of the β -ketoadipate pathway in *Moraxella calcoacetica* and *Pseudomonas putida* (Dagley, 1971)



An arrow directed from protocatechuate, muconate or β -ketoadipate towards an enzyme denotes that synthesis of this enzyme is derepressed by the compound designated.

β -ketoadipate pathway in P.putida, P.aeruginosa and P.multivorans was similar, suggesting that this mechanism of control may be typical of the whole genus Pseudomonas (Cánovas et al., 1967). Although M.calcoaceticus is similar to pseudomonads in many nutritional and physiological features, it differs in the base content of its DNA. Furthermore, the evolutionary divergence between Pseudomonas and Moraxella may be reflected in the different mechanisms of control of enzyme synthesis.

In conclusion, a comparison of the pathways of degradation of aromatic compounds and the control mechanisms used by different organisms may manifest evolutionary and taxonomic relationships. However, a single characteristic only provides limited information and ideally species should be characterized by complete descriptions of their phenotypes and genotypes. Only on consideration of structural, biochemical, physiological, ecological and genotypic properties can taxonomic and evolutionary relationships between organisms be fully elucidated.

1.3 TOLUENE-UTILIZING BACTERIA

1.3.1 Possible pathways of toluene catabolism

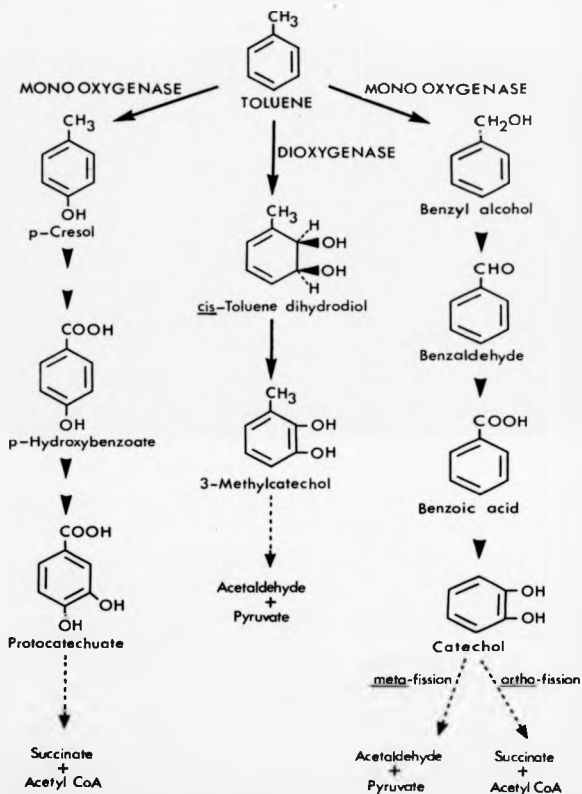
Bacteria have evolved several routes by which toluene can be metabolized to tricarboxylic acid cycle intermediates. The presence of the methyl group on the benzene ring provides an additional site for microbial oxidation alternative to the direct dioxygenation of the aromatic nucleus as in benzene metabolism. A summary of the possible pathways of toluene catabolism is given in Figure 1.9.

1.3.1.1 Oxidation by toluene monooxygenase

Kitagawa (1956) studied the oxidation of toluene and other aromatic compounds by a strain of Pseudomonas aeruginosa which had been grown in nutrient broth and then exposed to various aromatic compounds. Since induced cells oxidized toluene, benzyl alcohol, benzaldehyde, benzoic acid and catechol at approximately equal rates, he concluded that toluene was possibly oxidized through this series of intermediates.

More recently, Morsey & Williams (1975) described a different pseudomonad which also oxidized toluene via benzyl alcohol, benzaldehyde and benzoic acid. Pseudomonas putida (arvilla) at-2 carries genes for the catabolism of toluene, *m*-xylene, and *p*-xylene on a transmissible plasmid,

Figure 1.9 Pathways by which bacteria degrade toluene



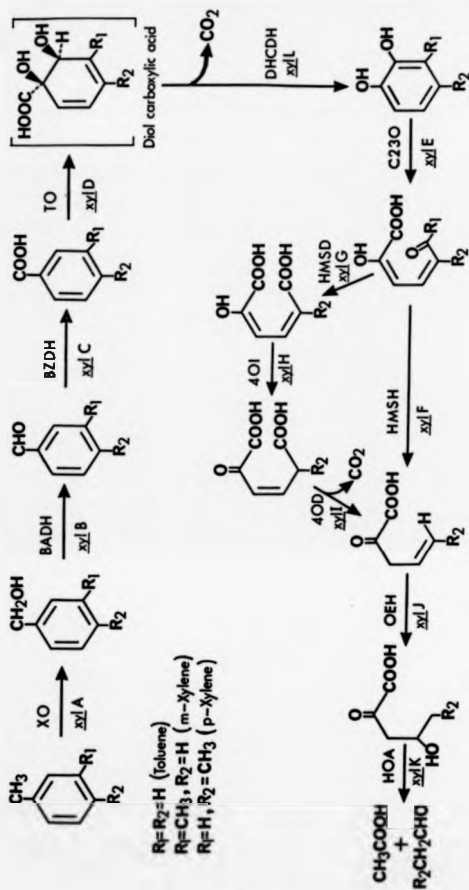
TOL. The TOL plasmid consists of two operons which encode the enzymes for the upper and lower pathways, and the use of meta-fission for the degradation of catechol intermediates. The first three steps in the catabolic sequence, which constitute the upper pathway, effect successive oxidations of the methyl group of toluene to form alcohol, aldehyde and carboxylic acid intermediates. TOL plasmids ranging in size from about 40 to 300 kb have been isolated from different strains of Pseudomonas (Duggleby et al., 1977 ; Williams & Morse, 1976). The TOL plasmid pNM0 (115 kb) of Pseudomonas putida at-2 encodes a set of inducible enzymes required for the complete degradation of toluene, m- and p-xylene, 3-ethyl toluene, and 1,2,4-trimethyl benzene (Harayama et al., 1984). These compounds are oxidised to the corresponding carboxylic acids, i.e. benzoate, m- and p-toluate, 3-ethylbenzoate, and 3,4-dimethyl benzoate, respectively, which are subsequently degraded to pyruvates and aldehydes via a meta-cleavage pathway (Figure 1.10). The meta pathway is necessary for the metabolism of methyl-substituted catechols which cannot be cleaved by ortho-fission since catechol 1,2-oxygenase has a very low affinity for methylcatechols or produces 'dead end' intermediates (Dagley, 1971). The pathway of metabolism was elucidated further by Whitel et al. (1986), who identified cis-diols as intermediates in the oxidation of aromatic acids by P.putida BB1.

Figure 1.10 Pathway for the catabolism of toluene by
bacteria that contain TOL plasmids

Enzyme abbreviations:

XO, xylene monooxygenase; BADH, benzyl alcohol dehydrogenase;
BZDH, benzaldehyde dehydrogenase; TO, toluate oxidase; DHCDH,
dihydroxycyclohexidione carboxylate dehydrogenase; C23O,
catechol 2,3 - oxygenase; HMSD, 2-hydroxymuconic semialdehyde
dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase;
4OI, 4-oxalocrotonate isomerase; 4OD, 4-oxalocrotonate
decarboxylase; DEH, 2-oxopent-4-enoate hydratase; HDA, 4-
hydroxy-2-oxovalerate aldolase.

xyl A to xyl L, genes for the degradative pathway enzymes.



The xyl A gene specifies xylene monooxygenase (Meraud et al., 1986), a relaxed specificity enzyme that monooxygenates or hydroxylates toluene and xylenes and their corresponding alcohols. This enzyme exemplifies the usual broad specificities of plasmid-encoded enzymes involved in hydrocarbon degradation (Reineke & Knacknuss, 1978; Timmis et al., 1985). Since a relatively large amount of DNA is transferred during plasmid-mediated conjugation, the genes for the complete degradation of several compounds can be gained when necessary. Consequently, plasmids are instrumental for rapid genetic transfer in bacteria (Farrell & Chakrabarty, 1979).

It has been observed that in the presence of benzoate, which can be degraded more quickly by the chromosomally encoded ortho pathway, the plasmid is lost or 'cured' (Williams & Murray, 1974; Clarke & Laverack, 1984). However, Stephens & Dalton (1987) recently observed that plasmid loss was too rapid to be explained simply by the proliferation of plasmid-free cells. They observed that lipophilic weak acids (benzoic, p-toluic, acetic and butyric acids) induced deletions in the TOL plasmid and disrupted segregation of deleted plasmid, thereby causing curing. However, no mechanism by which benzoate might disrupt plasmid partitioning was postulated since it is a poorly understood process.

An alternative monooxygenase-dependent pathway for toluene oxidation has been observed in Pseudomonas mendocina

(Richardson & Gibson, 1984). This organism metabolized toluene by initial oxidation to *p*-cresol. The subsequent oxidation leads to *p*-hydroxybenzoate and then protocatechuate, which serves as a substrate for *ortho* ring fission of the aromatic nucleus via β -ketoadipate (Figure 1.9). It is known that at least part of this toluene catabolic pathway is encoded by a self-transmissible plasmid (Yen *et al.*, 1986).

1.3.1.2 Purification and characterization of toluene monooxygenase

In 1975, Morsey and Williams stated that it had not been possible to develop an assay for toluene monooxygenase due to the toxicity of benzyl alcohol and other products. However, seven years previously Nozaka & Kusunose (1968a) described an assay system, albeit a lengthy process, for toluene monooxygenase. The assay required [14 C-methyl]toluene and subsequent detection of the radioactivity present in the benzyl alcohol and benzoic acid, which were separated using an alumina column.

Cell extracts prepared from *Pseudomonas aeruginosa* converted *p*-xylene to *p*-toluic acid and toluene to benzoic acid (Nozaka & Kusunose, 1968a). The enzyme system in crude extract required FAD and either NAD^+ or NADH as cofactors. Reduced sulphhydryl compounds were activators and the addition of 10mM glutathione to crude extracts

resulted in both activation and stabilization of the enzyme. The monooxygenase was partially purified using DEAE-cellulose chromatography and was resolved into three protein components (Nozaka & Kusunose, 1968b). When all three components were combined in the presence of NADH and FAD, activity was restored. Although FMN could replace FAD, lower activity was obtained.

Nozaka and Kusunose (1968a) suggested that toluene monooxygenase from Pseudomonas aeruginosa was similar to the alkane monooxygenases from P. desmolytica (Kusunose et al., 1967) and from P. oleovorans (Peterson et al., 1966). The enzyme involved in alkane hydroxylation was also comprised of three protein components, namely rubredoxin, NADH-rubredoxin reductase and alkane hydroxylase, and required NADH and FAD as cofactors. However, these properties are not unusual for monooxygenases and do not necessarily indicate that the same mechanism applies to both the toluene and alkane monooxygenase systems. Indeed, the enzymes certainly differed in requirements for ferrous ions which were necessary for maximum activity of the alkane monooxygenase but drastically inhibited toluene monooxygenase.

1.3.1.3 Oxidation by Toluene Dioxygenase

Toluene may be degraded by direct attack on the aromatic nucleus to produce, ultimately, 3-methylcatechol, which is

analogous to the oxidation of benzene via catechol (Marr & Stone, 1961; Wieland et al., 1958). Claus & Walker (1964) isolated a Pseudomonas sp. and an Achromobacter sp. from soil. Since 3-methylcatechol accumulated during growth, it was concluded that this compound was an intermediate and this was confirmed by simultaneous adaptation experiments. Subsequently, Nozaka & Kusunose (1969) showed that cell extracts of Pseudomonas aildenbergii oxidized toluene to 3-methylcatechol. Gibson et al. (1968 & 1970) demonstrated that P. putida degraded toluene via cis-toluene dihydrodiol, which was excreted by a mutant strain (strain 39/D). Further oxidation of cis-toluene dihydrodiol via a dehydrogenase-catalyzed reaction led to the formation of 3-methylcatechol (Rogers & Gibson, 1977), which is then degraded to intermediates of the tricarboxylic acid cycle via the meta-cleavage pathway.

The initial oxidation of toluene by toluene dioxygenase is the pathway of interest since an initial aim of the project was to produce cis-toluene dihydrodiol.

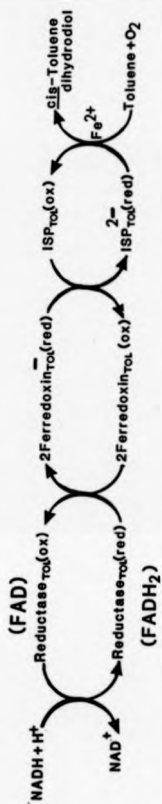
1.3.1.4 Purification and characterization of toluene dioxygenase

Studies with isolated enzyme preparations have demonstrated that dioxygenases which form cis-dihydrodiols from aromatic substrates are multi-component enzyme systems and

consequently are difficult to purify. An example of a two-component dioxygenase that hydroxylates the aromatic nucleus is benzoate dioxygenase (Yamaguchi & Fujisawa, 1978; Reineke & Knacknuss, 1978). Naphthalene dioxygenase (Ensley et al., 1983). benzene dioxygenase (Axcell & Beary, 1975) and pyrazon (5-amino-4-chloro-2-phenyl-2H-pyridazin-3-one) dioxygenase (Saubert et al., 1977) each contain three components, as does toluene dioxygenase. Toluene dioxygenase from *P. putida* F1 oxidizes toluene to *cis*-toluene dihydrodiol. The enzyme consists of a flavoprotein and 2 iron-sulphur proteins (Subramanian et al., 1985) and has been purified to homogeneity. As with many other oxygenases, two of the components serve to transfer electrons to the hydroxylase component which is responsible for converting toluene to *cis*-toluene dihydrodiol (Figure 1.11).

The ferredoxin_{ox} reductase functions by accepting two electrons from NADH and transferring them to the ferredoxin_{ox} component. During purification, FAD was removed from the ferredoxin_{ox} reductase, which is a flavoprotein that contains FAD as the only prosthetic group. The ferredoxin_{ox} reductase had a M_r of 46 000 Da and exhibited similar physical properties to flavoproteins that are required for other monooxygenase systems (Subramanian et al., 1980). These flavoproteins include putidoredoxin (Tsai et al., 1971), rubredoxin (Ueda et al.,

Figure 1.11 Scheme to show the pathway of electron transfer
in the toluene dioxygenase system
(Subramanian et al., 1985)



1972) and adrenodoxin (Chu & Kimura, 1973) reductases which function in (+)-camphor methylene hydroxylase, alkane hydroxylase, and adrenal steroid hydroxylase systems, respectively. All four reductases have similar molecular weights, absorption spectra and amino acid compositions. It is interesting to note that although putidaredoxin and adrenodoxin reductases are not interchangeable (Chu & Kimura, 1973), both ferredoxin_{yea} reductase (Subramanian et al., 1979) and rubredoxin reductases (Ueda et al., 1972) can be replaced by spinach ferredoxin reductase (reduced-NADP:ferredoxin oxidoreductase, EC 1.6.99.4).

The iron-sulphur protein designated ferredoxin_{yea}, which accepts electrons from the reductase, has also been purified to homogeneity (Subramanian et al., 1985). The molecular weight of ferredoxin_{yea} was 15 300 Da. Anaerobic reductive titrations revealed that ferredoxin_{yea} is a one-electron carrier that accepts electrons from NADH in a reaction mediated by ferredoxin_{yea} reductase. Ferredoxin_{yea} has many similar properties to adrenodoxin (Darru et al., 1966), putidaredoxin (Bunsulus & Lipscomb, 1973), and with the ferredoxin that participates in the benzene dioxygenase system (Axcell & Geary, 1975). All are one-electron carriers and each has a [2Fe-2S] cluster and similar molecular weight. All these proteins have similar amino acid compositions, being rich in acidic and hydrophobic amino acids. Although spinach ferredoxin reductase and NADPH could substitute for ferredoxin

reductase and NADH, it has been observed by Subramanian et al. (1985) that ferredoxin_{vol} cannot be replaced by either spinach ferredoxin, (Matsubaru et al. 1968), ferredoxin from Clostridium pasteurianus (Lovenberg et al. 1963), putidaredoxin or adrenodoxin. These observations suggest that there is a specific interaction between ferredoxin_{vol} and the terminal oxygenase component, ISP_{vol}, during the transfer of electrons.

The oxygenase component of the toluene dioxygenase enzyme system, in P. putida, is an iron-sulphur protein that has been designated ISP_{vol} (Subramanian et al. 1979). The molecular weight was 151 000 Da and SDS-denaturing polyacrylamide electrophoresis revealed the presence of two subunits with molecular weights of 52 500 Da and 20 800 Da.

Preliminary experiments revealed the presence of 2 g atoms of iron and 2 g atoms of acid-labile sulphur per mole of protein.

The structure of toluene dioxygenase purified by Yeh et al. (1977) is very similar to that of the benzene dioxygenase described by Crutcher & Geary (1979) (Table 1.1). Furthermore, both enzymes exhibited similar substrate specificities and studies with enzymes and whole cells showed that both can utilise benzene, toluene, ethylbenzene, chloro-, fluoro-, bromo- and iodo-benzene as substrates. Activity, however, was greatest towards the substrate on which the organism was grown. Both enzymes were isolated from strains of Pseudomonas putida and,

Table 1.1 A Comparison of toluene dioxygenase and benzene dioxygenase

	TOLUENE DIOXYGENASE	BENZENE DIOXYGENASE
Organism isolated from	<u>Pseudomonas putida</u> (Yeh <u>et al.</u> , 1977)	<u>Pseudomonas putida</u> (Crutcher & Seary, 1979)
Cofactors	NADH, Fe ²⁺	NADH, Fe ²⁺
Number of components	3	3
Mr Flavoprotein component (Da)	46 000	81 000
Mr Ferredoxin component (Da)	15 300	12 300
Mr Terminal oxygenase		
component (Da)	151 000	215 300 (168 000)*
subunits (Da)	52 500, 20 800	(54 500, 23 500)*

* as estimated by Zamanian & Mason (1987)

although isofunctional, appeared to differ in M_r . However, recently Zamanian & Mason (1987) estimated the native M_r of the benzene dioxygenase to be only 168 000 +/- 4 000 Da. The terminal oxygenase component of benzene dioxygenase from P. putida strain ML2 was also shown to contain 2 subunits of M_r 54 500 and 23 500 (similar to those of toluene dioxygenase) (Zamanian & Mason, 1987). The function of the 2 subunits is unknown but by analogy with other aromatic dioxygenases it has been proposed that the α subunit may contain a prosthetic group (2Fe-2S) (Yamaguchi & Fujisawa, 1982) and the β subunit may be involved in substrate recognition (Harayama et al., 1986). A further similarity between the benzene and toluene dioxygenases was observed after polyclonal antibodies were raised against each of the SDS-denatured subunits of the terminal oxygenase component of benzene dioxygenase. These polyclonal antibodies cross-reacted with 2 polypeptides in cell extracts of toluene-grown P. putida NCIB 11767 and these polypeptides had M_r values similar to those reported for the subunits of the ISP_{TOL} component of toluene dioxygenase (Zamanian & Mason, 1987). Conversely, no cross-reactivity was observed with subunits of the naphthalene dioxygenase or benzoate dioxygenase systems indicating that these aromatic dioxygenases probably developed independently from benzene and toluene

dioxygenase.

Despite all the similarities mentioned between the toluene dioxygenase from P.putida 11767 and the benzene dioxygenase from P.putida ML2, a difference in the mechanism of regulation has been observed (Zamanian & Mason, 1987). Toluene dioxygenase activity in P.putida 11767 was inducible, whereas benzene-oxidizing activity of P.putida ML2, grown on succinate, was 0.9% of that observed in benzene-grown cells. Furthermore, analysis of immunological cross-reactivity revealed that succinate-grown ML2 cells contained approximately 50% of the amount of the terminal oxygenase protein that was present when the organism was grown on benzene. Conversely, no terminal oxygenase protein was detected in succinate-grown cells of P.putida 11767. The question as to why there was such a high degree of apparently inactive protein in succinate-grown ML2 cells remains to be answered after further investigations.

The structural, functional and immunological similarities of toluene dioxygenase and benzene dioxygenase pose the question whether extensive regions of homologous amino acid sequences exist due to the presence of just one unspecific enzyme. A further comparison of the dioxygenases from a single organism grown separately on benzene and toluene would answer these questions and provide an insight as to whether the enzymes are indeed homologous. Drawing comparisons between enzymes from different organisms, often

purified by different procedures, although tempting, often provides only limited information which should be treated with caution. This is illustrated by comparing the two different M_r values obtained for benzene dioxygenase actually from the same organism, by Crutcher & Seary (1979) ($M_r=215\ 000$) and by Zamanian & Mason (1987) ($M_r=168\ 000$).

1.3.1.5 Purification and characterization of *cis*-toluene dihydrodiol dehydrogenase

Dehydrogenases catalysing the oxidation of *cis*-1,2-benzoic acid diol (Reiner, 1972), *cis*-benzene dihydrodiol (Axcell & Seary, 1973), *cis*-naphthalene dihydrodiol (Patel & Gibson, 1974), *cis*-toluene dihydrodiol (Rogers & Gibson, 1977) and chloridazodihydrodiol[5-amino-4-chloro-2-(2,3-*cis*-dihydroxy-4,6-cyclohexadienyl)-2H-pyridazin-3-one] (Eberspacher & Lingens, 1978) have been purified. These enzymes were all isolated from various mesophilic bacteria and they share similar properties. They are all tetramers, utilize NAD^+ as their primary electron acceptor and oxidise a range of *cis*-dihydrodiols.

The physicochemical and immunological properties of various bacterial *cis*-dihydrodiol dehydrogenases have been compared by Patel & Gibson (1974). *cis*-Naphthalene dihydrodiol dehydrogenases were isolated from *Pseudomonas putida* NP, *Pseudomonas* sp. NCIB 9816 and a *Nocardia* sp. All the dehydrogenases, except the one from *Nocardia* sp., exhibited

immunological cross-reaction with the anti-bodies (IgG) prepared against the enzyme purified from P.putida NP (Patel & Gibson, 1976). The enzymes from P.putida and Pseudomonas sp. NCIB 9816 were very similar (Table 1.2) and both showed strong cross-reactions with IgG and were inhibited by IgG to a similar degree. However, the enzyme from P.putida NP showed a spur formation in the Ouchterlony double-diffusion tests indicating the presence of antigenic determinants not present on the Pseudomonas sp. NCIB 9816 enzyme. Furthermore, the cis-naphthalene dihydrodiol dehydrogenase from P.putida NP was stable at 50°C for 90 min before losing activity, whereas the enzyme from Pseudomonas sp. NCIB 9816 lost all activity within 5 min. These differences may reflect minor alterations in their tertiary structures.

cis-Dihydrodiol dehydrogenases from P.putida biotype A grown separately on toluene and benzene exhibited similar pH values (pH 8.9, pH 8.8) and similar M_r values (155 and 160 kDa, respectively). However, the enzyme from toluene-grown cells retained 50% of its initial activity after 10 min at 50°C, whereas the enzyme isolated from cells grown on benzene was completely inactive after this time. Both gave weak precipitin bands with IgG (prepared against the purified cis-naphthalene dihydrodiol dehydrogenase from P.putida NP) (Patel & Gibson, 1976). When cell extracts from toluene- and benzene-grown P.putida biotype A were incubated in adjacent wells, they formed

Table 1.2 Comparison of cis-naphthalene dihydrodiol from
P.putida NP and Pseudomonas sp. NCIB 9816

	<u>Pseudomonas sp.</u> NCIB 9816 enzyme	<u>Pseudomonas putida NP</u> enzyme
Molecular weight (daltons)	112 000	102 000
pH optimum	9.0	9.0
K_m for the diol ($10^{-4}M$)	2.4	2.0
K_m for NAD ⁺ ($10^{-4}M$)	10.4	10.2
% Initial activity after 5 min. at 50°C	0	100

precipitin bands that were fused indicating that identical antigenic determinants were present. Therefore, the enzymes were closely homologous probably differing only slightly in primary structures. This could indicate that the mechanism of enzyme induction in benzene- and toluene-grown cells is similar.

It is noteworthy that the enzyme isolated from benzene-grown P.putida biotype A (Patel & Gibson, 1976) differed significantly from the cis-benzene dihydrodiol dehydrogenase isolated from a Pseudomonas sp. by Axcell & Geary (1973). The latter enzyme had a molecular weight of 440 000 Da and inactivation of the enzyme, observed during purification, was reversed by the addition of ferrous ions and reduced glutathione. Conversely the enzyme from P.putida biotype A, of molecular weight 160 000 Da, did not appear to require ferrous ions or glutathione. However it is possible that metal ions were very tightly bound to this enzyme.

The occurrence of the two different cis-benzene dihydrodiol dehydrogenases from two different Pseudomonas sp. may indicate that the two enzymes evolved independently or it may simply reflect the fact that different techniques were used by the two groups.

1.4 AROMATIC-UTILIZING BACILLI

1.4.1 Novel activities of mesophilic Bacillus spp.

During the course of the present study it became apparent that the thermotolerant, toluene-utilizing bacteria isolated belonged to the genus Bacillus. Although there have been no previously reported cases of toluene-utilizing Bacillus spp., certain other aromatic substrates are degraded by similar reaction sequences to those in other bacterial genera. Furthermore, work with bacilli could reveal new pathways of catabolism since it is already apparent that certain Bacillus strains degrade aromatic compounds by different routes (Crawford, 1975b).

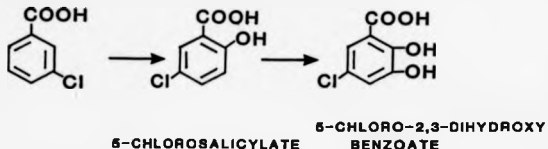
Certain bacilli behave similarly to other organisms, for example, Bacillus megaterius, Pseudomonas fluorescens, and Xanthomonas pruni all degrade tryptophan using the anthranilic acid pathway via N-formyl-L-kynurenine, kynurenine and anthranilic acid. The subsequent oxidation of anthranilate through catechol ultimately produces acetate and succinate (Bouknight & Sadoff, 1975; Brown & Wagner, 1970; Palleroni & Stanier, 1964). However, it is becoming increasingly evident that certain Bacillus spp. may possess unique activities not observed in other bacterial genera. For example, Spokes & Walker (1974b) investigated chlorophenol and chlorobenzoic acid co-metabolism by a Bacillus sp. previously grown on

benzoate. Interestingly, 3-chlorobenzoate was not converted to a chlorocatechol but to 5-chloro-2,3-dihydroxybenzoic acid via 5-chlorosalicylic acid. This seems to be a novel pathway not found in other organisms (Figure 1.12). The product resulting from the co-metabolism of 3-chlorobenzoate by a strain of Arthrobacter, isolated by Horvath & Alexander (1970), was characterized as 4-chlorocatechol. Conversely, 3-chlorocatechol was formed by the action of benzoate oxidase on 3-chlorobenzoate in Pseudomonas fluorescens (Ichiara et al., 1962) and in two Azotobacter spp. (Walker & Harris, 1970). A Pseudomonas sp. isolated by Johnston et al. (1972) actually utilized 3-chlorobenzoic acid as a source of carbon and energy and there was no evidence for the formation of either 3- or 4-chlorocatechol. The results obtained suggested a direct displacement of 3-chlorobenzoate to form 3-hydroxybenzoic acid, which was metabolized to 2,5-dihydroxybenzoic acid (gentisate) (Walker & Evans, 1952).

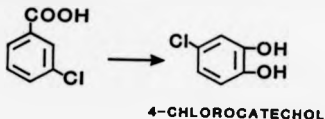
After observing the formation of 5-chloro-2,3-dihydroxybenzoic acid by co-metabolism of 3-chlorobenzoic acid by a benzoate-induced Bacillus sp., Spokes & Walker (1974b) examined the pathway of benzoate metabolism in this organism. The Bacillus isolates dissimilated benzoate via salicylic acid (2-hydroxybenzoate) and 2,3-dihydroxybenzoic acid but catechol was not an intermediate.

Figure 1.12 Pathways used for the metabolism of
3-chlorobenzoate

Bacillus sp. (Spokes & Walker, 1974a)

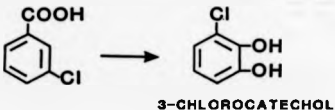


Arthrobacter sp. (Horvath & Alexander, 1970)

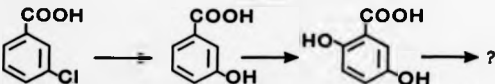


Azotobacter spp., Pseudomonas fluorescens

(Walker & Harris, 1970; Ichihara et al., 1962)



Pseudomonas sp. (Johnston et al., 1971)



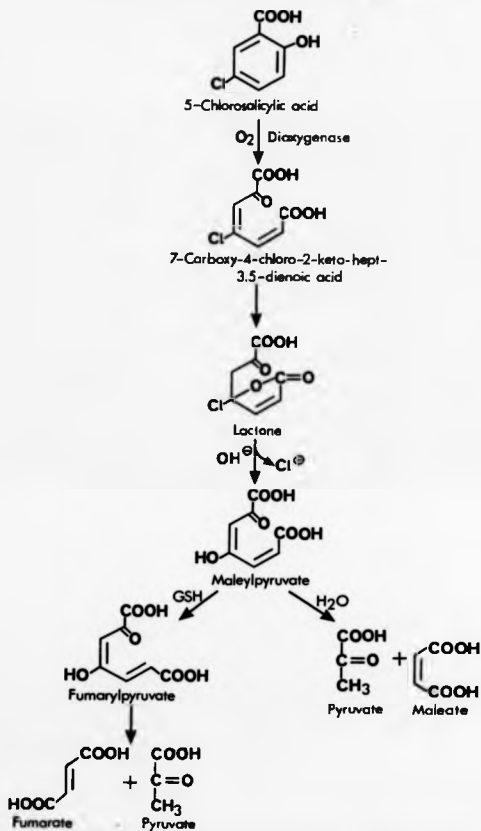
Most organisms degrade benzoate via catechol (Dagley, 1971) but exceptions include a non-sporing thermophilic bacterium which metabolized benzoate via 2,5-dihydroxybenzoate (gentisate) (Buswell & Twomey, 1974). The only bacterial species previously known to form salicylic acid from benzoate were Azotobacter vinelandii (Walker & Harris, 1970) and Pseudomonas convexa var. hippuricum (Bhat et al., 1959). However, in these organisms catechol was the next product to be formed. Therefore, the Bacillus sp. appears to oxidize benzoate by a novel pathway via salicylic acid and 2,3-dihydroxybenzoic acids before ring cleavage.

A strain of Bacillus brevis isolated from a polluted section of the Mississippi River was shown to utilize 5-chloro-2-hydroxybenzoate (5-chlorosalicylate) as the sole source of carbon and energy (Crawford et al., 1979b). Halogenated aromatic compounds are often particularly resistant to microbial degradation but there are a few known examples of degradative enzymes which are quite specific for chlorinated substrates. For example, pyrocatechase II, isolated from 3-chlorobenzoate-grown cells of Pseudomonas sp. B13, showed higher relative activities with 3- and 4-chlorocatechol than with catechol (Dorn & Knackeuss, 1978). Bacillus brevis synthesised a specific 5-chlorosalicylate, but not gentisate, as a substrate (Crawford et al., 1979b). This enzyme was unusual in that it cleaved the aromatic ring while it was

substituted with only one hydroxyl group. Usually, prior to ring fission, the aromatic nucleus must contain at least two hydroxyl groups (Dagley, 1971). The Bacillus brevis strain catabolized 5-chlorosalicylate via maleylpyruvate (Figure 1.13) which was degraded by both glutathione(GSH)-dependent and glutathione-independent pathways, since crude extracts contained maleylpyruvate hydrolase and fumarylpyruvate hydrolase activities. To observe degradation of maleylpyruvate via fumarylpyruvate, GSH must be added since insufficient quantities are present in cell extracts. It has been observed that both GSH-dependent and GSH-independent variations of the gentisate pathway are common among other representatives of the genus Bacillus, when grown on gentisate or hydroxybenzoate, and maleylpyruvate appears to be an important catabolic intermediate (Crawford et al., 1979a; Crawford & Frick, 1977).

Crawford et al. (1979b) have suggested that the pathway by which Bacillus brevis degraded 5-chlorosalicylate may provide an example of divergent evolution. Gentisate 1,2-dioxygenase, an enzyme that has been found in several other Bacillus spp. (Crawford, 1975b) may have evolved in Bacillus brevis so that 5-chlorosalicylate was attacked in preference to gentisate. The subsequent steps of the degradative pathway need not have changed immediately since loss of chlorine and formation of maleylpyruvate occur slowly nonenzymically. Further evolution could have

Figure 1.13 Degradation of 5-chlorosalicylic acid by
Bacillus brevis (Crawford et al., 1979b)



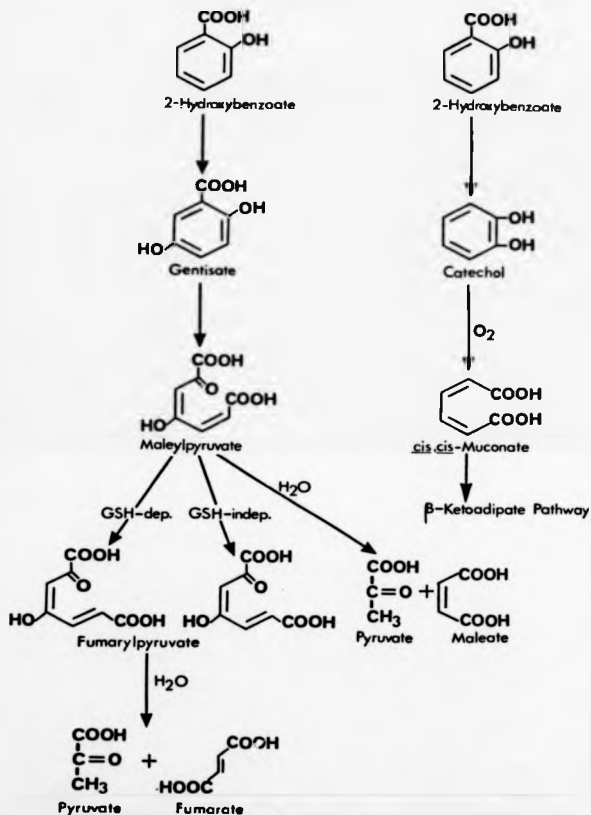
recruited an enzyme to catalyze the dehalogenation reaction. Furthermore, selective pressure for the evolution of enzymes able to degrade chlorinated aromatic compounds must exist since these compounds are often recalcitrant. However, no further experiments, such as amino acid sequencing of the enzymes, have been carried out to help confirm or contradict the suggestion that 5-chlorosalicylate-1,2-dioxygenase evolved from gentisate 1,2-dioxygenase.

Many bacilli appear to degrade 2-hydroxybenzoate (salicylate) via gentisate. Crawford & Olson (1979) investigated the catabolism of 2-hydroxybenzoate by three strains of Bacillus megaterium and eleven strains of Bacillus brevis. They concluded that four primary pathways were used by the bacilli to degrade salicylate (Figure 1.14). Only two of the strains degraded salicylate via the catechol branch of the β -ketoadipate pathway. The remaining strains degraded salicylate using the gentisate pathway, of which three variations have arisen, probably due to divergent evolution. This catabolic diversity illustrates that the bacilli are a genetically diverse evolutionary group. This is further borne out by the observation that three Bacillus species degrade 4-hydroxybenzoate by three different mechanisms (see Section 1.2.3) (Crawford, 1976).

Various other aromatic compounds which have been found to serve as the sole source of carbon and energy for Bacillus

Figure 1.14 Pathways used by various *Bacillus* strains to degrade 2-hydroxybenzoate (salicylate)

(Crawford & Olson, 1978)



species include nicotinic acid (Hirschberg & Ensign, 1972), phenylalanine (Wallnoffer & Engelhardt, 1971), pentachlorophenol (Chu & Kirsch, 1972) and various alkylbenzene sulphonate homologues (Willetts & Cain, 1972). Willetts (1974) investigated a Bacillus sp. which could grow on various aromatic compounds including alkylbenzene sulphonate detergents. In this organism, *p*-hydroxybenzoate was initially hydroxylated to protocatechuate, which was cleaved by a substrate-specific 3,4-oxygenase (ortho-cleavage). In strict contrast, the higher homologue *p*-hydroxyphenylacetate (PHPA), after initial hydroxylation to 3,4-dihydroxyphenylacetate, was oxidatively cleaved by a substrate-specific 2,3-oxygenase (meta-cleavage) forming 4-carboxymethyl-2-hydroxyisocinnic semialdehyde which the bacteria were unable to metabolize further. This provides an example of the occurrence of two distinct oxygenase-catalyzed cleavage mechanisms for two similar dihydroxy-substituted aromatic moieties within a single microorganism.

In conclusion, the bacilli are a diverse group of bacteria able to degrade a wide range of aromatic compounds. Indeed, certain Bacillus spp. can attack halogenated aromatics which are known to be particularly recalcitrant (Spokes & Walker, 1973). Consequently, on consideration of the abundance of bacilli in soil and water environments and their nutritional versatility, it seems almost inevitable that strains of Bacillus spp. should degrade toluene.

1.4.2 Aromatic-utilizing thermophilic bacteria

On the basis of the temperature range of growth, bacteria are frequently divided into broad groups: thermophiles grow at elevated temperatures (above 55°C); thermotolerant organisms grow at 40-55°C; and mesophiles grow below 40°C. Most of the literature on the catabolism of aromatic compounds concerns mesophilic microorganisms. However, thermophilic species are found in many bacterial genera (but predominantly bacilli) and often ferment similar carbohydrates, utilize similar nitrogen sources and have similar oxidative pathways to mesophilic bacteria (Ljungdahl, 1979).

Early experiments with enrichment cultures suggested that phenolic compounds could serve as growth substrates for thermophilic bacteria (Egorova, 1942 & 1946). More recently, Buswell and Twomey (1974) reported the oxidation of benzoic acid by an unidentified thermophilic bacterium. The bacterium was isolated from soil and had a temperature optimum of about 60°C. Subsequently Buswell & Clark (1976), reported that 4-hydroxybenzoate was converted to gentisate by a facultative thermophilic strain of Bacillus. This Bacillus sp., isolated from coke-oven washings from a steelworks, had a temperature optimum of 55°C. Bacteria grown in the presence of 4-hydroxybenzoate rapidly oxidized benzoate and gentisate but not catechol or protocatechuic acid. Also, gentisate 1,2-dioxygenase activity was detected in cell extracts. The implication of gentisate as an intermediate in 4-hydroxybenzoate metabolism is an example of intramolecular migration ("NIH shift") (see Section 1.2.2.1.3).

B.stearothermophilus, strain PH24, has been reported to grow on phenol and the isomeric cresols and has the ability to oxidize a wide variety of aromatic compounds at 55°C (Buswell & Twomey, 1975; Buswell, 1975). In this bacterium phenol and the isomeric cresols were catabolized via catechol and the methylcatechols, respectively. These compounds were then further degraded by the meta-cleavage route. Both NAD⁺-independent hydrolase and NAD⁺-dependent semialdehyde dehydrogenase activities were detected against 2-hydroxy-muconic semialdehyde with extracts prepared from phenol-grown B.stearothermophilus PH24 (Buswell, 1974) (see Figure 1.6). Initial experiments indicated that the semialdehyde-metabolizing enzymes from Bacillus PH24 are both fairly stable and high activity was readily detected at 50°C. Furthermore, temperature inactivation studies indicated that the hydrolase activity was the most stable. However, it was necessary to stabilize the catechol 2,3-oxygenase activity in crude extracts by the addition of 10%(v/v) acetone since the enzyme was unstable in the presence of air, as is the case for catechol 2,3-oxygenase preparations from mesophilic bacteria (Nozaki et al., 1963; Sala-Trepat & Evans, 1971a). This finding suggests that oxygenases, even those isolated from thermophilic organisms, are very unstable.

It appears that the thermophilic aromatic-utilizers previously studied were predominantly Bacillus sp. However, few generalizations can be made due to the paucity of information available, which illustrates the need for further research on thermophilic and thermotolerant aromatic-utilizing bacteria.

1.5. COMMERCIAL POTENTIAL OF cis-TOLUENE DIHYDRODIOL PRODUCTION

Specific intermediates formed during the degradation of aromatic compounds by microorganisms may have biotechnological potential. Certain intermediates in aromatic pathways have already been exploited commercially, eg. cis-benzene dihydrodiol, and bioconversions are being used increasingly to produce high value-added chemicals. Many bioconversions are extremely difficult to duplicate chemically, especially in a single step reaction.

Pseudomonas putida Biotype A has been exploited to convert toluene to muconic acid, an important high volume commodity in the chemical industry (Hsieh, 1984). The ortho-cleavage of catechol produces cis,cis-muconic acid which is then further transformed by a muconate lactonizing enzyme. Since all known naturally occurring microorganisms metabolize toluene by meta-cleavage pathways, but often use the ortho pathway for the metabolism of other substrates, a mutant strain of P.putida was constructed. The mutant strain metabolized toluene using ortho-cleavage and lacked muconate- lactonizing enzyme activity. Hence, when the mutant strain was grown in the presence of toluene, muconic acid accumulated in the culture medium.

A further example of the use of biotransformations in organic synthesis is the production of fluorochemicals, which may be applied in the plastics, agricultural and pharmaceutical industries. Novel fluoroaromatic compounds have been

produced from 3-fluorophthalic acid by a combination of biotransformations using mutants of Pseudomonas testosteroni defective in the phthalic acid catabolism, and chemical modification of the products (Martin et al., 1987).

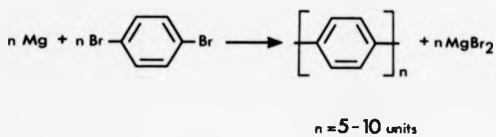
Another group of intermediates that have received some attention in recent years consists of the 3,5-dihydrocyclohexadienes (cis-dihydrodiols), which can be derived from aromatic hydrocarbons through the action of dioxygenases. The production of cis-benzene dihydrodiol may have great commercial potential since it can be polymerized to produce poly(1,4-phenylene). Plastics possess excellent physical and mechanical properties but typically are electrically insulating and so have not made an impact on the electronics industry except as dielectrics. However, organic polymers, such as polyphenylene, are good electrical conductors after the addition of metals and could be applied in the electronics industry (Greene & Street, 1984). Hence, there is currently great interest in electrically conducting organic solids which possess physical properties not found in inorganic conducting materials (White et al., 1984). Also, polyphenylene could be applied for both military and civil purposes as it will resist high temperatures and radiation levels. However, there are several difficulties associated with the chemical synthesis of polyphenylene. Condensation polymerization techniques (reaction 1, Figure 1.15) result in a polymer which is insoluble in known solvents and therefore only oligomers are formed. Also, the infusible polymer produced by reaction 1 is only capable of fabrication by

sintering and compression at high temperatures. Other approaches which do not involve polycondensation have been attempted to produce polyphenylene. Reaction 2 (Figure 1.15) illustrates the polymerization of cyclohexa-1,3-diene to give poly(cyclohexene) which involves the use of organometallic catalysts. However, attempts to aromatise the poly(cyclohexene) to give pure polyphenylene have failed due to the poor solubility of the partially aromatised product.

It was discovered that derivatives of cis-benzene dihydrodiol were readily polymerized, using radical initiators, to high molecular weight polyphenylene (Figure 1.16) (Ballard et al., 1983). The resulting polymer is an excellent insulator which, after the addition of metals, becomes a conductor. Furthermore, it has high temperature stability, is radiation-resistant, and is hydrophobic. However, the cis-benzene dihydrodiol required cannot be produced by any single chemical process (Nakajima et al., 1959). Consequently, the simplest route to produce cis-benzene dihydrodiol is the bacterial oxidation of benzene. ICI are currently investigating a process to produce cis-benzene dihydrodiol and other aromatic dihydrodiols using a mutant strain of Pseudomonas putida NCIB 11767 (Taylor, 1983 & 1984). The mutant (strain NG1) lacking cis-toluene dihydrodiol dehydrogenase was isolated by nitrosoquinidine mutagenesis and could no longer grow on benzene as the sole carbon and energy source. Therefore, an alternative growth substrate, such as glucose or ethanol, was required in addition to the substrate for product formation.

Figure 1.15 Chemical synthesis of polyphenylene

Reaction 1



Reaction 2

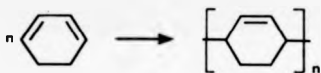
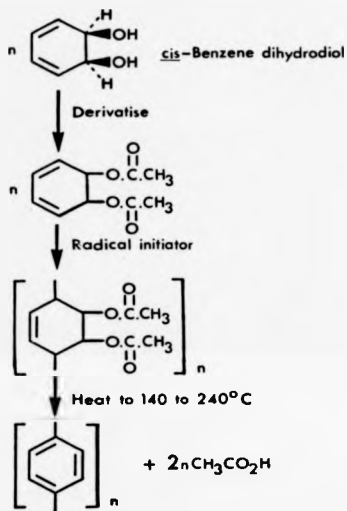


Figure 1.16 Synthesis of polyphenylene from cis-benzene dihydrodiol



$n = 600-1000$ units

Consequently, the bacteria can be grown on an inexpensive substrate while conserving the co-substrate (in this case benzene) primarily for product formation. The co-substrate is oxidized to carbon dioxide to regenerate NADH.

cis-Benzene dihydrodiol has other uses and may be used as a substrate for the chemical synthesis of a polyhydroxylated cyclohexane derivative, (+/-)-pinitol (Lay *et al.*, 1987). There is currently interest in the synthesis of cyclitol compounds, particularly in systems related to the cellular secondary messenger inositol-1,4,5-triphosphate (Osaki *et al.*, 1986).

On consideration of the great potential of cis-benzene dihydrodiol production, an initial aim of this project was to investigate whether this compound could be produced by thermotolerant organisms.

1.6. THERMOTOLERANT ORGANISMS IN INDUSTRIAL PROCESSES

The advantages of using thermophilic organisms in biotechnological processes have been discussed in reviews by Sonnleitner & Fiechter (1983) and Sharp & Munster (1986). The possible advantages of using thermotolerant organisms, as opposed to mesophilic organisms, in the production of cis-toluene dihydrodiol may include :

- i) The costs of cooling large-scale fermentations of thermotolerant organisms are reduced. The higher the temperature of cultivation, the larger is the temperature

gradient between culture and coolant and, hence, cooling is easier and cheaper.

- 2) Enzymes from thermotolerant organisms are, in general, more thermostable than homologous mesophilic enzymes (Singleton & Aselunxen, 1973). Therefore, purification of enzymes from thermotolerant organisms may result in higher enzyme recoveries owing to the enhanced stability. The enzyme thermolysin from the thermotolerant Bacillus thermoproteolyticus (an organism now considered to be a strain of Bacillus stearothermophilus) retains 86% of its activity after 30 hours at 70°C (Endo, 1961). This bacterium grows optimally at 53-55°C and although not a strict thermophile, the enzyme is certainly very thermostable.
- 3) Enzymes from organisms which grow at elevated temperatures tend to be more resistant to denaturing agents and more tolerant to higher substrate concentrations. For example, the aqualysins are proteases from a Thermus strain and are exceptionally stable towards denaturing agents, in addition to being very thermostable (Taguchi et al., 1983).
- 4) Immobilized enzyme systems would have a longer useful period of operation due to the greater stability of enzymes from thermotolerant organisms.
- 5) According to Arrhenius' law, an increase in temperature speeds up chemical and enzymatic reactions and consequently microbial growth and product formation. However, it must be concluded that, in general,

thermophiles do not have higher product formation rates. It was found, on comparing mesophilic and thermophilic ethanol producers, that the thermophiles, disappointingly, did not produce higher yields of product (Payton, 1984).

- 6) Operation at elevated temperatures may reduce the possibility of contamination by mesophilic organisms. However, it does not eliminate contamination by thermophilic spore formers (Zeikus, 1979) which may be fairly ubiquitous.
- 7) Another possible advantage of investigating thermophilic / thermotolerant organisms is that they may degrade certain compounds by novel reaction sequences which have hitherto not been discovered in mesophilic organisms. Hence, there is the possibility of obtaining new compounds which could have biotechnological applications.

On considering the viability of using organisms which grow at elevated temperatures to produce cis-toluene dihydriodiol, the possible disadvantages must also be weighed up. One disadvantage is that the solubility of gases decreases with increasing temperature (Sonnleitner, 1983). This can pose a problem for aerobic thermophilic or even thermotolerant cultures. The solubility of oxygen in water at 50°C is only 60% of the solubility at 20°C. Furthermore, the solubility of toluene is also reduced at higher temperatures. Consequently, greater agitation and aeration is required.

AIMS OF THE PRESENT WORK

There is a paucity of information on high-temperature bacteria which can utilize aromatic compounds as the sole carbon and energy source. Hence, a major aim of the present study was to isolate aromatic-utilizing, thermotolerant bacteria from various soil and water samples, including liquid samples collected from a waste treatment works, which should be rich in aromatic pollutants. An additional aim was to study the physiology of these isolates and elucidate the pathways used by them to degrade aromatic compounds.

The metabolism of toluene was studied specifically due to the current biotechnological interest in cis-benzene dihydrodiol and cis-toluene dihydrodiol production. Consequently, on obtaining isolates which catabolized toluene via cis-toluene dihydrodiol, a further aim was to produce mutants lacking the enzyme cis-toluene dihydrodiol dehydrogenase in an attempt to accumulate cis-toluene dihydrodiol. The catabolism of toluene was studied in preference to benzene catabolism due to the potential hazards of working with benzene, including its carcinogenic properties and explosive nature.

In summary, the metabolism of toluene by thermotolerant microorganisms was studied in order to gain an insight into the degradative pathways used and to investigate whether the advantages of using thermotolerant organisms in industrial processes could be exploited in the production of cis-toluene dihydrodiol.

CHAPTER 2 : MATERIALS AND METHODS

2.1 ISOLATION AND GROWTH OF BACTERIA

2.1.1 Media

A basic mineral salts medium (MS medium) was used throughout these studies for the routine growth of the organisms. Cells were grown at pH 6.8 (or pH 7.0 for strain AT50) on a medium containing (g.l^{-1}): KH_2PO_4 , 2; NH_4Cl , 3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4; trace-element solution, 2ml.l^{-1} . The trace-element solution was as described by Vishniac & Santer (1957) but containing only 0.22% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. Solid media was prepared by the addition of 20 g.l^{-1} Difco Bacto-agar to the medium before autoclaving.

2.1.2 Isolation of bacteria

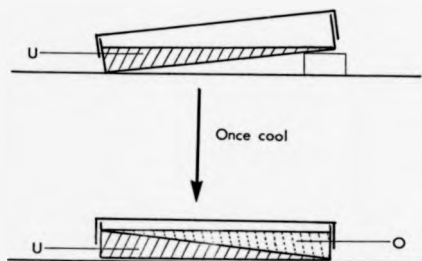
Toluene-utilizing bacteria were isolated from various soil samples and from effluent from a waste treatment plant. A loopful of soil or $100 \mu\text{l}$ of effluent was used to inoculate 250 ml flasks containing 50 ml MS medium. Liquid toluene was added to the medium to give final concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 % (v/v) and the flasks were closed with Suba-Seals. The flasks were incubated with shaking at a range of temperatures including 40°C , 45°C , 50°C , 60°C , 70°C . When turbidity developed the cultures were serially diluted to 1 in 10^6 and $20 \mu\text{l}$ samples of the 10^{-6} - 10^{-8} dilutions were spread

onto MS-agar plates. The plates were incubated at the appropriate temperature under toluene vapour. When small colonies had developed, loopfuls of the different colony types were streaked onto MS-agar plates and incubated again. The resulting colonies were used to inoculate flasks as before and the cycle described above was repeated at least twice more to ensure that the resulting colonies were pure. Culture purity was checked by careful examination of samples under the microscope and by ensuring that samples of diluted cultures gave rise to uniform colonies when spread on agar plates. If the isolates were still not pure then a further isolation step was carried out using antibiotic gradient plates. Gradients of 0-6 µg/ml chloramphenicol, 0-10 µg/ml streptomycin and 0-20 µg/ml kanamycin were used (Figure 2.1). A loopful of culture was streaked in a line across the centre of the plate and after incubation single colonies were picked off.

2.1.3 Maintenance and growth

The organisms were stored in L-broth containing 20% (w/v) glycerol at both -20°C and -70°C. Cultures were maintained by sub-culturing every 2 weeks on MS-agar plates. The plates were incubated in 10 litre paint tins fitted with air-tight lids. Prior to closing the lids, 5 ml of toluene was placed in a test-tube taped inside the tin. The containers were incubated at 30°C for Bacillus sp. AT50 and 45°C for Bacillus sp. HTB16.

Figure 2.1 Construction of antibiotic gradient plates



U = underlay (15 ml nutrient agar) poured and allowed to set before pouring the second medium as an overlay.

O = overlay (15 ml nutrient agar containing 6 $\mu\text{g/ml}$ chloramphenicol, 10 $\mu\text{g/ml}$ streptomycin or 20 $\mu\text{g/ml}$ kanamycin)

Large quantities of whole cells of the isolates were grown in continuous culture in a 2.5 litre working volume fermenter (LH Engineering Ltd., Stoke Poges, Bucks, UK) on MS medium. Toluene vapour was the sole carbon source and was supplied by bubbling the air stream through a reservoir of liquid toluene. The dilution rate was maintained at 0.1h^{-1} , with the growth limitation being toluene. The culture was maintained at pH 6.8 by automatic titration with 1M KOH. Air was supplied at $0.1\text{v.v}^{-1}\text{.a}^{-1}$ and the cultures stirred at 400 rpm. Flooding was prevented by the periodic addition of 3%(v/v) polypropylene glycol (PPG 2000) antifoam. For pyruvate-limited cultures 4 g.l⁻¹ pyruvic acid was added to the MS medium, which was supplied at a dilution rate of 0.1h^{-1} . Ammonium-limited cultures were grown on MS medium containing only 0.2 g.l⁻¹ NH₄Cl.

2.1.4 Measurement of cell density and growth rates

Cell density was measured routinely as the optical density at 540 nm. The total carbon content of cell suspensions were determined using a Beckmann Model 915-B total carbon analyzer (Beckmann Instruments Inc., Fullerton, California, USA). Samples were washed twice in K₂HPO₄ (0.01% w/v) before resuspending in distilled water. The cell carbon content was assumed to be 47% of the dry weight (Van Dijken & Harder, 1975) and thus the dry weight for the cell suspensions could be estimated. By dilution

of chemostat-grown cells, a curve of optical density at 540nm against dry weight was prepared for Bacillus sp. AT50. This gave an average dry weight value of an A_{540} of 1 of 0.3 mg.ml^{-1} .

Growth weights were determined by aseptically removing 1 ml samples at appropriate intervals and immediately measuring the optical density at 540nm. The growth rates were calculated from graphs of $\log_{10}(x/x_0)$ versus time, where x is the cell density and x_0 the initial cell density.

2.1.5 Identification tests

2.1.5.1 Gram stain

Several types of ostensibly Gram-positive bacteria demonstrate Gram variability. A positive Gram reaction is a feature of relatively young bacterial cells of some species and as cells age they may lose this characteristic. Therefore, Gram stains were performed on young cultures before the end of logarithmic growth. The method used was a modification of Kapeloff & Bearman (1922) as given by Cruickshank (1975).

2.1.5.2 Spore stain

The Schaeffer-Fulton spore stain was used (Cowan, 1974). Spores stain green and vegetative cells appear red.

2.1.5.3 Acid-Fast stain

Acid-fastness is shown when an organism resists decolouration by strong acids or mixtures of ethanol and mineral acid. This is a characteristic shown by a few bacteria and when positive is diagnostic of mycobacteria. The Ziehl-Neelsen's method was used (Cowan, 1974). Acid-fast organisms appear red, other organisms appear blue.

2.1.5.4 Catalase test

This test demonstrates the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide. A pure colony was placed on a clean, glass slide by means of a sterile platinum loop. A drop of 30% hydrogen peroxide solution was placed over the organism. The immediate production of bubbles indicates a positive result.

2.1.5.5 Oxidase test

This test depends upon the presence of certain oxidases that will catalyse the transport of electrons between electron donors in the bacteria and a redox dye, tetramethyl-p-phenylene diamine.

A strip of Whatman no. 1 filter paper soaked with freshly made reagent (1% w/v) was used immediately by rubbing culture into it using a clean platinum loop. A purple colour develops in 5-10 s if the result is positive.

2.1.5.6 Oxidation-Fermentation (OF) test

The principle of this test is to determine the oxidative or fermentative metabolism of a carbohydrate or its non-utilization. Some bacteria are capable of metabolizing a carbohydrate (as exhibited by acid production) only under aerobic conditions, while others produce acid both aerobically and anaerobically. The medium described by Hugh & Leifson (1953) was used. For each carbohydrate used, two stabs were performed for each isolate. One was sealed with liquid paraffin and the other left open. The tubes were incubated for up to 14 days. The results were interpreted as illustrated in Table 2.1. This medium can also be used for detecting motility.

2.1.6 Photomicrography of bacteria

A drop of molten Noble Agar (Difco, Detroit, Michigan, USA) was placed on a microscope slide and squashed flat before 10 μ l of bacterial culture was added and a cover slip placed on the agar. The culture was examined under a Leitz Ortho-Dialux microscope fitted with a camera. Kodak Panatonic-X film was used, developed in Kodak D19 developer for 3 min and fixed in Kodafix for 5 min.

Table 2.1 Interpretation of results for the OF test

(Cowan, 1974)

	Open	Closed
Oxidation	Yellow	Green
Fermentation	Yellow	Yellow
Neither	Blue or Green	Green

2.2 ELUCIDATION OF THE PATHWAY OF TOLUENE METABOLISM

2.2.1 Tests of growth substrate specificity

The ability of the various isolates to utilize a wide variety of substrates was tested by their ability to grow on the substrates in liquid MS medium. Cultures were incubated for up to 10 days to test for growth and any that grew were checked under the microscope for contaminants. The substrate concentrations were varied (as given in the text) because some of the substrates were toxic.

2.2.2 Preparation of cell suspensions and cell extracts

Cells grown in continuous culture were harvested at 4°C by centrifugation for 10 min at 20 000g. After washing the cells once with 20mM potassium phosphate buffer, pH 6.8, the cells were resuspended in the same buffer (1g wet wt cells/ml buffer). When necessary, cells were stored at -70°C.

Cell-free extracts were prepared using cell suspensions as prepared above, except that the cells were resuspended in a stabilization buffer of 20mM potassium phosphate, pH 7.2, containing 10% ethanol and 10% glycerol. The cell suspension was disrupted by 3 passages at 1.37×10^6 Pa through a chilled French pressure cell (Aminco, Maryland, USA). Debris and unbroken cells were removed by

centrifugation at 100 000xg for 30 min. This gave a clear brown supernatant, which was decanted off, and a dark brown pellet, which was discarded. The soluble extract was then immediately used for assays or frozen in pellet form by drop-wise addition to liquid nitrogen. The pellets were stored at -70°C.

2.2.3 Respiration studies

The ability of the isolates to oxidize various substrates was investigated by measuring the stimulation of oxygen uptake on addition of the substrate to cell suspensions. Oxygen consumption was measured using a Clark-type polarographic oxygen electrode (Rank Brothers, Bottisham, Cambridge, UK) polarized at -0.6V. The temperature was controlled by circulating heated water through a water jacket. The dissolved oxygen concentration in air-saturated buffer was determined by the method of Robinson & Cooper (1970). The fall in oxygen concentration is measured on addition of NADH to a reaction mixture containing phenazine methosulphate and catalase due to the following reaction sequence :-



The measured decrease in oxygen concentration was therefore proportional to the quantity of NADH added.

The electrode was calibrated to zero O_2 content by the addition of a few granules of sodium dithionite to the

reaction vessel containing 3 ml phosphate buffer.

Assays were routinely carried out at 45 °C in a stirred reaction mixture containing 2.85 ml pre-equilibrated 20mM potassium phosphate buffer, pH 6.8, 50 µl cell suspension and 100 µl substrate solution. In the case of substrates readily soluble in water, 100 µl of 150mM solution was added to give a final concentration of 5mM in the reaction mixture. Saturated solutions were used where it was impossible to obtain concentrations of 150mM, or the substrates were dissolved in ethanol. Rates of oxygen uptake were corrected for the endogenous respiration rate.

2.2.4 Enzyme assays

All enzyme assays were routinely performed at 45°C, unless otherwise stated, using a Pye Unicam double beam SPB-200 spectrophotometer with integral chart recorder. Cuvettes of 1cm light path were used throughout. One unit of enzyme activity was defined as the amount of enzyme required to transform 1 µmole of substrate per min.

2.2.4.1 Toluene dioxigenase

Three different assay systems were used in the study of this enzyme.

a) Polarographic assay

Activity of cell extracts was determined polarographically by measuring toluene-dependent oxygen uptake by a modification of the procedure of Beechey & Ribbons (1972). The

reaction mixture (3 ml) contained extract; 0.3 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 1.0 μmol NADH; 100 μl toluene-saturated buffer, in 50mM potassium phosphate buffer (pH 7.2). The extract was incubated with FeSO_4 for 5 min in the dark prior to addition to the reaction vessel. The order of addition was treated enzyme, NADH and then toluene. The rates of oxygen consumption were corrected for the endogenous rate in the absence of toluene.

b) Spectrophotometric indole assay

The method of Jenkins & Dalton (1985) was used. The assay is based on the finding that several bacteria able to oxidize aromatic hydrocarbons to cis-dihydrodiols also oxidize indole to cis-indole dihydrodiol which then undergoes spontaneous elimination of water to form indoxyl, which is a yellow dye and its formation can be followed at 400nm. Reaction mixtures (3 ml) in 25mM potassium phosphate buffer, pH 7.2, contained 0.3 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 1.0 μmol NADH; cell extract; 17 μmol indole. Absorbance at 400nm was measured against a blank, placed in the spectrophotometer reference beam, containing all ingredients except indole.

c) Assay using [^{14}C]-toluene

Attempts were made to assay toluene dioxygenase activity by measuring the formation of radioactive cis-toluene dihydrodiol, as devised by Yeh et al (1977). Reaction mixtures in a total volume of 0.2ml PEG buffer (pH 7.2) contained 0.12 μmol NADH, 0.15 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 μmol

unlabelled cis-toluene dihydrodiol, 0.014 μmol [^{14}C] CH_3 1-toluene and cell extract. The reaction was initiated by the addition of [^{14}C] CH_3 -1-toluene. After 12 min, enzymic reactions were terminated by the addition of 10 μl of 1M NaOH. 10 μl of the reaction mixture was applied directly to the base of a silica gel thin layer chromatography sheet with fluorescent indicator (Merck, Darmstadt, Germany). Chromatograms were developed in chloroform : acetone (80 : 20) and the reaction product, cis-toluene dihydrodiol, was located by viewing under ultraviolet light. The area containing the cis-toluene dihydrodiol was cut out and put in a vial containing 3 ml of scintillation fluid. Radioactivity in each sample was detected in a LKB liquid scintillation counter (model Wallac 1212 mini beta).

2.2.4.2 cis-Toluene dihydrodiol dehydrogenase

The enzyme was assayed by a modification of the method of Reiner (1972). Assays were routinely carried out at pH 8.0 and 45°C. Reaction mixtures in a total volume of 3 ml 20mM Tris-HCl buffer (pH 8.0) contained: 1.5 μmol cis-toluene dihydrodiol, 1.5 μmol NAD $^{+}$ and extract or purified enzyme. NADH formation was followed at 340nm. The blank lacked cis-toluene dihydrodiol. For pH stability and pH optima studies the following buffers were substituted for the Tris-HCl buffer : 50mM citrate buffer (pH range 3-6), 50 mM Tris-HCl buffer (pH range 6-9) and 50mM carbonate-bicarbonate buffer (pH range 9-10.4). The change in buffer had minimal effect on the activity of the

enzyme. The stability studies were performed by incubating the enzyme at the selected pH value for 30 min at 4°C and then titrating back to pH 8.0 and assaying at pH 8.0.

2.2.4.3 Alcohol dehydrogenase (EC 1.1.1.1)

The same method as above was used but 660 μ mol ethanol was substituted for the cis-toluene dihydrodiol.

2.2.4.4 Catechol 2,3-oxygenase (EC 1.13.1.2)

The method of Feist & Hegeman (1969) was used. Reaction mixtures in a total volume of 3ml 33mM Tris-HCl buffer (pH 7.6) contained 0.2 μ mol catechol and extract. The rate of accumulation of 2-hydroxymuconic semialdehyde was followed at 375nm.

2.2.4.5 Catechol 1,2-oxygenase (EC 1.13.11.1)

The assay for this enzyme was based on the method of Hegeman (1966). Reaction mixtures (3ml) contained 4 μ mol EDTA, 0.3 μ mol catechol and extract, in 67mM potassium phosphate, pH7.2. The formation of cis,cis-muconate was followed at 260 nm in quartz cuvettes.

2.2.4.7 Isocitrate dehydrogenase [L. Isocitrate: NADP oxidoreductase, (decarboxylating) EC 1.1.1.42]

The assay method was essentially that reported by Daron et al. (1966), as described by Norris & Ribbons (1971). The reaction mixture, which consisted of a total volume of 3mls of 30mM Tris-HCl (pH 7.5), contained 120 μ mol MnCl₂,

30 μ mol NADP⁺, 30 μ mol sodium isocitrate and extract. The reaction was followed at 340nm.

2.3 ENZYME PURIFICATION AND CHARACTERIZATION

2.3.1 Purification of cis-toluene dihydrodiol dehydrogenase from *Bacillus* sp.AT50

The enzyme was purified from cell extracts using a 4 step purification procedure described below. Steps 2-4 were carried out at 4°C.

Step 1: Heat treatment

The cell extract was heated for 90 min at 75°C in a water bath, centrifuged at 38,000xg for 30 min and the precipitate discarded.

Step 2: DEAE-Sephacrose

The supernatant from step 1 was applied to a column (2cm x 28cm) of DEAE-Sephacrose CL-6B (Sigma, Poole, Dorset, UK) previously equilibrated with 20mM potassium phosphate buffer, pH 7.2. The column was washed with 20 ml of the same buffer after which the cis-toluene dihydrodiol dehydrogenase activity was eluted using a linear gradient of 0.05M sodium chloride in the same buffer and 5ml fractions were collected.

Step 3: Hydroxylapatite column chromatography

The peak fractions (37-39) from step 2 were diluted 1:1 in 20mM potassium phosphate buffer (pH 7.2) to reduce the concentration of sodium chloride in solution. The resulting protein solution was applied to a column (2.5cm

x 5cm) of hydroxylapatite (Bio-Rad Ltd, Watford, Herts, UK). The column was washed with 40ml buffer before the enzyme activity was eluted with 400ml of a linear gradient of 20-500mM potassium phosphate buffer, pH 7.2. 5ml fractions were collected.

Step4: 5' cAMP-Sepharose column chromatography

The peak fractions (24-26) from step 3 were pooled and further purified by affinity chromatography on a 5' cAMP Sepharose (Sigma, Poole, Dorset, UK) column (2cm x 11cm). The column was washed with 20mM potassium phosphate buffer (pH 7.2) until protein could no longer be detected in the eluate. The *cis*-toluene dihydrodiol dehydrogenase was eluted by passing 100ml 10mM NADH through the column and 5ml fractions were collected. The peak fractions (24 & 25) were pooled and concentrated by ultrafiltration through a PM10 membrane (Amicon Ltd) to remove NADH.

2.3.2 Molecular Weight Determination by Gel Filtration

The molecular weight of *cis*-toluene dihydrodiol dehydrogenase from *Bacillus* sp. AT50 was determined by gel filtration on a column (2.5cm x 62cm) of Ultragel AcA34 (LKB, Croydon, Surrey, UK). Catalase (M_r 232 000), lactate dehydrogenase (M_r 140 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 63 000) and chymotrypsinogen A (M_r 25 000) were used as standards. A 1ml sample containing *cis*-toluene dihydrodiol dehydrogenase and marker proteins was applied to the column at pH 9.0, eluted at a flow rate of 20 ml. h^{-1} and 5ml fractions

collected. Proteins were located by measuring the absorbance of the fractions at 280nm and by assaying fractions for enzyme activity.

2.3.3 Polyacrylamide gel electrophoresis (PAGE)

2.3.3.1 Slab gels

The procedure of O'Farrell (1975) was followed for the preparation and running of 18 x 15 x 0.2 cm slab polyacrylamide gels. The discontinuous buffering system of Laemmli (1970) was used. 10-30% (w/v)-acrylamide gradient-gels were used with the resolving gel prepared in 3.0M Tris-HCl, pH 8.8, and the stacking gel in 0.5M Tris-HCl, pH 6.8. The running buffer was Tris-glycine, pH 9.5 (0.025M Tris base, 0.192M glycine). For SDS-dissociating gels, the stacking gel, resolving gel and running buffer contained SDS at 0.1% (w/v) and 2-mercaptoethanol at 10mM. For non-dissociating gels, electrophoresis was carried out for 2000Vh. For SDS-dissociating gels, a constant current of 7mA was applied until the tracking dye, bromophenol blue, reached the bottom of the gel.

2.3.3.2 Sample preparation

Prior to loading on to non-dissociating gels, bromophenol blue (BPB) was added to each sample [10 μ l 0.1% (w/v) BPB/0.5ml sample]. For SDS-dissociating conditions, the protein samples were routinely denatured in the presence

of SDS and mercaptoethanol using Laemmli sample buffer (Laemmli, 1970). When added to an equal volume of protein sample, this gave final concentrations of: Tris-HCl, pH 6.8, 62.5mM; SDS, 2%(w/v); 2-mercaptoethanol, 5%(v/v); glycerol, 10%(v/v) and BPB, 0.01%(w/v). Complete denaturation was effected by heating at 100°C for 2 min before application to the gel.

2.3.3.3 Photography of polyacrylamide gels

Gels were photographed on a light box using Kodak Panatomic-X (32 ASA) 35mm monochrome film which was developed for 3 min in Kodak D19 developer and fixed in Kodafix for 5 min. A yellow filter (Pentax) was used to improve the contrast with Coomassie blue stain.

2.3.4 Isoelectric focusing

2.3.4.1 Tube gels

Isoelectric focusing was carried out as described by O'Farrell (1975). Gels were made in glass tubes (130mm x 2.5mm) and were run under urea-dissociating conditions. The pI value was determined using standards, pH range 3-10, obtained from Pharmacia (Hounslow, Middlesex, UK) by measuring the pH in the gradient at the position of the major band.

2.3.4.2 Slab gels

The pI value for the native protein was determined using

LKB (Bromma, Sweden) Ampholine PAG plates. The gel was placed on a Multiphor (LKB, Bromma, Sweden) which was cooled by circulating water and the power was supplied at 40mA for 4h. The pI markers used, in the range 5.65-8.3, were obtained from LKB (Bromma, Sweden).

2.3.5 Staining of Polyacrylamide Gels

2.3.5.1 Coomassie blue staining

Gels were immersed in a staining solution consisting of 45% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.1% (w/v) Coomassie Brilliant Blue R250 for 4-5h on a shaking platform. Destaining was effected by 3-4 successive soaks in 45%(v/v) methanol, 10% (v/v) glacial acetic acid.

2.3.5.2 *cis*-Toluene dihydrodiol dehydrogenase activity stain

cis-Toluene dihydrodiol dehydrogenase activity was located by activity staining using a modification of the method of Rogers & Gibson (1977). The gels were immersed in a mixture of 20mM Tris-HCl, pH 8.0; PMS, 25 $\mu\text{g} \cdot \text{l}^{-1}$; nitro blue tetrazolium, 200 $\mu\text{g} \cdot \text{l}^{-1}$ and NAD^+ , 1.16 $\text{g} \cdot \text{l}^{-1}$. After 3 min, *cis*-toluene dihydrodiol (3 $\text{mg} \cdot \text{ml}^{-1}$) was added and a blue/black stain indicated *cis*-toluene dihydrodiol dehydrogenase activity.

2.3.5.3 Silver staining

The isoelectric focusing gels were stained with silver stain since this method is up to 100 times more sensitive than Coomassie blue staining.

For silver staining, all solutions were made up in deionised water. Gels were transferred to Analaar methanol overnight on a gently shaking platform and then placed in up to 5 changes of Analaar methanol over 3h. The gels were then washed twice in deionised water for 1h. The staining solution was as follows:

Solution A: 1.6g silver nitrate (Johnson Matthey Ltd) dissolved in 8 ml deionised water.

Solution B: 42ml of 0.36%(w/v) sodium hydroxide containing 2.5ml of "0.880" ammonia solution (BDH).

Solution A was added dropwise to solution B with constant stirring and then made up to 200ml. The gel was immersed in the staining solution for 15 min with gentle agitation and then washed in 3 changes of deionised water for 5 min each. The protein bands were visualised with developer solution, prepared by the addition of 2.5ml of 1% (w/v) citric acid and 0.4ml of 37%(v/v) formaldehyde solution to 500ml deionised water. The staining process was stopped by transferring the gel into 45%(v/v)methanol-10%(v/v) acetic acid.

2.3.6 Determination of the stoichiometry of the reaction catalyzed by *cis*-toluene dihydrodiol dehydrogenase

To investigate the stoichiometry of the reaction, the assay was carried out at 45°C and pH 7.0 with 0.5mM *cis*-toluene dihydrodiol, 0.5mM NAD⁺ and 40 µg of purified enzyme. The amount of NAD⁺ converted into NADH was monitored at 340nm in

a Pye-Unicam spectrophotometer. After 30 min, a sample of the assay mixture was removed and the amount of 3-methylcatechol produced and cis-toluene dihydrodiol consumed was determined using HPLC (Jenkins & Dalton, 1985).

2.4 ENZYME IMMOBILIZATION

2.4.1 Immobilization of cis-toluene dihydrodiol dehydrogenase onto CNBr-activated Sepharose 4B

Purified cis-toluene dihydrodiol dehydrogenase was immobilized by covalent bonding using CNBr-activated Sepharose 4B (Sigma, Poole, Dorset, UK), which couples primary amino groups.

1g of CNBr-activated Sepharose 4B was resuspended in 1mM HCl solution and washed in the same solution on a sintered glass filter for 15 min before washing thoroughly with 0.1M NaHCO₃-0.5M NaCl, pH 8.3. About 1.2 mg of purified enzyme in solution was added to the slurry and stirred end-over-end for at least 1h at 4°C. The resulting suspension was washed on a sintered column with 0.1M NaHCO₃-0.5M NaCl, pH 8.3, before the slurry was resuspended in 10ml of 100mM Tris-HCl, pH 8. The suspension was stirred end-over-end for 1h at 4°C before washing in 100mM Tris-HCl (pH 8.0) to ensure all groups on the activated Sepharose had reacted with H⁺ ions.

2.4.2 Immobilization of cis-toluene dihydrodiol dehydrogenase onto alginate

Purified cis-toluene dihydrodiol dehydrogenase was immobilized by entrapment using sodium alginate. Alginates are polysaccharides obtained from seaweed. They gel in the cold in the presence of divalent cations, particularly calcium.

A 4% sodium alginate solution was added to an equal volume of enzyme solution, containing 0.6mg protein, and mixed with a glass rod. The mixture was dropped slowly from a syringe and needle into cold 0.1M CaCl_2 solution. The beads, which form on contact, were collected and washed in buffer containing 0.01M CaCl_2 for 1h.

2.5 GENETICS

2.5.1 Competence medium (CM medium)

Competence medium was used to grow cells of Bacillus sp. AT50 prior to transformation by plasmid DNA. The medium contained (g.l^{-1}): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.4; $(\text{NH}_4)_2\text{SO}_4$, 2; sodium citrate $\cdot 2\text{H}_2\text{O}$, 1.9; acid-hydrolysed casein, 0.2. The phosphates, K_2HPO_4 (14g.l^{-1}) and KH_2PO_4 (6g.l^{-1}) were autoclaved separately and added after cooling. Histidine, tryptophan and glucose were added at a final concentration of 5g.l^{-1} after being made up at 10 times concentration and filter sterilized.

2.5.2 Strains

Bacillus subtilis PY143 contains a heat-sensitive plasmid, pTV1, which encodes chloramphenicol (Cm) and erythromycin (Em) resistance. The bacteria were grown on Difco Penassay Broth (PAB medium) (Difco antibiotic medium No.3, Difco, Detroit, Michigan, USA) containing 20 µg/ml Em and 5 µg/ml Cm.

Bacillus subtilis 168 contains a small (2.9kb) plasmid, pC194, which encodes Cm resistance. This bacterium was used as a control when attempting plasmid isolation from other Bacillus strains. Since Bacillus subtilis 168 is unable to synthesize tryptophan or histidine, the PAB medium used for growth contained (µg/ml) : Cm,25; tryptophan,50; histidine,50.

2.5.3 Mutagenesis

Several different mutagenic procedures were attempted with Bacillus sp.AT50 and HTB16.

2.5.3.1 N-Methyl-N'-nitro-N-nitrosoguanidine

(NTG) mutagenesis

NTG mutagenesis of HTB16 was achieved by the exposure of a 50ml liquid culture to 20 µg/ml NTG for 70 min during exponential growth on 0.5% (w/v) pyruvate, resulting in 99.9% kill. For AT50, 99.9% kill was achieved by the addition of 40 µg/ml NTG for 80 min. After incubation with NTG, the cells were harvested by

centrifugation, washed in MS medium and grown overnight in 0.5% (w/v) pyruvate. The culture was serially diluted and plated onto nutrient agar. The resulting colonies were picked off and replica plated onto MS plates containing 0.5% (w/v) pyruvate and MS plates incubated in the presence of toluene vapour. Any mutants able to grow on pyruvate but not toluene were subjected to further characterization.

2.5.3.2 Ultraviolet (UV) light mutagenesis

A single colony from a NA plate was used to inoculate 50ml of MS medium containing 0.1% (v/v) toluene and incubated overnight at 45°C. 100 μ l aliquots were spread onto NA plates. The plates were exposed to UV light (an Anderson short wavelength UV lamp was used at a height of 15cm above the petri-dish) for various lengths of time and then immediately placed in the dark in a 45°C incubator. After overnight incubation, mutants amongst the survivors were detected by replica plating. Presumptive mutants were further checked to determine their phenotype.

2.5.3.3 Transposon mutagenesis

The plasmid pTV1, which contains the transposon Tn917, was isolated from B. subtilis PY143. The plasmid DNA was used to transform cells of Bacillus sp. AT50.

Bacillus sp. AT50 cells were transformed essentially as described by Bott and Wilson (1967). A single colony from a PAB plate (Difco antibiotic medium 3, Difco, Detroit,

Michigan, USA) at least 3 days old was inoculated into 50ml of PAB broth. After overnight incubation at 50°C, the culture was diluted to an OD_{600nm} of 0.1 with competence medium. When the cells became competent (achieved 3h after the culture had left log phase), 1ml aliquots of the culture were removed and stored at -70°C until required.

To transform the cells, 100 µl DNA solution was added to 1ml competent cells and incubated with vigorous agitation at 37°C for 30-45 min. The cells were then grown up in PAB broth before plating onto selective media.

2.5.4 Isolation of plasmid DNA

2.5.4.1 Small scale isolation of plasmid DNA

In order to rapidly screen cells for the presence of extrachromosomal DNA, mini plasmid preparations were performed as described by Ish-Horowitz & Burke (1981). 2ml of an overnight culture of cells were pelleted by centrifugation in a microcentrifuge (MicroCentaur, MSE, Sussex, UK), and resuspended in 100 µl solution I [glucose, 50mM; Tris-HCl (pH8.0), 25mM; Na₂EDTA, 10mM] containing 10 mg/ml lysozyme. After 10 min at 37°C, 200 µl freshly-made solution II [NaOH, 0.2M; SDS, 1% (w/v)] was added and the sample left for 5 min at room temperature after mixing gently by inversion. 150 µl of an ice-cold solution of 5M potassium acetate, pH 4.8 (made by adding 11.5ml glacial acetic acid and 28.5 ml H₂O to 60 ml of 5M potassium acetate) was added,

mixed gently and then left on ice for 5 min in a microcentrifuge. The supernatant fluid was extracted with 2.5 volumes of cold ethanol. The resulting DNA pellet was vacuum dried and analysed on agarose gels.

2.5.4.2. Large scale isolation of plasmid DNA

Plasmid DNA was isolated as detailed by Bryczan (1978). Cells from 1 litre of overnight broth (containing appropriate antibiotics) were harvested, washed and then resuspended in 40 ml of 50mM Tris-HCl (pH 7.4) containing 25%(w/v) sucrose, and 100mM sodium chloride. 1ml of lysozyme solution (200mg/ml) was then added and the cells were incubated at 37°C for 15 min. The following solutions were then added with gentle mixing after each addition:

9.6 ml NaCl, 5M

2.4 ml EDTA, 0.5M

50 ml SDS, 2% (w/v) in 0.7M NaCl

The lysate was mixed and left overnight at 4°C. Cell debris was removed by centrifugation (45 000g for 45 min at 4°C). The supernatant fluid was adjusted to 1M NaCl to remove chromosomal DNA by alkaline denaturation. Plasmid DNA was precipitated by the addition of polyethylene glycol (PEG 6000) to a final concentration of 10% (w/v). After leaving on ice for 1h, the DNA was pelleted by centrifugation (30 000g for 15 min at room temperature), and resuspended in 10mM Tris-1mM EDTA buffer, pH 8 (TE buffer). The lysate was then subjected to isopycnic centrifugation.

2.5.4.3 Isopycnic centrifugation and extraction of DNA

The DNA pellet was dissolved in 32 ml TE buffer (10mM Tris-1mM EDTA buffer, pH 8.0), to which was added 32g of caesium chloride and 2ml of ethidium bromide solution (5mg/ml in TE buffer). Centrifugation was carried out at 45 000 rpm for 16h at 20°C using a Beckmann VTi50 vertical rotor (Palo Alto, California, USA).

After centrifugation, the plasmid (lower) and chromosomal (upper) DNA bands were visualized with a UV transilluminator (Black-ray, Ultra-Violet Products Inc., California, USA), and the plasmid DNA band removed. This was done by puncturing the side of the centrifuge tube with a hypodermic needle and withdrawing 2 to 3ml into a syringe. Ethidium bromide was removed from a 3ml sample by extracting with 1.2ml Sarkosyl, 1% (v/v); 1.2 ml sodium acetate, pH5.6, 3M; 6.6ml 10mM Tris-0.1mM EDTA, pH 8.0; 2 volumes of 95% (v/v) ethanol (pre-cooled to -20°C). After 3h at -70°C or overnight at -20°C, the DNA was pelleted by centrifugation at 12 000xg for 10 min at 4°C. The pellet was washed in 75% ethanol and re-centrifuged. The supernatant was poured off and the pellet vacuum-dried and resuspended in 200 µl of TE buffer.

2.5.4.4 Treatment of DNA with restriction endonucleases

The DNA was cut according to the manufacturer's instructions. After incubation at 37°C for 5h, the restriction endonuclease

was inactivated by heating at 70°C for 10 min. If necessary, digestion with a second enzyme followed. If the second enzyme required a different assay buffer, the concentration of the buffer constituents was altered accordingly. If the size of the DNA fragments was to be estimated, λ DNA, digested with EcoRI and HindIII (Amersham, Bucks, UK) was used.

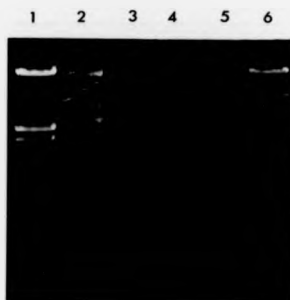
2.5.4.5 Agarose gel electrophoresis

'Mini-gels' were poured in mini-gel tanks (Cambridge Biotechnology Laboratories, London, UK) and were routinely used for the rapid analysis of DNA samples. 7% (w/v) agarose was used with Tris-borate buffer, pH 8.3 containing (g.l⁻¹) : Tris, 10.8; boric acid, 5.5; disodium EDTA, 0.95. Before loading the gel, 0.25 volume tracking dye containing Bromophenol blue and glycerol (50%v/v) was added to each DNA sample. Electrophoresis was carried out at a constant 40mA for about 30 min. After electrophoresis, gels were stained using ethidium bromide (about 10 µg/ml buffer, final concentration) and photographed using a UV transilluminator with a Polaroid camera.

2.5.4.6 Sizing plasmid pTV1

Plasmid pTV1 was isolated from Bacillus subtilis PY143. After isopycnic centrifugation, the plasmid band was extracted and purified. The plasmid was cut with various restriction endonucleases (Figure 2.2) to determine its size

Figure 2.2 Restriction endonuclease-digested pTV1 DNA
after agarose gel electrophoresis



Lane 1 : HindIII-EcoRI-digested λ DNA

Lane 2 : EcoRI-digested λ DNA

Lane 3 : XbaI-PvuII-digested pTV1 DNA (10.5, 1.4Kb)

Lane 4 : PvuII-digested pTV1 DNA (12.9Kb)

Lane 5 : XbaI-digested pTV1 DNA (11.5, 1.4Kb)

Lane 6 : HindIII-digested λ DNA

and confirm that it was pTV1 (which has been reported to be 12.4 Kb) (Youngman et al., 1983). pTV1 has only one PvuII recognition site. Since one of the XbaI recognition sites is very close to the PvuII site, a small fragment of DNA is lost when the DNA is digested with XbaI and PvuII. The purified plasmid appeared to have the same restriction sites as those reported for pTV1. The size was calculated to be approx. 12.9 Kb providing further evidence that the plasmid was pTV1.

2.6 ANALYTICAL DETERMINATIONS

Pyruvate was measured using a Sigma diagnostic kit (Sigma, Poole, Dorset, UK). Ammonia was assayed by the Chaney & Marbach (1962) method. cis-Toluene dihydrodiol and 3-methylcatechol were determined by HPLC using a Lichrosorb RP18 column (4 x 250mm) and a 254 nm UV-monitor (LKB, Bromma, Sweden). The solvent used was 60% methanol with a flow rate of 0.5 ml/min.

Protein was determined by a Biorad assay with bovine serum albumin as standard.

For the extraction of indigo, cells were grown in MS media containing indole (1mM), pyruvate [0.5%(w/v)] and toluene [0.1% (v/v)]. On dark blue colouration of the culture supernatant, an equal volume of chloroform was added and the indigo extracted overnight. The chloroform

phase was reduced to approx. one tenth the original volume and applied to the base of an aluminium backed TLC cellulose plate (Merck, Darmstadt, Germany). Chromatograms were developed in ethyl acetate before viewing the spots.

2.7 CHEMICALS

Most chemicals were obtained from the following manufacturers: Sigma (London) Chemical Co. Ltd., Poole, Dorset, UK; Fisons Scientific Apparatus, Loughborough, Leics, UK; Aldrich Chemical Co. Ltd., Gillingham, Dorset, UK; Kodak Ltd., Kirby, Liverpool, UK; Koch-Light Laboratories Ltd, Colnbrook, Bucks., UK; Pharmacia (Great Britain) Ltd., Hounslow, Middlesex, UK; BDH Chemicals Ltd., Poole, Dorset, UK.

3-Methylcatechol was obtained from Phase Separations Ltd., Queensferry, Clwyd, UK. [^{14}C]-Toluene was bought from Amersham International, Amersham, Bucks., UK. cis-Toluene dihydrodiol and cis-benzene dihydrodiol were gifts from Dr. S.C. Taylor, ICI, Billingham, UK. cis-Naphthalene dihydrodiol was a gift from Dr. R.D. Jenkins, University of Warwick, Coventry, UK. The organism Bacillus subtilis PY143 was a gift from Dr. J. Dultram, University of Aberystwyth, UK.

CHAPTER 3 : ISOLATION AND IDENTIFICATION OF
TOLUENE-UTILIZING BACTERIA

INTRODUCTION

Organisms that grow on aromatic compounds are very widespread and can be isolated from various soil or water samples, particularly in areas polluted with chemical waste. Consequently, soil samples obtained from polluted areas and liquid samples from a chemical waste treatment plant would be expected to be rich in aromatic-utilizing bacteria.

The principal aim of isolating new strains of bacteria which could grow on toluene was to obtain thermophilic or thermotolerant organisms capable of degrading toluene via cis-toluene dihydrodiol, a compound of commercial importance. For ease of metabolic studies, the bacteria isolated should have fast growth rates, grow well in continuous culture and possess high tolerance to aromatic compounds.

RESULTS AND DISCUSSION

3.1 Isolation of toluene-utilizing, thermotolerant bacteria

Toluene-utilizing bacteria were isolated from various locations in Warwickshire. A soil sample from farmland in Ashow, Warwickshire, appeared to be rich in humus and decomposing organic matter and was thought to be a likely source of aromatic-utilizing bacteria. Soil samples were also obtained from a Scottish Leach dump and from an ICI site in Billingham, Durham, where waste from various plants and refineries is ploughed into the soil and allowed to degrade naturally. Both these sites provided a rich supply of aromatic-degrading organisms. Lastly, liquid samples were obtained from a synthol works (Croda Hydrocarbon Chemicals) in Knottingley, Yorkshire. The effluent is the waste formed during the synthesis of various hydrocarbons. This effluent feed is kept at about 40°C and pumped to a large aeration tank which overflows to a small aeration tank. This in turn overflows to a settlement tank where the top layer of treated effluent is allowed to overflow into lagoons. Some of the settled sludge in the settlement tank is pumped back to the large aeration tank. Samples from each tank were used for isolation of bacteria but the small aeration tank proved to be the best source of microorganisms.

Continuous enrichment was used to isolate bacteria since theoretically the strain most suited to the culture conditions will be selected for and other slower growing bacteria will be washed out of the fermenter vessel. A small fermenter (25 ml working volume) was inoculated with a sample of soil. The culture was grown in batch mode at 45°C, with toluene as the sole carbon and energy source, before switching to continuous operation at a dilution rate of 0.05h^{-1} . The toluene was added by sparging the air through a toluene reservoir before it entered the fermenter vessel. Although some pure isolates were obtained using continuous enrichment (Table 3.1), they were not selected for further metabolic studies since they had relatively low growth rates on 0.1% toluene at 45°C.

Flask enrichments were also used to isolate bacteria (see Materials and Methods subsection 2.1.2), which were purified by the conventional technique of serial dilutions followed by plating out onto agar plates to give single colonies. Enrichment cultures were set up at a range of temperatures between 45-70°C. Growth was observed in the flasks set up at 45, 50 and 55°C but no growth was observed at 60, 65 and 70°C over 14 days. The cultures at 55°C exhibited poor growth within 14 days and no pure isolates were obtained. However, various isolates were obtained from the enrichment

Table 3.1

The origins of the various isolates

Strain number	Enrichment method	Isolation temperature (°C)	Source
AR4	Continuous enrichment	45°C	Soil sample from farmland at Ashow, Warwickshire
ICI9	Continuous enrichment	45°C	Soil sample from ICI chemical plant, Billingham
HTB16	Flask enrichment	45°C	Soil sample from Scottish leach dump
CN9	Flask enrichment	45°C	As above
AT50	Flask enrichment	50°C	Liquid sample from small aeration tank from Croda Hydrocarbon Chemicals, Knottingley

flasks incubated at 45°C and 50°C (Table 3.1).

If isolates were still not pure after successive serial dilutions of flask cultures followed by plating onto agar plates, then antibiotic gradient plates were used as an additional purification step. This purification procedure exploits the fact that all bacteria differ in their natural resistance to antibiotics. Since the purpose was purely to separate bacteria and not to select for antibiotic resistant mutants, low concentrations of antibiotic were sufficient. Consequently, gradients of 0-6 µg/ml chloramphenicol, 0-10 µg/ml streptomycin or 0-20 µg/ml kanamycin were used.

3.2 Selection of strains for further studies and their identification

Several isolates were obtained from various sources (Table 3.1). Strains HTB16 and AT50 were selected for further study since they grew more rapidly on 0.1% (v/v) toluene at 45°C than the other isolates (Table 3.2). Strain AT50 had a faster growth rate and higher temperature optimum (50°C) than strain HTB16, which had a temperature optimum of 45°C.

The strains HTB16 and AT50 were both Gram-positive, aerobic, rod-shaped bacteria. Although the rods of strain HTB16 were larger than those of strain AT50 (Figure 3.1), the strains share many common properties. They were both non-motile and

Table 3.2

Doubling times of the various isolates
on 0.1 % (v/v) toluene at 45°C

Isolate	Doubling time (h)
HTB16	7.5
IC19	8.5
AR4	12
CM9	8.5
AT50	5.2

Figure 3.1 The morphology of strains AT50 and HTB16

The bacteria were photographed as described in Materials and Methods subsection 2.1.6.

The photographs show the cultures at an enlargement of 2800-fold and the bars represent 5 μ m.

Strain AT50



Cells from:

(a) continuous culture (b) a stationary flask culture

Strain HTB16



formed oval sub-terminal spores but did not contain metachromatic granules. Positive results were obtained with both catalase and oxidase tests. Neither of the strains were capable of fermentative metabolism nor were they acid-fast. In addition they did not form irregularly-shaped, branched cells at any phase of growth. This suggested that they did not belong to the genera Mycobacterium, Cornebacterium or Actinomyces. The characteristics suggested that they were Bacillus species, according to the criteria of Bergey's Manual of Determinative Bacteriology (Gibson & Gordon, 1974).

3.3. DISCUSSION

The nature of enrichment cultures depends upon the environmental conditions imposed by the medium, nature of carbon and energy source, temperature etc. On inoculation of the medium with a mixed population of microorganisms, such as a sample of soil, then the organisms most adapted to the particular growth conditions provided will outgrow the other organisms. The use of continuous enrichment thereby enables the organism best suited to the conditions imposed to remain in the fermenter vessel whereas the slower growing organisms are washed out of the vessel with the overflow. Conversely, enrichments using flask cultures followed by serial dilutions do not necessarily lead to the isolation of

only the most highly adapted strain and several different isolates may be obtained. Consequently, continuous enrichment was used initially as it is supposedly a better method of isolation. However, using a very small laboratory fermenter (working volume of 25 ml) several failings of the apparatus became apparent. Firstly, the fermenter lacked automatic pH and antifoam control. In addition, due to insufficient mixing, it proved difficult to get sufficient toluene dissolved in the culture medium. Only very small amounts ($< 0.2\text{mM}$) of toluene were detected in the culture medium using gas chromatography and significant amounts were leaving with the outflowing air. Although toluene gas is very difficult to measure since it is not a true gas, toluene could be identified in the exhaust gas outlet due to its smell. Furthermore, this problem was magnified at higher temperatures when toluene was more volatile. As a consequence, flask enrichments which were simpler and quicker, were used subsequently.

Using both methods of enrichment, the various soil and liquid waste samples were found to be rich in thermotolerant, aromatic-degrading bacteria but no thermophiles were isolated at $60-70^{\circ}\text{C}$. The lack of thermophiles may simply reflect the fact that there were no thermophilic toluene-utilizers in the samples investigated. Alternatively, this finding may be due to the decreased

solubility of toluene at these temperatures. A large proportion of the toluene may have been present in the vapour phase above the medium. Unfortunately, toluene is very difficult to measure quantitatively and it was not possible to compare the amounts of toluene dissolved in the medium and the amounts in the vapour phase above the liquid.

The isolates selected for further studies were thermotolerant, aerobic, Gram-positive rods which were tentatively characterized as belonging to the genus Bacillus. Most of the literature on toluene-utilizing bacteria concerns pseudomonads. However, there have been a number of references to bacilli, including some thermophilic strains, capable of growth on phenolic compounds, benzoates, alkyl benzene sulphonate detergents and even halogenated aromatic compounds (see Section 1.4). Thermophilic and thermotolerant Bacillus species are fairly ubiquitous in both soil and water samples and have even been isolated from freshly fallen snow (Wolf & Sharp, 1981). Consequently, it is hardly surprising that thermotolerant Bacillus species capable of degrading toluene have been isolated. Indeed, the absence of reports on thermotolerant, toluene-utilizers probably simply reflects the fact that there have been no previous attempts to isolate these organisms.

CHAPTER 4 : GROWTH OF STRAINS HTB16 AND AT50 ON TOLUENE

INTRODUCTION

Due to the paucity of information on thermotolerant aromatic-utilizing bacteria (see Section 1.4.2), there is a general interest in the physiology of thermotolerant toluene-utilizers. There have been no reports in the literature on toluene-utilizing bacilli or indeed on any thermotolerant organisms capable of metabolizing toluene.

Shake-flask cultures have certain limitations since it is not possible to obtain cells in exactly the same physiological state from day to day. Secondly, it is difficult to produce sufficient quantities of cells to facilitate metabolic studies. Consequently, the growth of isolates, particularly Bacillus sp. AT50, in continuous cultures were investigated to enable sufficient quantities of cells in the exponential phase to be produced. The theory of gas-limited continuous culture is also discussed.

RESULTS AND DISCUSSION

4.1 Growth on toluene in shake-flask cultures

The growth conditions of strains HTB16 and AT50 have not been fully optimized but some observations were made which led to improvements in growth. Since iron is known to be required for several enzymes responsible for the degradation of toluene, attempts were made to optimize the trace element concentration of the medium. Various concentrations of Vishniac trace element solution (Vishniac, 1956) were investigated using strain HTB16 (Table 4.1). Halving the trace element concentrations (1ml Vishniac/litre medium) decreased the growth rate but on doubling the concentrations no increase in growth rate was observed. However, when the final concentration of ferrous sulphate was maintained at 0.01g.l^{-1} (as in the original Vishniac recipe), but all the other trace element concentrations halved, then no decrease in growth rate occurred. This illustrates the importance of ferrous ions, which are known to be required for various aromatic dioxygenases.

The optimum pH for growth was pH 6.8 for strain HTB16 and pH 7.0 for strain AT50. Both strains were thermotolerant organisms and the optimum temperature for growth of strain HTB16 was 45°C , the minimum and maximum temperatures being 30°C and 53°C respectively (Figure 4.1a). However, when

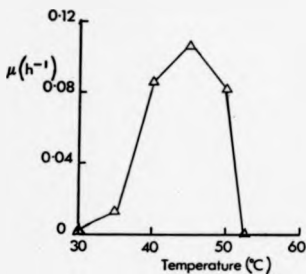
Table 4.1

The effect of altering the trace element concentration
with strain HTB16 grown on 0.1% (v/v) toluene

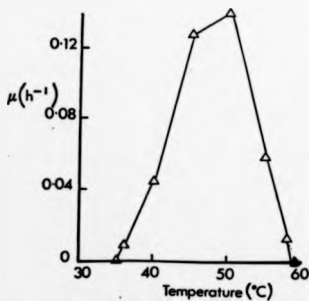
Trace element solution (ml.l ⁻¹)	Doubling time (h)
1	12
2 (Vishniac, 1956)	7.5
4	7.5

Figure 4.1 Effect of temperature on growth of strains HTB16
and AT50 on 0.1% (v/v) toluene

a) Strain HTB16



b) Strain AT50



grown below 40°C a long lag of about 30 hours was observed. The optimum growth temperature of strain AT50 was 50°C and it could not be grown below 34°C or above 58°C (Figure 4.1b). Therefore, strain HTB16 was subsequently grown at 45°C and pH 6.8, whereas strain AT50 was grown at 50°C and pH 7.0.

The optimum toluene concentration for growth of strain HTB16 was found to be 0.1% (v/v) toluene, giving a doubling time of 7.5h (Figure 4.2a). Since doubling time (t_d) is related to specific growth rate (μ) by the equation :

$$\mu = \frac{\ln 2}{t_d}$$

a doubling time of 7.5h corresponds to a growth rate of 0.092h^{-1} . The optimum toluene concentration of strain AT50 at 50°C was 0.2% (v/v) (Figure 4.2b). At this concentration a doubling time of 5h ($\mu = 0.139\text{h}^{-1}$) was observed. The growth rate dropped off with higher toluene concentrations indicating that high levels of toluene were toxic to the cells. Furthermore, growth of both strains HTB16 and AT50 on 0.5% (w/v) pyruvate was completely inhibited by the addition of 1% (v/v) toluene.

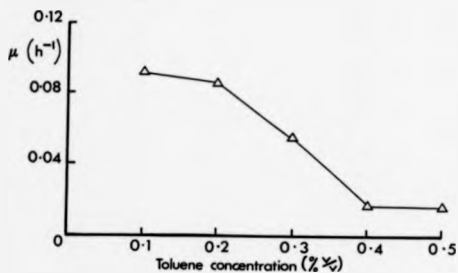
4.2 Growth of strain AT50 on toluene in continuous culture

4.2.1 Theory of continuous culture

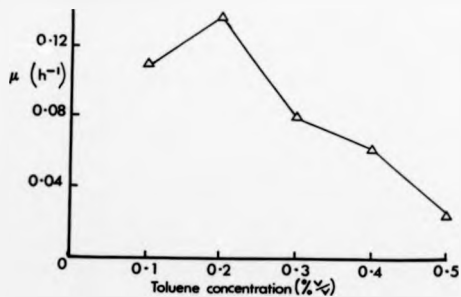
The theory of continuous culture has been described by

Figure 4.2 Optimum toluene concentrations for growth of strain HTB16 at 45°C and strain AT50 at 50°C

a) Strain HTB16



b) Strain AT50



Herbert et al. (1956).

The increase in the biomass of a culture is given by the biomass balance,

net increase in biomass = growth - output

and $\frac{dx}{dt} = (\mu - D)x$ (1)

dt where μ = specific growth rate

D = dilution rate

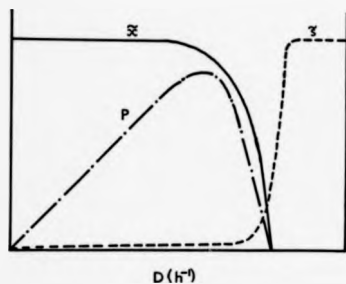
In a steady state, when $dx/dt = 0$, then $\mu = D$. Herbert et al. (1956) plotted out theoretical dilution rate and productivity curves from a continuous culture (Figure 4.3).

4.2.2 Theory of gas-limited continuous cultures

Strictly speaking, a continuous culture limited by a gas (in the gas phase) can neither be described as a chemostat nor a turbidostat. The main difference between a chemostat and a gas-limited culture is that in a chemostat the medium flow rate (i.e. dilution rate) is proportional to the rate of supply of the limiting substrate, whereas in a gas-limited system the rate of supply of the limiting substrate remains constant whatever the dilution rate (Stanley, 1977; Pirt, 1975).

Consequently, equations containing (or derived from) S_0 (limiting substrate concentration in inflowing medium) have no relevance to gas-limited cultures (Stanley, 1977).

Figure 4.3 Steady-state relationships in a continuous culture (theoretical)



x = Biomass

s = Substrate concentration (~ represents steady state values)

P = Productivity

$$\text{Since } F = DV \quad (2)$$

where F = flow rate, V = culture volume and productivity (P) is the amount of organisms leaving the fermenter per unit time, $P = xF$, then :

$$P = x \cdot DV \quad (3)$$

This is based on the following assumptions :-

- 1) The characteristics of the organism do not change with changes in dilution rate
- 2) the culture is able to utilize a constant amount of limiting gas per unit time over a whole range of dilution rates.

From these assumptions, if the rate of limiting substrate utilization is constant and the yield is also constant then the rate of biomass production will also be constant.

Equation (3) becomes : $xDV = \text{constant}$

but V is also a constant. Therefore,

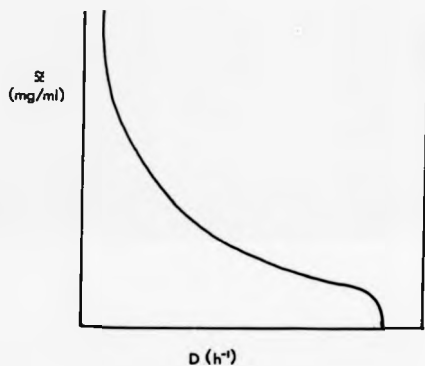
$$xD = \text{constant} \quad (4)$$

$$\text{or } x = \frac{1}{D} \text{ constant} \quad (5)$$

D

The concentration of biomass in the culture is therefore inversely proportional to the dilution rate (Figure 4.4). Clearly, this curve bears no relation to the dilution rate curve of Herbert et al. (1956). However, it is noteworthy that the assumptions may not hold true. The efficiency of

Figure 4.4 Theoretical dilution rate curve for a gas-limited continuous culture



growth may increase with an increase in dilution rate, imperfect mixing may occur and the amount of limiting gas consumed may not be constant over a wide range of dilution rates.

4.2.3 Toluene-limited continuous culture of strain AT50

Strain AT50 was grown as a batch culture for approx. 24h in a fermenter (2.5 litre working volume) to an optical density at 540 nm of 6-7. The fermenter was then switched to continuous operation with a dilution rate of 0.1h^{-1} . The fermenter was operated at 50°C with toluene as the sole source of carbon and energy. Air was sparged through a toluene reservoir before entering the fermenter vessel and consequently the rate of supply of toluene could not be measured. Due to the low solubility of toluene in water at 50°C , toluene was the growth-limiting factor and the levels of toluene in the medium were practically zero, as measured by gas chromatography. This was supported by the finding that pulsing the culture with liquid toluene (2 ml) caused the cell density to increase temporarily. Consequently, a relatively high air flow rate ($0.1\text{ v.v.}^{-1}\text{.a}^{-1}$) and high agitation (400 rpm) were necessary to improve the rate of gas transfer. Higher air flow rates and higher agitation did cause an increase in biomass but also had the adverse effect of increasing foaming due to shear forces. Since the fermenter lacked antifoam control, the compromise

values were reached and high dissolved oxygen concentration (approx. 95%) was maintained.

To investigate the best dilution rate for biomass production and efficient substrate utilization, a dilution rate curve was obtained for growth of Bacillus sp. AT50 in continuous culture (Figure 4.5). For each dilution rate studied a steady state was obtained by allowing time for 5 volume changes (Pirt, 1975). The plot of dilution rate (D) against culture density (x) did follow fairly closely to the predicted curves as did the plot of 1/D against x.

To estimate μ_m (maximum specific growth rate), the dilution rate was increased above D_c (critical dilution rate) and the rate of decline of culture density was followed. The μ_m was then calculated from the equation :

$$\ln x = (\mu_m - D)t + \ln x_0 \quad (\text{Pirt, 1975})$$

where the slope of the plot of $\ln x$ against t is equal to $(\mu_m - D)$.

The maximum growth rate was determined by increasing the dilution rate to 0.15 h^{-1} for several hours and following the decline in culture density (Figure 4.6). Since the slope was -0.0272 , the μ_m was calculated to be 0.123 h^{-1} ($D = 0.15 \text{ h}^{-1}$).

Consequently, the cells were routinely grown at a dilution rate of 0.1 h^{-1} (as described in Materials and Methods

Figure 4.5 Dilution rate curves from continuous cultures of *Bacillus* sp. AT50 grown on toluene

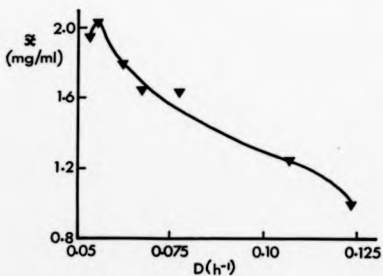
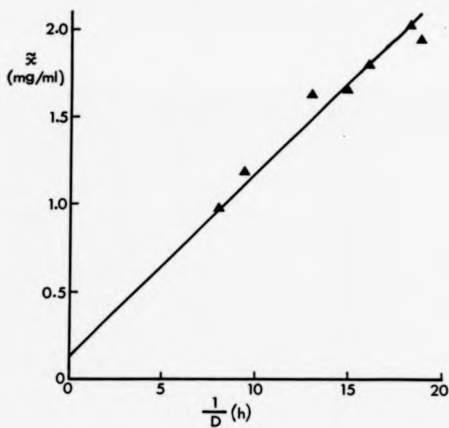
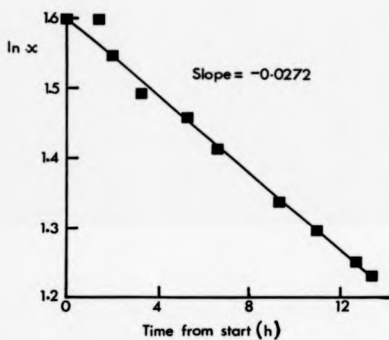


Figure 4.6 Calculation of μ_m of *Bacillus* sp. AT50 in continuous culture on toluene



subsection 2.1.3), which resulted in a steady state biomass concentration of 1.3mg/el.

4.3 DISCUSSION

The growth of strains HTB16 and AT50 on toluene in flask cultures was dependent upon the presence of ferrous sulphate in the medium. It is known that ferrous ions are required for both toluene dioxygenase and catechol 2,3-oxygenase activity in Pseudomonas putida (Subramanian *et al.*, 1985; Nakai *et al.*, 1983). However, catechol 1,2-oxygenase activity in P. putida requires ferric ions which are probably produced from ferrous ions after being taken up by the cells. Consequently, further experiments are necessary to elucidate the pathway of toluene metabolism and the organism's requirement for iron simply reflects the fact that several enzymes responsible for toluene oxidation require iron. There is evidence for a decreased iron requirement for both strains HTB16 and AT50 during growth on non-aromatic substrates since halving the iron concentration in the medium did not affect growth on 0.5%(w/v) pyruvate.

The optimum toluene concentration for growth of strain HTB16 at 45°C was 0.1% (v/v) toluene, whereas strain AT50 exhibited an optimum of 0.2% (v/v) toluene during growth at 50°C. However, due to the low solubility of toluene in water and the difficulty in measuring toluene gas, it was

not possible to measure the actual amounts of toluene present in the culture medium. It is possible that at 50°C more toluene remains in the vapour phase above the medium than at 45°C. Consequently, the higher toluene tolerance of strain AT50 may be due to a greater intrinsic tolerance to toluene or it may reflect the fact that more toluene is in the vapour phase at 50°C thereby creating a reduction in toxicity. Liquid toluene is known to be toxic to certain organisms and experiments have shown that the cell envelope of Gram-negative bacteria is damaged by toluene (De Saet et al., 1978). Toluene does appear to inhibit growth of both strains HTB16 and AT50 since lower growth rates are observed at toluene concentrations greater than 0.1% (v/v) or 0.2% (v/v) respectively (see Figure 4.1). Furthermore, growth of both strains on 0.5% (w/v) pyruvate was completely inhibited by the addition of 1% (v/v) toluene.

On comparing flask cultures and continuous cultures of strain AT50, it was observed that the specific growth rate (μ) in flask cultures (0.14h^{-1}) was greater than in continuous cultures (0.12h^{-1}). This illustrates that, in the continuous culture, toluene was supplied below the saturation constant, K_m (Pirt, 1975). In the small laboratory fermenter, toluene was limiting the growth of the culture and the effective concentration of the gas was practically zero in the culture itself. However, although

it could not be measured quantitatively, it was possible to smell that some toluene was leaving with the exhaust gas. This was due to the factors which affect the transfer of the toluene from the gas phase to the liquid phase and hence to the organism. These factors include the gas transfer rate (dependent on the partial pressure of the toluene in the gas and dissolved phases) as well as the growth rate of the organism. Although the growth of strain AT50 in continuous culture was not fully optimized, the use of continuous cultures did enable growth conditions to be monitored, quantified and repeated under identical conditions. Furthermore, the main purpose of working with continuous cultures was to obtain efficient substrate utilization and produce sufficient biomass to enable physiological studies of the bacteria. However, the optimization of culture conditions required for the bioconversion of toluene to *cis*-toluene dihydrodiol will, of course, be necessary once a suitable mutant is obtained.

CHAPTER 5 : ELUCIDATION OF THE PATHWAY OF TOLUENE METABOLISM

BY STRAINS HTB16 AND AT50

INTRODUCTION

There are several routes by which bacteria can metabolize toluene and three types of oxygenase exist (see Figure 1.9). There are two different toluene monooxygenase enzymes. Consequently, initial attack by toluene monooxygenase may give rise to benzyl alcohol (Kitagawa, 1956) or *p*-cresol (Richardson & Gibson, 1984). Toluene dioxygenase converts toluene to cis-toluene dihydrodiol (Gibson et al., 1968, 1970) and therefore the subsequent attack is by a different cleavage pathway. The toluene dioxygenase pathway was of primary interest since an initial aim of the present work was to produce cis-toluene dihydrodiol using thermotolerant bacteria. Consequently, after isolating bacteria capable of growth on toluene, it was necessary to elucidate the pathway by which toluene was metabolized. This was attempted using studies with both whole cells and cell extracts.

RESULTS AND DISCUSSION

5.1 Growth of strains HTB16 and AT50 on potential intermediates of toluene metabolism

The ability of strains HTB16 and AT50 to grow on intermediates of the three different pathways of toluene metabolism was tested (Table 5.1). *p*-Cresol, which is an intermediate of the toluene monooxygenase pathway described by Richardson & Gibson (1984), did not support growth of either strain. To ensure that the negative result was not due to its toxicity, this substrate was tested for toxicity by determining whether it inhibited growth of either strain HTB16 or strain AT50 on toluene at the original test concentration (0.1%v/v). No inhibition was observed. Since *p*-cresol was not toxic, it appeared that this substrate did not support growth simply because it was not oxidized by either strain. The most obvious explanation for this is that strains HTB16 and AT50 did not oxidize toluene via this monooxygenase pathway and did not possess the enzymes necessary for *p*-cresol oxidation. However, other possible explanations are that *p*-cresol was an ineffective inducer of the necessary enzyme activities required for growth on this substrate (eg. the activities might be induced by toluene coordinately with toluene monooxygenase) or that cells were unable to transport *p*-cresol across the cell membrane.

Table 5.1 Growth of strains HTB16 and AT50 on potential
intermediates of toluene metabolism

Carbon source	Concentration %(v/v)	Strain HTB16	Strain AT50
Toluene	0.1	+	+
Benzene	0.1	+	+
Phenol	0.05	+	-
p-Cresol	0.05	-	-
o-Cresol	0.05	-	-
Benzyl alcohol	0.05	-/i	-/i
Benzaldehyde	0.05,0.01	-/i	-/i
Benzoic acid	0.05(w/v),0.01	-/i	-/i

+ indicates growth

- indicates no growth after 10 days

i indicates inhibitory during growth on 0.5%(w/v) pyruvate

Further experiments, with both whole cells and cell extracts, were necessary to determine which explanation was correct.

Neither benzyl alcohol, benzaldehyde nor benzoic acid supported growth of strains HTB16 and AT50, when added at concentrations of 0.05%(v/v) or 0.01%(v/v). However, 0.05%(w/v) benzoate completely inhibited growth of strains HTB16 and AT50 on 0.1% toluene or 0.5%(v/v) pyruvate. Some inhibition even occurred with 0.01%(w/v) benzoate (with 0.1%(v/v) toluene), giving only 25% of the growth rate determined in the absence of benzoate. Growth of strain AT50 was also completely inhibited by 0.05% benzaldehyde whereas 0.05% benzyl alcohol only caused partial inhibition (approx. 25% inhibition). Similarly, benzyl alcohol and benzaldehyde (0.05%v/v) both caused partial inhibition (approx. 50% inhibition) of growth of strain HTB16. Since these possible intermediates were toxic to cells of strain HTB16 and AT50, this alternative monooxygenase pathway via benzyl alcohol cannot be ruled out.

Due to the instability of both cis-toluene dihydrodiol and 3-methylcatechol (which tends to polymerize above pH 6.0) at pH 7.0, neither of these compounds were investigated as growth substrates. However, both strains were capable of growth on benzene. Since it does not possess the additional methyl group, benzene must be oxidized by direct attack on

the ring. Therefore, it is likely that benzene is oxidized by a dioxygenase reaction via cis-benzene dihydrodiol to give catechol. Consequently, it may be predicted that bacteria capable of oxidizing benzene also oxidize toluene via cis-toluene dihydrodiol. This prediction is based on the assumption that benzene is not oxidized via phenol to catechol. This route is certainly uncommon, but was suggested by O'Connor et al. (see Mc Kenna & Kallio, 1965) and it should be noted that strain HTB16 did indeed grow well ($t_m = 7h$) on 0.05%(v/v) phenol. Therefore, further experiments are necessary to elucidate the pathway of toluene metabolism in strains HTB16 and AT50.

5.2 Oxidation of potential intermediates of toluene and benzene metabolism by whole cell suspensions

The oxidation rates of various potential intermediates of toluene metabolism by whole cell suspensions of strains HTB16 and AT50, after growth on toluene in continuous culture, were investigated (Table 5.2). According to the principle of simultaneous adaptation (Stanier, 1947), growth on one substrate endows cells with the ability to oxidize specifically that compound and the intermediates which are produced during its degradation. Conversely other compounds that are utilized via metabolically distinct pathways will not be oxidized immediately. Oxygen consumption by strains HTB16 and AT50 was minimal or non-existent with both

Table 5.2

Oxidation of potential intermediates of
toluene metabolism by whole cell suspensions

Substrate	Final Conc (mM)	Oxidation Rate	
		HTB16	AT50
Toluene	Sat.	81	105
<u>cis</u> -Toluene dihydrodiol	5	99	109
3-Methylcatechol	5	80	122
p-Cresol	Sat.	10	0
p-Hydroxybenzoate	5	5	0
Benzyl alcohol	Sat.	0	12
Benzaldehyde	Sat.	6	8
Benzoic acid	5	3	0
Catechol	5	58	90

Oxidation rates are quoted as oxygen consumption
($\text{nmol. min}^{-1}.\text{mg dry wt}^{-1}$).

Sat. indicates that 100 μl of a saturated solution
was added to the 3ml reaction volume.

The oxidation rates are the average of several determinations
using different preparations of cells. The average endogenous
rate was 14 $\text{nmol O}_2.\text{min}^{-1}.\text{mg dry wt}^{-1}$

p-cresol and p-hydroxybenzoate. This finding suggested that toluene was simply not oxidized by this particular monooxygenase pathway. Similarly, the metabolism of toluene via the alternative monooxygenase pathway did not seem likely in either strain HTB16 or AT50 since very low oxidation rates were observed with benzyl alcohol, benzaldehyde or benzoic acid. It is noteworthy that the low oxidation rates may be due to the toxicity of these compounds. However, relatively high oxidation rates were observed with intermediates of the toluene dioxygenase pathway (3-methylcatechol and cis-toluene dihydrodiol) by both strains. These results suggest that toluene is metabolized by the dioxygenase pathway by both strains HTB16 and AT50. Similarly, benzene appears to be oxidised via cis-benzene dihydrodiol and catechol since high rates of oxygen consumption were observed with these compounds by both strains HTB16 and AT50 (Table 5.3).

5.3 Oxidation of indole by whole cells

It has been shown that the ability to convert indole to indigo is a property of bacterial enzymes that form cis-dihydrodiols from aromatic hydrocarbons (Jenkins & Dalton, 1985; Ensley et al., 1983; Clarke & Laverack, 1984). In whole cells, toluene dioxygenase and naphthalene dioxygenase are thought to be responsible for the oxidation of indole to cis-indole 2,3-dihydrodiol. Spontaneous

Table 5.3

Oxidation of potential intermediates of benzene metabolism
by whole cell suspensions

Substrate	Final Conc (mM)	Oxidation Rate	
		HTB16	AT50
Benzene	Sat.	58	77
<u>cis</u> -Benzene dihydrodiol	5	70	77
Phenol	5	ND*	0
Catechol	5	58	90

Oxidation rates are quoted as oxygen consumption
 (nmol. min⁻¹.mg dry wt⁻¹).

Sat. indicates that 100µl of a saturated solution
 was added to the 3ml reaction volume.

ND indicates not determined.

* Phenol will serve as a growth substrate.

elimination of water then forms indoxyl and subsequent air oxidation produces indigo (Figure 5.1). Recently it has also been shown that toluene monooxygenase / xylene monooxygenase can convert indole directly to indoxyl with the subsequent production of indigo (Hermad *et al.*, 1986; Keil *et al.*, 1987).

Strains HTB16 and AT50 streaked onto agar plates containing 1mM indole and 0.5% (w/v) pyruvate and incubated in the presence of toluene, formed colonies with dark blue/black centres. Also, although indole concentrations greater than 2mM were inhibitory, whole cell suspensions of both strains HTB16 and AT50 oxidized 0.5mM indole with rates of 18 and 27 nmole O_2 min^{-1} mg dry wt $^{-1}$ respectively. Furthermore, a dark blue, water-insoluble pigment was formed during growth of either strain in liquid medium containing 1mM indole, 0.5% (w/v) pyruvate and 0.1% (w/v) toluene. The pigment produced by strain AT50 was extracted into chloroform, as described in Materials and Methods subsection 2.6, before being applied to the base of an aluminium-backed silica gel chromatography sheet. Thin-layer chromatography of the purified pigment (Figure 5.2) revealed that it was a mixture of compounds similar to those produced by *Pseudomonas putida* NCIB 11767, an organism known to metabolize toluene via cis-toluene dihydrodiol (Jenkins & Dalton, 1985). This finding may provide additional evidence that toluene is

Figure 5.1 Proposed pathway for indigo formation
from indole (Ensley *et al.*, 1983)

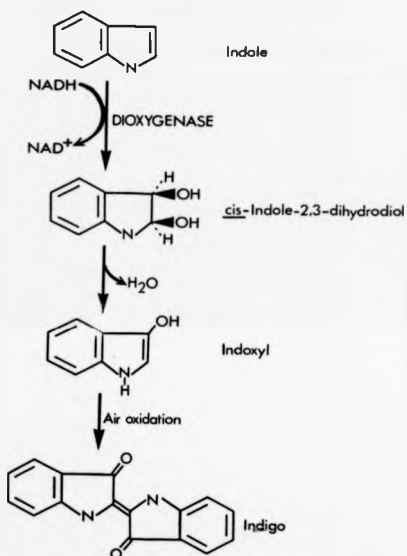
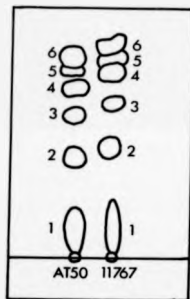


Figure 5.2 Coloured products produced during growth of
Bacillus sp. AT50 and Pseudomonas putida
NCIB 11767 in the presence of indole

The cells were grown on 1mM indole, 0.5%(w/v) pyruvate and 0.1%(v/v) toluene. The coloured products were extracted before being applied to a silica gel chromatograph sheet which was developed in ethyl acetate.



The colour of the spots were : 1, blue; 2, pink; 3, purple; 4, yellow; 5, pink; 6, blue.

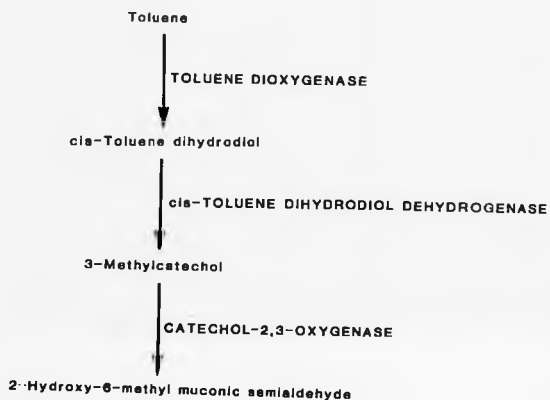
oxidized via cis-toluene dihydrodiol in strains HTB16 and AT50. Stephens & Dalton (personal communication) have shown that the coloured products formed from indole by the dioxygenase enzyme are different from those produced by the monooxygenase. Thin-layer chromatography of the pigment produced by cells of P. putida mt-15, which contains a TOL plasmid specifying toluene/xylene monooxygenase, revealed only 2 coloured compounds. These compounds corresponded to spots 5 and 6 produced by the dioxygenase enzyme. Consequently, it appeared that the reaction catalyzed by the dioxygenase enzyme produced several other compounds in addition to indigo, whereas the monooxygenase-catalyzed reaction produced only indigo.

5.4 Enzyme activities in crude extracts of HTB16 and AT50

The results of substrate specificity studies and simultaneous adaptation experiments suggested that strains HTB16 and AT50 oxidized toluene via cis-toluene dihydrodiol. However, the detection of the various enzyme activities (Figure 5.3) in crude extracts was necessary to confirm the pathway of metabolism since lack of oxidation may have been due to permeability barriers.

In order to investigate which enzyme activities were present in crude extracts it was first necessary to optimise extract preparation. Cell extracts were prepared from cells of

Figure 5.3 Enzymes involved in the catabolism of toluene
via cis-toluene dihydrodiol



strain MTB14 using two different physical cell breakage methods. A pressure drop from 137 MPa in a French pressure cell was relatively inefficient since microscopic observations revealed that a large percentage (>90%) of the cells were still intact. The extract was cooled at 4°C before repeating the treatment. The optimum breakage conditions appeared to be 3 passages through a French pressure cell at 137 MPa. After this treatment, microscopic observations revealed that few cells remained intact (<20%) and 20 mg.ml⁻¹ protein was obtained from 50 mg.ml⁻¹ dry weight of cells. Sonication proved less effective for the preparation of extracts. Sonication for 10s, followed by 10s cooling intervals, for a total time of 3 min yielded an extract containing only 4mg.ml⁻¹ protein and a large proportion of the cells appeared intact. Sonication for longer time periods (20s or 30s) over 5 min gave little improvement in the protein yield and very low enzyme activities. Consequently, cells were routinely disrupted using 3 passages through a French pressure cell at 137 MPa. Cell debris was then removed by centrifugation at 100 000 g for 30 min. Consequently, the enzyme activities measured represented soluble enzymes.

5.4.1 Toluene dioxygenase

5.4.1.1. Polarographic assay

Initially toluene oxygenase activity was detected in crude

extracts of HTB16 and AT50 by the polarographic assay, as described in Materials and Methods subsection 2.2.4.1a. This assay method is simple as it measures the stimulation of oxygen uptake on addition of the substrate to cell suspensions. However, the major disadvantage of this method is its low sensitivity.

Toluene oxygenase activity was detected in toluene-grown cells (Table 5.4), whereas no activity was observed with crude extracts prepared from uninduced cells of HTB16 or AT50 grown on pyruvate (0.5% w/v). Maximal enzymic activity was obtained when the extract (prepared from cells grown on toluene) was preincubated with 1.5mM FeSO_4 , for 5 min in the dark, prior to addition to the reaction vessel. This was to be expected since toluene dioxxygenase from Pseudomonas putida also requires ferrous ions for full activity (Yeh et al., 1977; Jenkins & Dalton, 1985). The chelating agent ethylenediamine tetraacetate (EDTA), which reversibly binds divalent cations, caused total inhibition of the toluene oxygenase activity present in crude extracts prepared from cells of HTB16. This finding suggested that toluene dioxxygenase requires ferrous ions for full activity. Although some toluene oxygenase activity was detected, this assay could not be used routinely since low sensitivity was a major problem. Very high endogenous oxygen consumption rates were often obtained, particularly with

Table 5.4

Activity of toluene dioxygenase in extracts as
measured using a polarographic assay

Extract pre-treatment	Specific Activity (nmole O ₂ .min. ⁻¹ .mg prot ⁻¹)	
	HTB16	AT50
Untreated extract	24	20
Treated with FeSO ₄	71	30
Treated with EDTA	0	ND

ND indicates not determined

crude extracts prepared from cells of strain AT50, and this interfered with the measurement of oxygen consumption rates on addition of toluene.

5.4.1.2-Spectrophotometric indole assay

The use of indole as a spectrophotometric assay substrate for toluene dioxygenase is based on the reaction sequence shown in Figure 5.1 (Jenkins & Dalton, 1985). The assay measures the formation of indoxyl, a yellow dye, which can be followed at 400nm. Although this procedure was supposedly a rapid, sensitive method, several shortcomings became apparent immediately. Assays were carried out at 45°C with 3mg protein and indole dioxygenase activity could be detected, even at low levels, but the spectrophotometric trace obtained was difficult to interpret. Using extracts prepared from either strain HTB16 or AT50, there was an initial increase in absorbance at 400nm, immediately followed by a drop to just above the basal level before the rate began to increase again. Conversely, when the assay was performed at 30°C with extracts of Pseudomonas putida (for which the assay was devised) there was no initial peak but a lag of 2-3 min was observed, as reported by Jenkins & Dalton (1985). The initial peak observed with extracts of HTB16 or AT50 may indicate the presence of a transient compound which then rapidly disappeared. Attempts were made to determine the cause of this initial rise in absorbance at

400nm. When toluene was substituted for indole in the assay mixture, no rate was measured at 400nm. Similarly, using extracts prepared from uninduced cells previously grown on pyruvate, no activity was detected. Furthermore, the absence of the initial rapid absorbance increase indicated that this was formed from a reaction with indole. Neither dialyzing the extract overnight in 20mM potassium phosphate buffer (pH 7.2) nor de-salting the extract by passing it through a 8-25 Sephadex column, had any effect on the trace or rates obtained. Therefore, small solute molecules, which may have been present in the extract were not interfering with the assay. Although the reason for the transient peak was not discovered, rates were calculated from the large peak formed after approx. 2 min.

Several control experiments with extracts prepared from strains HTB16 and AT50 suggested that the assay was indeed an indicator of toluene dioxygenase activity (Table 5.5). No reaction rate was detected when indole or NADH was omitted or when the assay was performed under anaerobic conditions. Only approx. 30% of the maximum enzyme activity was detected when 100 μ l toluene-saturated buffer was added to the reaction mixture. This finding suggested that the toluene was competing with indole as the substrate for the dioxygenase enzyme, assuming the toluene was not responsible for inactivation of protein. Furthermore, the addition of

Table 5.5

Toluene dioxygenase activity present in extracts
prepared from cells of HTB16 and AT50

The enzyme was assayed using a spectrophotometric indole assay, as described in Materials and Methods subsection 2.2.4.1b. The assay conditions were as described in Jenkins & Dalton (1985) unless otherwise stated.

Reaction mixture	Activity ($A_{400} \cdot \text{min}^{-1} \cdot \text{mg prot}^{-1}$)	
	AT50	HTB16
	0.08	0.009
100 μ l toluene-saturated buffer added	0.03	0.003
50mM <u>cis</u> -toluene dihydrodiol added	0.015	0.002
5 mM <u>cis</u> -toluene dihydrodiol added	0.053	ND
No ferrous sulphate added	0.04	0.007
Anaerobic assay conditions	0	0

ND indicates not determined.

cis-toluene dihydrodiol caused inhibition of the rates presumably due to product inhibition of toluene dioxygenase.

To elucidate the products of the assay, the contents of the cuvettes were scanned after the reaction had proceeded. A peak at 400nm was observed, which could be loosely interpreted as suggesting the presence of indoxyl. Furthermore, several hours later the reaction mixture turned blue-black indicating that indigo had been formed from the air oxidation of indoxyl. Also, the indoxyl produced was detected by the method of Fe^{3+} entrapment (Rosero, 1951) but difficulties associated with obtaining a standard solution of indoxyl prevented quantitation of the assay. In conclusion, it was difficult to determine exactly what the reaction product(s) of the assay was or how it was formed.

Despite the fact that the assay procedure was unsatisfactory, it was used to determine some characteristics of toluene dioxygenase present in cell extracts of strain AT50. When NAD^+ was substituted for NADH, approx. 90% of the original activity was obtained. This finding may be explained by the fact that the extract used was prepared in a stabilization buffer containing 10%(v/v) ethanol. Ethanol dehydrogenase present in the crude extract may have reduced the NAD^+ thereby enabling the indole dioxygenase reaction to proceed. When extract was freshly prepared in potassium phosphate buffer (without

ethanol) then no rate was observed with NAD⁺ suggesting that NADH was the necessary cofactor for indole dioxygenase. Initial investigations revealed that the temperature optimum of toluene dioxygenase from AT50 was around 40°C and the pH optimum was around 6.8. A non-linear relationship was observed between activity and protein (Table 5.6). This finding illustrates the difficulty in quantification of the assay but may reflect the fact that the enzyme is multicomponent. Also, the apparent decrease in specific activity at protein concentrations above 3mg/assay may be due to the rapid formation of indoxyl which is spontaneously oxidized to indigo non-enzymically when present at high concentrations.

5.4.2 cis-Toluene dihydrodiol dehydrogenase

Activity of cis-toluene dihydrodiol dehydrogenase was relatively easy to detect in crude extracts prepared from cells of strain HTB16 and AT50. The optimum pH values were 10.2 and 9.8 for strain HTB16 and strain AT50 respectively (Figure 5.4). The assays were routinely performed at pH 8.0 (see Materials and Methods, subsection 2.2.4.2) and subsequent studies with the enzyme from AT50 revealed it was most stable at this pH value. Both enzymes exhibited high temperature optimum values of around 80°C but the enzyme from strain HTB16 appeared to be unstable at this temperature and only 50% of the initial activity remained

Table 5.6

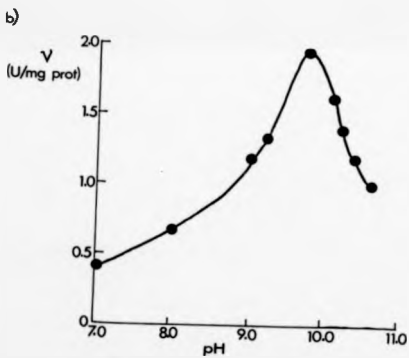
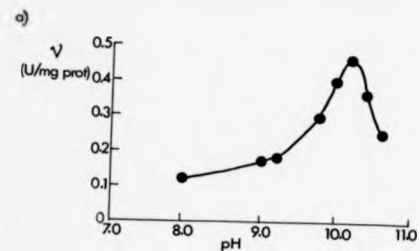
Effect of extract concentration on the
spectrophotometric indole assay

The assay was carried out at 45°C with extracts prepared from strain AT50 (see Materials and Methods subsection 2.2.4.1b).

Extract added per assay (mg protein)	Activity ($A_{400} \cdot \text{min.}^{-1} \cdot \text{mg prot}^{-1}$)
0.75	0.049
1.5	0.071
2	0.08
3	0.084
7	0.054

Figure 5.4

pH optimum of *cis*-toluene dihydrodiol dehydrogenase
present in crude extracts of (a) HTB16 and (b) AT50



after 10 min at 80°C. Conversely, the enzyme obtained from strain AT50 did not appear to lose any activity after 30 min at 80°C. The cis-toluene dihydrodiol dehydrogenase present in crude extracts of AT50 exhibited an apparent K_m of 240 μ M for cis-toluene dihydrodiol. No cis-toluene dihydrodiol dehydrogenase activity was detected in extracts prepared from uninduced cells grown on pyruvate (0.5%w/v). The cis-toluene dihydrodiol dehydrogenase from strain AT50 was subsequently purified and further characterized (see Chapter 6).

The presence of several other dehydrogenases in crude extracts of strains HTB16 and AT50 were investigated using the same assay procedure but the cis-toluene dihydrodiol (0.5mM) was substituted with 0.5mM cis-benzene dihydrodiol, 0.5 mM benzyl alcohol, 0.5 mM benzaldehyde or 0.22 M ethanol (Table 5.7). No activity was detected with benzyl alcohol or benzaldehyde but ethanol dehydrogenase activity was detected in both strains. cis-Benzene dihydrodiol dehydrogenase activity was detected in strain AT50 but higher activity was detected when cis-toluene dihydrodiol was the substrate present.

5.4.3. Fission enzymes

The catechol-fission enzymes were assayed by simple procedures, as described in Materials and Methods

Table 5.7

Dehydrogenase activities present in extracts
of HTB16 and AT50

The assays were carried out at 45°C as described in Materials and Methods subsection 2.2.4.2, but the other substrates were substituted for cis-toluene dihydrodiol.

Substrate	Activity(U.mg protein ⁻¹)	
	HTB16	AT50
0.5mM <u>cis</u> -Toluene dihydrodiol	0.09	0.75
0.5mM <u>cis</u> -Benzene dihydrodiol	ND	0.39
0.5mM Benzyl alcohol	0	0
0.5mM Benzaldehyde	0	0
0.22M Ethanol	0.002	0.045

ND indicates not determined

subsections 2.2.4.4. and 2.2.4.5. Extracts prepared from cells of strain HTB16 and strain AT50 previously grown on toluene in continuous culture, exhibited approx. 10-fold greater catechol-2,3-oxygenase activity than catechol-1,2-oxygenase activity (Table 5.8). This indicates that the 3-methylcatechol produced during the catabolism of toluene, is further metabolized via a meta-cleavage pathway. The catechol-1,2-oxygenase activity detected may simply reflect co-induction of this enzyme by toluene, or lack of specificity of the assay. The temperature optimum for catechol-2,3-oxygenase in extracts of HTB16 was 45°C and 50°C for the enzyme from strain AT50. Catechol-2,3-oxygenase from strain HTB16 was not stable when frozen at -20°C and only 10% of the initial activity remained after 48 h but the enzyme was stable for at least 2 months when stored at -70°C. The enzyme present in crude extracts of AT50 was more stable and no activity was lost after 48h at -20°C. Furthermore, the enzyme activity in extracts of AT50 appeared to be relatively thermostable and 85% of the initial activity remained after 4h at 50°C.

5.5 Substrate specificity of toluene dioxygenase in whole cells

Due to the shortfalls of the assay systems described previously, the development of a new assay for toluene

Table 5.8

Enzyme activities in crude extracts prepared from cells
of HTB16 and AT50

Enzyme	Activity(U.mg protein ⁻¹)	
	HTB16	AT50

Catechol-2,3-oxygenase	0.45	3.3
Catechol-1,2-oxygenase	0.05	0.4

One enzyme unit (U) represents the activity produced per
umole product per min.

dioxygenase was attempted. Compounds which are substrates for the dioxygenase enzyme but are not further metabolized, could be used as an assay substrate. Furthermore, knowledge of the substrate specificity of the toluene dioxygenase from strain AT50 would provide an insight as to which dihydrodiols could be produced once a suitable mutant is obtained.

Various compounds were tested as possible substrates for the dioxygenase enzyme. The oxidation rates of the compounds by whole cell suspensions of strain AT50, after growth on toluene in continuous culture, were investigated as an indicator of toluene dioxygenase activity (Table 5.9). Relatively low oxygen consumption rates were observed with most of the compounds investigated but it was apparent that the dioxygenase has wide substrate specificity. In general, before ring fission can occur the benzene nucleus must carry two hydroxyl groups situated ortho or para to one another (Dagley, 1971). Presumably most of the compounds investigated would be attacked by a dioxygenase enzyme to produce a dihydrodiol and subsequently the catechol structure necessary for ring cleavage. However, compounds already possessing one hydroxyl group on the aromatic nucleus, eg. 2-ethylphenol, are likely to be attacked by a monooxygenase reaction to introduce a further hydroxyl group. This fact may explain why no rate was observed with

Table 5.9

Oxidation of various compounds by whole cell suspensions of strain AT50

Substrate	Final Conc (mM)	Growth substrate	Oxidation Rate (moles O ₂ /min/mg dry wt ⁻¹)
4,4'-Biphenol	5	✓	5.9
Biphenyl	5	✓	16.1
Ethyl benzene	1.67*	ND	91.6
2-Ethyl phenol	1.67*	ND	0
m-Xylene	1	ND	24.6
Phenethyl-1-ol	5	ND	0
4-Picoline	1.67	ND	29.5
Naphthalene	5	✓	9.1
Chlorobenzene	1	ND	14.8
Fluorobenzene	1	ND	6.1
p-Chlorotoluene	1	ND	6.8
o-Fluorotoluene	1	ND	5.8
2,5-Dichlorotoluene	1	ND	8.0
2,6-Dichlorotoluene	1	ND	13.1
Toluene	1.67*	✓	80.2
Benzene	1.67*	✓	49.0

ND indicates not determined.

✓ indicates growth occurred within 10 days

* indicates compound was made up in dimethyl formamide

2-ethylphenol.

Secondly, the compounds were tested to determine whether they supported growth of the organism. Ideally, a suitable assay substrate would be oxidized by the dioxygenase enzyme but would not be further degraded, i.e. would not support growth of the organism. When a few granules of biphenol were placed into the lid of a petri dish streaked with strain AT50, no growth was observed after 10 days at 45°C. However, subsequent investigations revealed that when 0.5mM biphenol was incorporated into agar plates, some growth occurred after several days. Similarly, agar containing 0.5mM naphthalene or 0.5mM biphenyl supported some growth of strain AT50. Consequently, these compounds were deemed unsuitable as an assay substrate for the dioxygenase enzyme. Unfortunately time did not permit further studies with all the compounds which appeared to be oxidized. However, after further work there is the possibility of developing an assay. After obtaining a substrate which did not support growth it would then be necessary to ensure that the compound was not further partially metabolized. This could be determined using GC or HPLC by confirming that the diol was the compound produced.

DISCUSSION

Several different approaches may be used to determine the pathway of catabolism of a particular compound. Whole cell oxidation studies can be used to predict pathways according to the principle of simultaneous adaptation (Stanier, 1947).

This principle states that growth at the expense of a single substrate enables cells to oxidize specifically that compound and catabolites thereof, whereas compounds which are metabolized via different pathways are not oxidized immediately. Of course, this principle is based on the assumption that inducible enzymes are synthesized at high rates only when they serve a necessary function during growth. From investigations of substrate specificities and whole cell oxidation studies, it appeared that both strains HTB16 and AT50 metabolized toluene using the dioxygenase pathway. Very low rates of oxygen consumption were observed with intermediates of the two described monooxygenase pathways, whereas high oxidation rates were measured with cis-toluene dihydrodiol and 3-ethylcatechol. Similarly, benzene appeared to be oxidized via the dioxygenase pathway. The results of the enzyme assays also supported the finding that the dioxygenase pathway was operative in both strains. The use of extracts avoids problems with permeability barriers but since the enzymes are no longer in their natural environment, they may display distorted activities

or become inactivated. Indeed, difficulties arose when attempting to measure the toluene dioxygenase activity. Strains HTB16 and AT50 exhibited similar rates of toluene-stimulated oxygen uptake by both whole cells and cell extracts. However, extracts of HTB16 exhibited only approx. 10% of the toluene dioxygenase activity measured in cell extracts of AT50 by the indole spectrophotometric assay. Similarly, with assays for the catechol-fission enzymes and cis-toluene dihydrodiol dehydrogenase, approx. 10-fold greater activity was detected in extracts of AT50. Consequently, the polarographic assay may be inaccurate. It is possible that the high endogenous rates measured with extracts of AT50 interfered with the assay. Consequently the activities measured may have been lower than the true value. Alternatively, the toluene-stimulated oxygen uptake by extracts of HTB16 may have been higher than the true value due to the presence of a compound which increased the reaction rate. The values obtained from the spectrophotometric assay and the polarographic assay cannot be compared since a standard solution of indoxyl cannot be obtained. Therefore it is difficult to draw any conclusions as to which assay is more accurate. The polarographic assay was intrinsically less sensitive but the spectrophotometric assay produced curious traces. The spectrophotometric indole assay was devised using extracts of Pseudomonas putida and the assay did not appear to be applicable to

Bacillus extracts. Furthermore, using various concentrations of extract from strain AT50, it became apparent that there was not a linear relationship between activity and amount of protein. This non-linear relationship was also observed with extracts of Pseudomonas putida, on which the spectrophotometric assay was devised (Jenkins & Dalton, 1985). The authors reported that a linear relationship was only observed with approx. 2-4mg protein per assay. This effect is typical of multicomponent enzyme systems and may explain why different rates were obtained with extracts from HTB16 and AT50 since it is unlikely that the toluene dioxygenase represents the same proportion of the total protein in the two organisms. However, since this phenomenon would also apply to the polarographic assay, no conclusions can be drawn. A [^{14}C -methyl]toluene assay devised by Yeh *et al.* (1977), based on measuring the formation of cis-[^{14}C -methyl]-toluene dihydrodiol from [^{14}C -methyl]toluene, was attempted but was unsuccessful. Consequently, it did not reveal whether the polarographic assay or the spectrophotometric assay was more accurate.

As already mentioned, the enzyme activities present in extracts of AT50 were generally approx. 10-fold greater than the corresponding enzyme activities measured in extracts of strain HTB16. This finding suggests that the enzymes

partially inactivated whereas the enzymes in strain AT50 appeared to be more stable. Initial investigations revealed that cis-toluene dihydrodiol dehydrogenase present in extracts of AT50 was very stable, a finding which suggested that this enzyme should be relatively easy to purify (Chapter 6). Furthermore, catechol-2,3-oxygenase from AT50 also appeared to be more thermostable than the analogous enzyme from HTB16. The greater stability of enzymes from AT50 reflects the higher optimum growth temperature (50°C) of this organism compared to that of strain HTB16 (45°C). A number of enzymes from thermophilic/thermotolerant bacteria have been shown to be more thermostable and tolerant to chemical denaturing agents than comparable preparations obtained from mesophiles (eg. Amelunxen & Murdock, 1978). Isolation and characterization of enzymes from Bacillus sp. AT50 may provide evidence to support this finding (Chapter 6).

Although time did not permit the development of a new assay for toluene dioxygenase, the oxidation studies provided an indication of the substrate specificity of the enzyme. A comparison of the oxidation rates obtained with ethylbenzene, toluene and benzene revealed that ethylbenzene was the preferred substrate, followed by toluene. This finding suggests that the dioxygenase has a greater affinity for substrates with side groups which may contribute towards slightly increased hydrophobicity. Conversely, a strain of

slightly increased hydrophobicity. Conversely, a strain of Pseudomonas putida (Gibson, 1972), after growth on toluene, oxidized toluene and benzene at approximately equal rates. Ethylbenzene was oxidized more slowly and isopropylbenzene was oxidized at only half the rate observed with toluene. With extracts of Bacillus sp. AT50, chlorobenzene was oxidized at a slightly greater rate than fluorobenzene, possibly due to the fact that fluorine groups would tend to repel the nucleophilic groups on the enzyme more strongly. An investigation of the enzyme specificity for chlorinated aromatic compounds indicated that the following compounds are oxidized in order of preference: chlorobenzene, 2,6-dichlorotoluene, 2,5-dichlorotoluene, p-chlorotoluene. Therefore, it appears that the presence of substituents in the position para (position 4) to the methyl group of toluene, may result in steric hindrance, i.e. 2,6-dichlorotoluene is preferred to p-chlorotoluene. Similarly, substituent groups at positions 3 and 5 may also contribute to steric hindrance thus explaining why toluene is the preferred substrate compared to m-xylene; also 2,6-dichlorotoluene is preferred to 2,5-dichlorotoluene. A similar pattern of results appeared to be obtained by Axcell & Geary (1975) on studying the substrate specificity of partially purified benzene dioxygenase from P. putida ML2. Toluene was oxidized whereas p-xylene with the extra methyl group at position 4, was not. Axcell & Geary (1975)

reported that this partially purified benzene dioxygenase also oxidized fluorobenzene, chlorobenzene and bromobenzene, but not phenol or naphthalene. However subsequently Zamanian & Mason (1987), working with crude extracts of the same organism revealed that it did oxidize naphthalene. This highlights the fact that the substrate concentration and protein concentration are important when determining substrate specificities of enzymes. For example, certain aromatic compounds may be toxic at high concentrations but if the substrate concentration is too low, the assay method may not be sufficiently sensitive to detect any activity. Indeed, the low oxidation rate obtained with crude extracts of AT50 with 5mM biphenol is curious since no rate was observed with phenol or 1.67mM ethylphenol. Since the dioxygenase present in crude extracts of AT50 appeared to exhibit wide substrate specificity, isolation of a suitable mutant should yield a wide variety of diols from this organism.

In summary, the combination of different approaches enabled the elucidation of the pathway of toluene metabolism by strains HTB16 and AT50. Both organisms appeared to oxidize toluene using the dioxygenase pathway. Ten-fold greater catechol-2,3-oxygenase activity than catechol-1,2-oxygenase activity was detected in both strains. Hence it appears that the 3-methylcatechol, produced during the catabolism of

toluene, is further metabolized via the meta-cleavage pathway giving rise to a hydroxymuconic semialdehyde. Since both strains oxidized toluene via cis-toluene dihydrodiol, there is the potential to produce this compound using a suitable mutant from either strain. Furthermore, mutagenesis followed by the identification of mutants blocked at various stages of the pathway, may provide further confirmation of the pathway of toluene catabolism (Chapter 7). The isolation of two potential cis-toluene dihydrodiol producers may indicate that the majority of organisms in the samples obtained did degrade toluene using the dioxygenase pathway. Alternatively, the strains may have been isolated purely by luck; a systematic survey is required before any predictions on the relative occurrence of the different pathways of metabolism in thermotolerant organisms can be made.

CHAPTER 6 : PURIFICATION AND PROPERTIES OF CIS-TOLUENE

DIHYDRODIOL DEHYDROGENASE

INTRODUCTION

Toluene was metabolized via cis-toluene dihydrodiol in Bacillus sp. AT50, as indicated by whole cell studies and assays on crude extract preparations (Chapter 5). Further studies of the enzymes responsible for toluene metabolism in Bacillus sp. AT50 would help to confirm the pathway and may reveal whether these enzymes are similar to those previously isolated from toluene-utilizing, mesophilic organisms. However to enable further investigation of the enzymes, purification was necessary. Attempts were made to purify toluene dioxygenase but the enzyme was not amenable to study due to the lack of a suitable assay procedure (see Chapter 5). Consequently, purification and subsequent characterization of the next enzyme in the pathway, cis-toluene dihydrodiol dehydrogenase, was attempted. Inhibitors of the enzyme were investigated since a possible route to cis-toluene dihydrodiol production is to specifically inhibit the dehydrogenase. Consequently, growth of Bacillus sp. AT50 on pyruvate, in the presence of toluene and a specific inhibitor of cis-toluene dihydrodiol dehydrogenase, could lead to the accumulation of cis-toluene dihydrodiol in the culture supernatant.

The following section outlines the purification of cis-toluene dihydrodiol dehydrogenase from Bacillus sp. AT50 and describes the physical characteristics and properties of the enzyme.

RESULTS AND DISCUSSION

6.1 Purification of *cis*-toluene dihydrodiol dehydrogenase

Crude extract was prepared from Bacillus sp. AT50 cells grown at 50°C with toluene vapour as the sole source of carbon and energy. Preliminary investigations with crude extracts had revealed that the enzyme was extremely thermostable. This thermostability was exploited in the first step of the purification procedure, which involved heat treatment at 75°C for 90 min. Initially, a five step procedure was used involving gel filtration, with an Ultrogel AcA34 column, after the initial DEAE step. Although gel filtration is supposedly a relatively mild method of purification, much activity was lost and this step was subsequently omitted with no significant detrimental effect on the purification of the enzyme.

The purification protocol adopted is shown in Table 6.1 and described in Materials and Methods, subsection 2.3.1 There have been reports of several forms of diol dehydrogenases in other organisms (Jenkins et al., 1987; Eberspächer & Lingens, 1978). However, enzymes eluted from DEAE-sepharose CL-6B (Figure 6.1), gel filtration (Figure 6.2) and hydroxylapatite (Figure 6.3) columns as a single peak of activity indicated on the basis of both charge and molecular size, the presence of only one *cis*-toluene dihydrodiol dehydrogenase in Bacillus sp. AT50.

Table 6.1 Summary of purification

Step	Volume (ml)	Protein (mg/ml)	Enzyme units	Specific activity	Purification factor	Recovery (%)
Cell extract	30	54	636	0.39	0	100
1. Heat treatment	20	32.5	578.4	0.89	2	91
2. DEAE- sepharose CL-6B	20	8.95	265.2	1.48	4	42
3. Hydroxyapatite	10	0.81	130.2	16.1	41	20
4. 5' cAMP- sepharose	2	0.5	80.0	81.0	210	13

Figure 6.1 Elution of *cis*-toluene dihydrodiol dehydrogenase from a column of DEAE-sepharose CL-6B

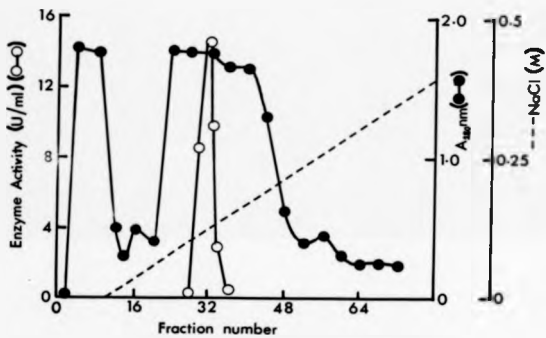


Figure 6.2 Elution profile of Ultragel AcA34 gel
filtration column for the purification of
cis-toluene dihydrodiol dehydrogenase

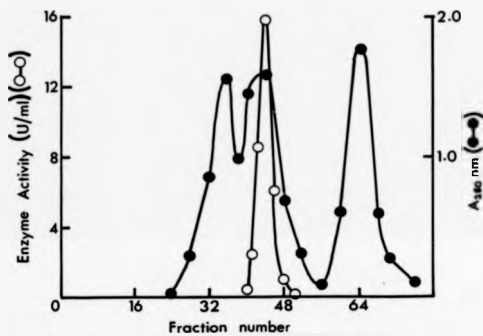
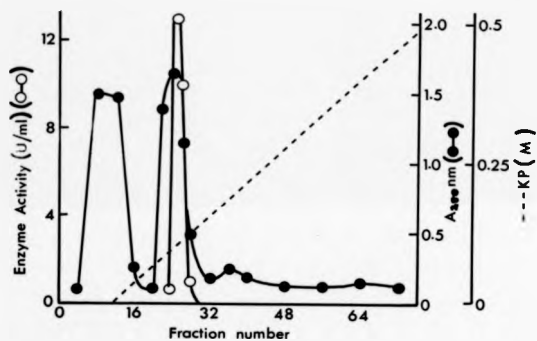


Figure 6.3 Elution of *cis*-toluene dihydrodio] dehydrogenase
from a Hydroxylapatite column



cis-Toluene dihydrodiol dehydrogenase was purified 210-fold with 13% recovery of activity. Analysis of 25 μ g of protein from the purified enzyme preparation by 10-30%(w/v)-polyacrylamide-gradient-gel electrophoresis resulted in the detection of one major protein band when stained with either Coomassie Blue or glycol dehydrogenase activity stain (Rogers & Gibson, 1977). One major band and several minor bands were observed with 81 μ g protein. A polyacrylamide-gradient-gel of various preparations obtained during the purification of the enzyme is shown in Figure 6.4.

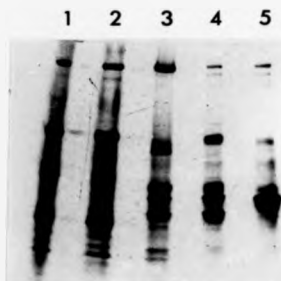
6.2 Properties of purified cis-toluene dihydrodiol dehydrogenase

6.2.1 M_r and subunit structure

cis-Toluene dihydrodiol dehydrogenase purified by Rogers and Gibson (1977) was found to have a M_r of 104 000 Da when determined at pH 9.7 by PAGE but when determined by gel filtration at pH 7.2 the M_r was 135 000 Da, suggesting that a rearrangement of the quaternary structure of the enzyme occurred with changing pH. Conversely the M_r of cis-toluene dihydrodiol dehydrogenase from Bacillus sp. AT50 was approximately 172 000 Da as determined by gel filtration at both pH 7.2 and pH 9.0, indicating that no such rearrangement occurred with this enzyme. The M_r determined by measuring the electrophoretic mobility of the

Figure 6.4

Polyacrylamide-gradient-gel electrophoresis (10-30%w/v) of various preparations obtained during the purification of *Bacillus* sp. AT50 *cis*-toluene dihydrodiol dehydrogenase



Track 1: 500 μ g crude extract

Track 2: 500 μ g protein after heat treatment

Track 3: 270 μ g protein after DEAE column chromatography

Track 4: 98 μ g protein after hydroxylapatite column
chromatography

Track 5: 81 μ g protein after 5'cAMP column chromatography

protein on a non-denaturing polyacrylamide-gradient-gel was approximately 166 000 Da (Figure 6.5). A polyacrylamide denaturing gel run in the presence of SDS indicated that the subunit M_r was 29 500 Da (Figure 6.6). The above results suggest that the enzyme consisted of six apparently identical subunits.

6.2.2 Isoelectric focusing

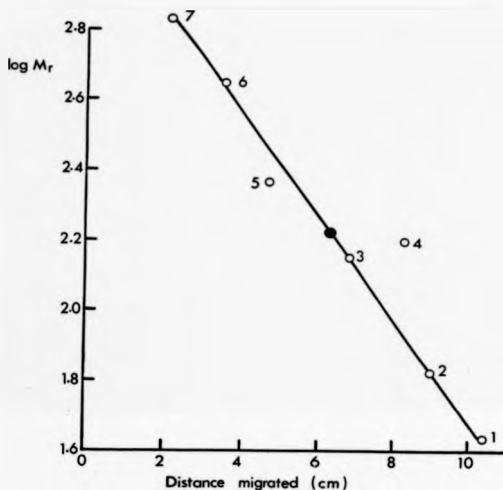
Isoelectric focusing run under SDS-denaturing conditions in tube gels revealed one major band and three very minor bands (Figure 6.7). The presence of only one major band indicated that only one type of subunit was present. The pI of the denatured protein was 6.5 and using slab-gels the pI for the native protein was found to be 6.4.

6.2.3 Temperature optimum and stability

Activity of the purified cis-toluene dihydrodiol dehydrogenase was assayed over the temperature range 30-88°C, and the optimum was found to be 80°C. From an Arrhenius plot (Figure 6.8), the activation energy of the reaction was calculated to be 36 kJ.mol⁻¹. The Arrhenius plot was linear up to 65°C, at which point the curve levelled off, suggesting some type of conformational change above 65°C which lowers the activation energy to approx. 4.41 kJ.mol⁻¹.

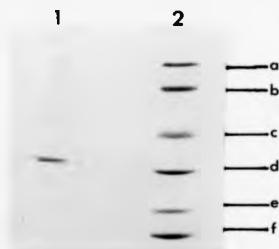
When stored at -20°C the purified enzyme appeared to be

Figure 6.5 M_r determination of purified *cis*-toluene dihydrodiol dehydrogenase using a non-denaturing gel



Protein standards represented by open circles (○) were :
 (1) ovalbumin, (2) bovine serum albumin, (3) lactate dehydrogenase, (4) aldolase, (5) catalase, (6) ferritin and (7) thyroglobulin. The closed circle (●) represents *cis*-toluene dihydrodiol dehydrogenase.

Figure 6.6 SDS-polyacrylamide gradient-gel (10-30% w/v)
electrophoresis of purified protein

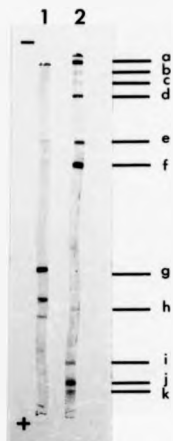


Track 1: SDS-denatured cis-toluene dihydrodiol dehydrogenase

Track 2: Protein standards were (a) phosphorylase b (94 000 Da); (b) albumin (67 000Da); (c) ovalbumin (43 000Da); (d) carbonic anhydrase (30 000Da); (e) trypsin inhibitor (20 100 Da); (f) α -lactalbumin (14 400Da).

The values in parentheses represent the subunit M_r .

Figure 6.7 Isoelectric focusing of purified enzyme
on SDS-polyacrylamide gels



Track 1: Purified enzyme (15 μ g protein)

Track 2: Standards (a) trypsinogen (pI=9.3); (b), (c), (d) lentil lectin (pI=8.65, 8.45, 8.15); (e) horse myoglobin (pI=7.35); (f) horse myoglobin α (pI=6.85); (g) human carbonic anhydrase B (pI=6.55); (h) bovine carbonic anhydrase B (pI=5.85); (i) β -lactoglobulin (pI=5.2); (j) soya bean trypsin inhibitor (pI=4.55); (k) amyloglucosidase (pI=3.5)

Figure 6.8 Arrhenius plot for purified enzyme

From Arrhenius' equation :

$$\log k = \log A - \frac{E}{19.14} \cdot \frac{1}{T}$$

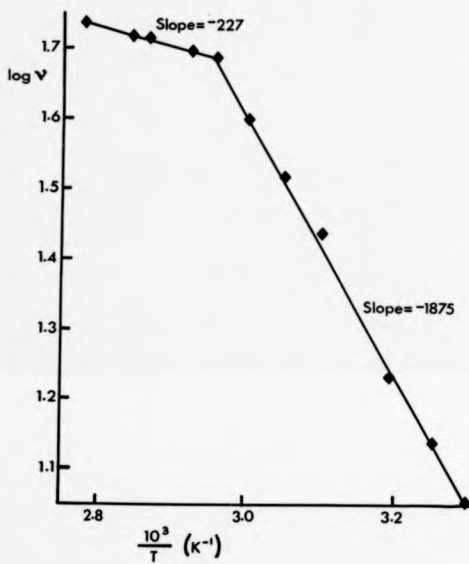
where k = specific rate constant (min^{-1})

A = frequency factor (min^{-1})

E = Activation energy (J)

T = temperature (K)

$$\therefore \text{Slope of line} = \frac{-E}{19.14}$$



stable for at least 5 months. Furthermore, a filter-sterilized solution of enzyme was stable at both 4°C and room temperature for at least 10 days with no loss of enzyme activity. The thermostability of the enzyme was investigated at both 75°C and 80°C and the half-lives were found to be 140 min and 110 min, respectively. Indeed, it was consistently observed that, after 5 min at 80°C, the activity increased slightly. When the enzyme was incubated at 80°C in the presence of either NAD⁺ or cis-toluene dihydrodiol the enzyme was less stable, with the half-life falling to 30 min.

4.2.4 pH optimum and pH stability

The pH optimum of the purified enzyme was 9.8 with both cis-toluene dihydrodiol and cis-benzene dihydrodiol as substrate. When purified enzyme was incubated at 4°C for 30 min at pH 6.0, 7.0 and 9.0, and then assayed for activity at pH 8.0, little effect on enzyme activity was observed. However, beyond this range, activity was rapidly lost until, at pH 3.0, the enzyme lost 87% of activity in 30 min. The enzyme was most stable at pH 8.0.

4.2.5 Absorption spectrum of cis-toluene dihydrodiol dehydrogenase

The absorption spectrum of the enzyme exhibited absorption maxima at 220nm and 280nm. All proteins absorb in the far-UV and the peak at 280nm was presumably caused by the

absorption of tryptophan residues. There was no significant absorbance in the visible part of the spectrum indicating that no chromophoric prosthetic groups were present.

6.2.6 Stoichiometry

The stoichiometry of the enzyme-catalyzed reaction was determined as described in Materials and Methods, subsection 2.3.6. Purified enzyme reduced one equivalent of NAD^+ for each equivalent cis-toluene dihydrodiol converted into 3-methylcatechol.

6.2.7 Inhibitors

A range of possible inhibitors of cis-toluene dihydrodiol dehydrogenase was investigated since a possible route to cis-toluene dihydrodiol production is to specifically inhibit the dehydrogenase in cultures of Bacillus sp. AT50. Of the compounds tested, mercuric chloride was found to be the best inhibitor. Its effect on enzyme activity was investigated at concentrations of 3mM and 0.1mM, which gave 100% and 50% inhibition respectively. Another mercuric compound, p-hydroxymercuribenzoate, also inhibited the enzyme, albeit at such higher concentrations (>10mM). Metal-ion chelators such as EDTA, KCN, α, α' -dipyridyl and bathophenanthroline were relatively inefficient inhibitors of the enzyme.

giving approx. 15% inhibition when added at a concentration of 3mM. The inefficiency of metal-ion chelators on the enzyme suggests that the activity does not depend on metal ions.

4.2.8 Substrate specificity

Various other diols were tested as possible substrates for cis-toluene dihydrodiol dehydrogenase. Reaction mixtures were similar to those used routinely to assay cis-toluene dihydrodiol dehydrogenase, except that cis-toluene dihydrodiol was replaced by the test substrate at a final concentration of 0.5mM. The compounds tested included pentane-1,4-diol, propane-1,3-diol, butan-2-ol, butan-1-ol, glycerol and ethanol. No activity was detected with these substrates. None of these compounds, when added at 10mM to standard assay mixtures was found to inhibit cis-toluene dihydrodiol dehydrogenase. Activity was observed when either cis-naphthalene dihydrodiol or cis-benzene dihydrodiol were used as substrates. The enzyme was specific for NAD⁺ since no activity was detected when NADP⁺, phenazine methosulphate, cytochrome c, potassium ferricyanide or 2,4-dichlorophenol indophenol were substituted as electron acceptors for the enzyme.

4.2.9 Michaelis constants

The K_m of cis-toluene dihydrodiol dehydrogenase for cis-toluene dihydrodiol was 92 μ M, as determined by means

of double-reciprocal plots (Figure 4.9a). To determine the apparent K_m for cis-naphthalene dihydrodiol the reactions were performed at pH 7, since at higher pH values the autoxidation of the reaction product interfered with the enzyme assay (Patel & Gibson, 1974). The apparent K_m values of the enzyme for cis-toluene dihydrodiol and cis-naphthalene dihydrodiol were 330 μ M and 51 μ M respectively. This suggests that the enzyme has greater affinity for substrates with side groups which contribute towards increased hydrophobicity. The K_m of cis-toluene dihydrodiol dehydrogenase for NAD^+ was 82 μ M (Figure 4.9b).

4.2.10 Product inhibition

Product inhibition can be classified as competitive, uncompetitive, or non-competitive, depending on whether it affects the slope of the Lineweaver-Burk plot, the intercept, or both (Engel, 1981).

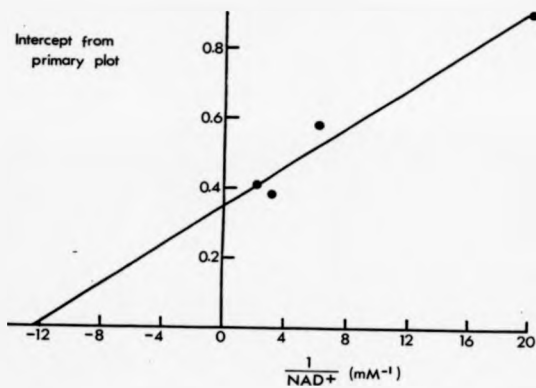
Non-competitive or 'mixed' inhibition was observed with either 3-methylcatechol [$K_{i,m}$ (slope)=3.6mM; $K_{i,m}$ (intercept)=1.7mM] or NADH ($K_{i,m}$ =0.21mM; $K_{i,m}$ =0.14mM) using 0.5 mM NAD^+ with cis-toluene dihydrodiol concentrations ranging from 0.05 to 0.5 mM. When the NAD^+ concentration was varied from 0.25-2.5 mM (TCB concentration was 0.5mM), mixed inhibition was observed with 3-methylcatechol and the $K_{i,m}$ and $K_{i,m}$ values were 2.7mM and 5.2mM respectively (Figure 4.10). However, at

Figure 6.9 Secondary plots for K_m determination of
cis-toluene dihydrodiol dehydrogenase

(a) Calculation of K_m for NAD^+ . Data was obtained from primary Lineweaver-Burk plots in which the *cis*-toluene dihydrodiol concentration was varied at four NAD^+ concentrations.

(b) Calculation of K_m for *cis*-toluene dihydrodiol (TCG).

a)



b)

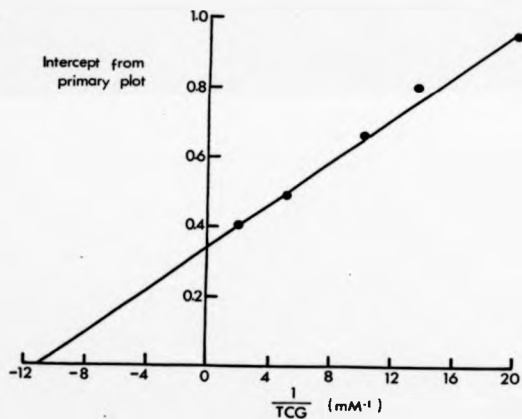
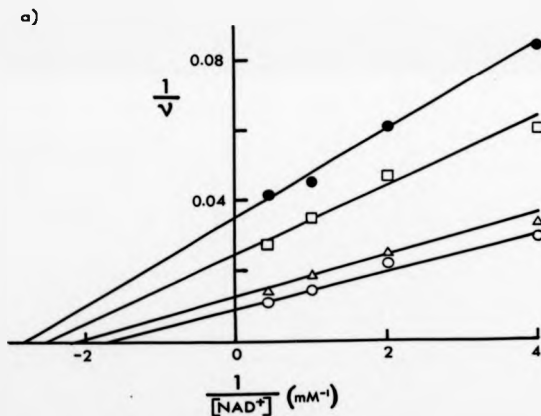


Figure 6.10 Determination of K_i of *cis*-toluene dihydrodiol dehydrogenase for 3-methylcatechol

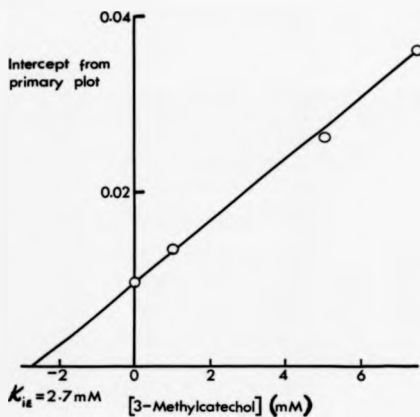
(a) Primary plot of the effect of adding 3-methylcatechol at concentrations of 1mM (Δ); 5mM (\square); 7.5mM (\bullet) and no inhibitor (\circ). *cis*-Toluene dihydrodiol was present at a concentration of 0.5mM.

(b) Secondary plot of the intercepts

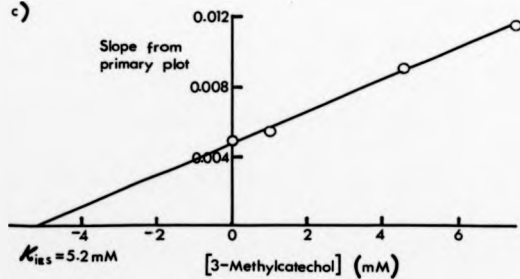
(c) Secondary plot of the slopes



b)



c)



saturating concentrations of the diol (5 mM), uncompetitive inhibition was observed (Figure 6.11). Over varied concentrations of NAD^+ , competitive inhibition was observed with NADH (Figure 6.12) and a K_s of 80 μM was determined.

6.2.11 Enzyme mechanism

The primary Lineweaver-Burk plots and the product-inhibition patterns can both be used to predict the reaction mechanism.

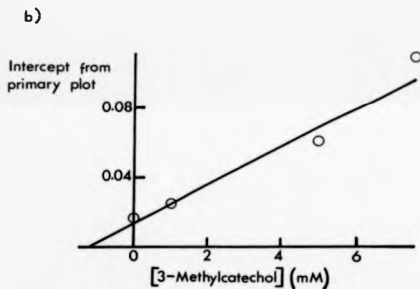
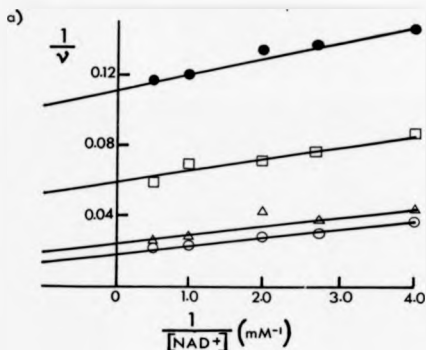
The primary Lineweaver-Burk plots intersect in the third quadrant suggesting that the mechanism is sequential. However, the product-inhibition, which is summarized in Table 6.2a, indicates that the mechanism is ordered Bi Bi (see Table 6.2b). If the mechanism is ordered Bi Bi, with NAD^+ binding first, then the K_m for NAD^+ should be similar when different substrates are used. The K_m values using either cis-toluene dihydrodiol or cis-benzene dihydrodiol (80 μM and 90 μM respectively) indicated that NAD^+ binds first in an ordered Bi Bi mechanism. The fact that inhibition by 3-methylcatechol with respect to NAD^+ became uncompetitive at saturating concentrations of the diol provides further evidence for an ordered Bi Bi mechanism.

6.2.12 Immobilization of cis-toluene dihydrodiol dehydrogenase

6.2.12.1 Advantages of enzyme immobilization

The great stability of purified cis-toluene dihydrodiol dehydrogenase should facilitate ease of immobilization.

Figure 6.11 Determination of K_i of *cis*-toluene dihydrodiol dehydrogenase for 3-methylcatechol at saturating concentrations of the diol



(a) Primary plot of the effect of adding 3-methylcatechol at concentrations of 1mM (Δ); 5mM (\square); 7.5mM (\bullet) and no inhibitor (\circ). *cis*-Toluene dihydrodiol was present at a concentration of 5mM.

(b) Secondary plot of the intercepts

Figure 6.12 Determination of K_i of *cis*-toluene dihydrodiol
dehydrogenase for NADH

(a) Primary plot of the effect of adding NADH at concentrations of 0.1mM (Δ); 0.2mM (\square); 0.4mM (\bullet) and no inhibitor (\circ). *cis*-Toluene dihydrodiol was present at a concentration of 0.5mM.

(b) Secondary plot of the slopes

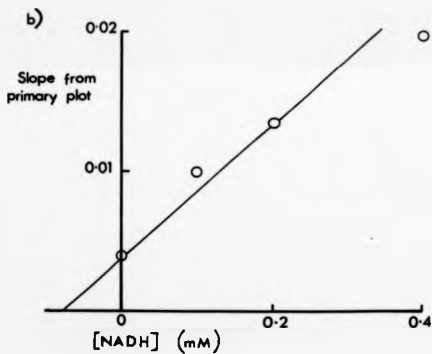
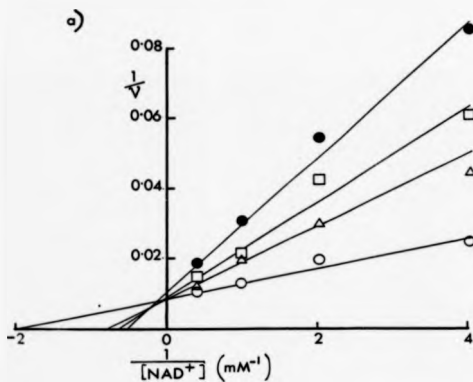


Table 6.2 (a). Summary of the product-inhibition pattern of
cis-toluene dihydrodiol dehydrogenase

Product Inhibitor	Vary [NAD ⁺]		Vary[TCB] Unsat.[NAD ⁺]
	Saturated[TCB]	Unsat.[TCB]	
NADH		C	NC
3-Methylcatechol	UC	NC	NC

Table 6.2 (b). Product-inhibition patterns observed for
different enzyme mechanisms

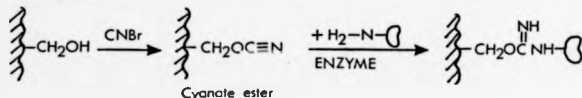
Mechanism	Product Inhibitor	Vary [NAD ⁺]		Vary[TCB]	
		Sat[TCB]	Unsat[TCB]	Sat[NAD ⁺]	Unsat[NAD ⁺]
ORDERED	NADH	C	C	-	NC
BI BI	3-Methylcatechol	UC	NC	NC	NC
RANDOM	NADH	NC	NC	NC	NC
BI BI	3-Methylcatechol	NC	NC	NC	NC
PING PONG	NADH	C	C	-	NC
BI BI	3-Methylcatechol	-	NC	C	C

Consequently, attempts were made to immobilize the enzyme and to determine whether the stability was maintained. Two different procedures were investigated, immobilization by covalent bonding and by matrix-entrapment, and the effectiveness of the two methods was compared.

Various catechols can be produced by chemical synthesis using the Dakin reaction (Carruthers, 1986). However, a dihydrodiol dehydrogenase could be used to produce certain catechols if they could not be synthesized by conventional chemical methods. In this situation, it would be advantageous to use immobilized p-toluene dihydrodiol since enzyme immobilization effectively renders an enzyme water-insoluble, thereby facilitating recovery and re-use of the enzyme.

4.12.2.2 Immobilization onto CNBr-activated Sepharose

Immobilization onto CNBr-activated Sepharose involves the formation of covalent linkages. The main advantages of this method of immobilization are that there is little limitation of access of substrate to the bound enzyme. Secondly, leaching of the enzyme is not a problem. CNBr activation of hydroxyl groups on the support to form cyanate esters enables subsequent coupling of the enzyme (Axén *et al.*, 1967).



After immobilizing the enzyme on CNBr-activated Sepharose (as described in Materials and Methods subsection 2.4.1), relatively high activity was detected in the supernatant collected. This indicated that a relatively high proportion of the enzyme (approx. 50%) did not bind to the Sepharose but was washed straight through the column. This inefficiency of binding to CNBr-activated Sepharose implied that there may be a lack of amino groups on the protein available for binding.

The effectiveness of the column was investigated by continually circulating 10mM NAD⁺ and 10mM cis-toluene dihydrodiol through the column at room temperature for 12 hours. After 5h and after 12h a sample of the eluate was removed and analysed by HPLC using a Lichrosorb C18 column (see Materials and Methods section 2.6) but no attempt was made to extract the 3-methylcatechol. After 5h, 4.8mM 3-methylcatechol and 1.5mM cis-toluene dihydrodiol were detected, whereas after 12h only 0.02mM cis-toluene dihydrodiol remained and 6.3mM 3-methylcatechol was detected. The column was stored at 4°C for 28 days to investigate the stability of the immobilized enzyme. After 14 days and 28 days the experiment was repeated and similar results to those above were obtained (Table 6.3) indicating that the immobilized enzyme was stable for at least 4 weeks.

6.2.12.3 Immobilization onto alginate beads

Alginates are polysaccharides obtained from seaweeds and

Table 4.3 Production of 3-methylcatechol by immobilized
cis-toluene dihydrodiol dehydrogenase

Method of immobilization	Storage period of column (days)	TCB remaining* after 12h (mM)	3-MC produced after 12h (mM)
CNBr-activated sepharose	0 14 28	0.02 0.1 0.03	6.3 6.0 7.1
Alginate beads	0 14	2.5 7	3 0.5

* : Starting concentrations were 10mM TCB and 10mM NAD⁺

TCB = cis-toluene dihydrodiol

3-MC = 3-methylcatechol

they gel in the cold in the presence of divalent cations, particularly calcium. The immobilization procedure which was used is described in Materials and Methods, subsection 2.4.2. Entrapment into a gel matrix is a mild method of immobilization but reactions may be diffusion-limited and substrate size may limit the applicability of the method.

After re-circulating 10mM cis-toluene dihydrodiol and 10mM NAD⁺ through the column for 12 h, only 3mM 3-methylcatechol was detected (Table 6.3). This finding could indicate that the entrapped enzyme was not accessible to the substrate. Furthermore, after storing the column for 14 days at 4°C, the activity deteriorated indicating that the enzyme had become unstable or that the alginate was being degraded. It is possible that the alginate became contaminated with microorganisms capable of growing on the alginate.

6.3 DISCUSSION

To investigate the enzymes involved in toluene metabolism in Bacillus sp. AT50, it was necessary to purify the enzymes. Initially, attempts were made to purify toluene dioxygenase, the enzyme responsible for converting toluene into cis-toluene dihydrodiol. However, the efforts to purify toluene dioxygenase only served to highlight the need for a new assay system for this enzyme (Chapter 5). Using DEAE-cellulose chromatography, the method used by Yeh et al., (1977) to purify toluene dioxygenase from Pseudomonas putida, some activity was detected with the

spectrophotometric indole assay after combining various fractions. However, further purification of the ferredoxin-reductase component using DEAE-Sephadex was unsuccessful and no activity was detected. Subsequent attempts to purify the various components of toluene dioxygenase with a p-toluic acid affinity column (Subramanian *et al.*, 1979, 1980 & 1985) were also unsuccessful. Therefore, the production of *cis*-toluene dihydrodiol using purified toluene dioxygenase was not possible. Consequently, purification of the next enzyme in the pathway, *cis*-toluene dihydrodiol dehydrogenase, was attempted.

cis-Toluene dihydrodiol dehydrogenase was previously purified by Rogers & Gibson (1977). The enzyme was purified from *Pseudomonas putida* and consisted of four identical subunits of M_r 27 000 and was specific for NAD⁺ as the electron acceptor. The enzyme was inhibited by reducing agents and had a high affinity for *cis*-toluene dihydrodiol and indeed was specific for *cis*-dihydrodiols. Dehydrogenases that catalyze the oxidation of *cis*-diols from benzoic acid (Reiner, 1972), benzene (Axcell & Geary, 1973), naphthalene (Patel & Gibson, 1974) and chloridazon (Eberspacher & Lingens, 1978) have also been purified. A comparison of these enzymes is summarised in Table 6.4. Apart from the enzyme reported here, all the other *cis*-dihydrodiol dehydrogenases described were isolated from mesophilic organisms and most work has been carried out

Table 6.4

A comparison of purified cis-diol dehydrogenases

Substrate	Organism	Characteristics of enzyme			
		M _r (kDa)	Subunits (kDa)	K _m (μM) Diol	K _m (μM) NAD ⁺
<u>cis</u> -Toluene dihydrodiol	<u>Bacillus</u> sp. A750 (This work)	172	6x29.5	92	80
<u>cis</u> -Toluene dihydrodiol	<u>Pseudomonas putida</u> (Rogers & Gibson, 1977)	104	4x27	<2	460
<u>cis</u> -Benzene dihydrodiol	<u>Pseudomonas putida</u> (Axcell & Seary, 1973)	440	4x110	286	43.5
<u>cis</u> -Naphthalene dihydrodiol	<u>Pseudomonas putida</u> (Patel & Gibson, 1974)	102	4x25.5	29	800
Chloridazadi- hydrodiol	Unidentified bacterium (Eberpacher & Lingens, 1978)	220	4x50	250*	200 250
<u>cis</u> -1,2 Benzoic acid diol	<u>Alcaligenes eutrophus</u> (Reiner, 1972)	94.6	4x24	200	150

* Two iso-enzymes exist which differ in K_m values
for substrate

with the enzyme from Pseudomonas putida. The enzyme purified from Bacillus sp. AT50 exhibits some unusual characteristics including remarkable thermostability. The enzyme exists as a hexamer and not, as in the case of previously reported enzymes, as a tetramer. Furthermore, although very little work on the kinetics of the dehydrogenases has been carried out, it is notable that the K_m values all differ significantly.

The K_m of cis-toluene dihydrodiol dehydrogenase for NADH (80 μ M) was comparable to the K_m for NAD⁺ (82 μ M) which suggests that NADH may have a physiological role for the control of activity of the enzyme. The dioxygenase which converts toluene into cis-toluene dihydrodiol requires NADH as substrate and, therefore, intracellular accumulation of NADH could indicate a lowered activity of toluene dioxygenase.

Since toluene dioxygenase and benzene dioxygenase require NADH as a substrate (Yeh et al., 1977), which is a common product of the respective dehydrogenases, it has been suggested that a dioxygenase-dehydrogenase complex may exist in the intact organism (Rogers & Gibson, 1977). This coupling would permit a recycling of electrons from one enzyme to the other. There is evidence of a coupled aryl monooxygenase-epoxide hydrolase system in mammalian microsomes (microsomal fractions) (Desch & Daly, 1972). Also, it has been suggested that, at least under certain growth

conditions, there is a direct coupling between methane monooxygenase and methanol dehydrogenase in cells of Methylococcus capsulatus (Leak & Dalton, 1986). It is possible that in cells of Bacillus sp. AT50 a dioxxygenase-dehydrogenase complex may exist with recycling of electrons between the two enzymes. Consequently, NADH would indeed have a physiological role for the control of activity of cis-toluene dihydrodiol dehydrogenase. Likewise, levels of NAD⁺ may control the activity of toluene dioxxygenase. It is noteworthy that the cis-toluene dihydrodiol dehydrogenase purified by Axcell & Geary (1973) was inhibited by 1,10-phenanthroline and contained loosely bound ferrous ions that were essential for full enzyme activity. The benzene dioxxygenase also required ferrous ions for activity (Yeh et al., 1977) providing further evidence which suggested that the two enzymes may be coupled. However, although toluene dioxxygenase from Bacillus sp. AT50 required ferrous ions, it appeared that cis-toluene dihydrodiol dehydrogenase did not require metal-ions for activity. Metal-ion chelators, such as EDTA, KCN, α,α' -dipyridyl and bathophenanthroline, were relatively inefficient inhibitors of the dehydrogenase. Therefore, the enzyme described here differs from the cis-benzene dihydrodiol dehydrogenase (Axcell & Geary, 1973) with respect to metal-ion requirements.

Although metal-ion chelators were not good inhibitors, the

purified cis-toluene dihydrodiol dehydrogenase was significantly inhibited by mercurials such as, mercuric chloride and p-hydroxymercuribenzoate. Although this finding is not conclusive, it appears possible that thiol groups are essential for catalytic competence. It was hoped to find a specific inhibitor of cis-toluene dihydrodiol dehydrogenase, which could result in the accumulation of cis-toluene dihydrodiol in cultures of Bacillus sp. AT50, but unfortunately all the compounds which inhibited this enzyme also inhibited toluene dioxygenase activity, as measured in crude cell extracts. Consequently, it was not possible to produce cis-toluene dihydrodiol by specifically inhibiting the dehydrogenase.

The purified cis-toluene dihydrodiol dehydrogenase was isolated from an organism which grew optimally at 50°C but the temperature optimum of the enzyme was 80°C. The temperature optimum is, of course, only a balance between the rate of reaction and the rate of enzyme inactivation but it does illustrate that this enzyme is more active at high temperatures than the majority of enzymes. Furthermore, the enzyme was extremely thermostable, with a half-life of 110 min at 80°C. When the incubation at 80°C was carried out in the presence of cis-toluene dihydrodiol or NAD⁺, then the stability of the enzyme was decreased. This finding suggests that, on binding substrate or cofactor, a conformational change of enzyme makes it more susceptible to inactivation. The activation energy of the reaction was 36

kJ.mol^{-1} which is relatively high for an enzymic reaction (Morris, 1974). The Arrhenius plot levelled off at 65°C , suggesting some type of conformational change above this temperature which lowers the activation energy to 4.41 kJ.mol^{-1} . The free energy of activation is the amount of energy required to bring all the molecules in one mole of a substrate at a given temperature to the transition state at the top of the activation barrier. Above 65°C less energy is required to bring the molecules to the transition state, indicating that the conformation of the enzyme in the transition state must be different than at the lower temperatures. It has been shown by Matsunaga and Nosoh (1974) that a conformational change occurs in glutamine synthetase of Bacillus stearothermophilus, well below the temperature at which the catalytic site is destroyed.

The stability of the cis-toluene dihydrodiol dehydrogenase purified from Bacillus sp. AT50 indicated that this enzyme should be relatively easy to immobilize. Immobilization onto CNBr-activated sepharose was more successful than immobilization onto alginate beads. Enzyme immobilized by the latter method was not very active and proved unstable after 14 days at 4°C . Conversely, immobilization by covalent coupling provided a relatively stable enzyme preparation with no loss of activity after 28 days at 4°C . This finding illustrates the importance of choosing the right method of immobilization even for enzymes which are

apparently very stable. cis-Toluene dihydrodiol dehydrogenase immobilized onto CNBr-activated sepharose could potentially be applied to produce catechols which cannot be synthesized by conventional chemical methods.

It has been observed that several enzymes from thermophilic organisms have a high content of acidic amino acids and exhibit acidic isoelectric points (Singleton & Amelunxen, 1973). Indeed the first intracellular enzyme crystallized from a thermophilic bacterium was glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) which exhibited marked thermostability at 80°C and had an acidic isoelectric point (Amelunxen, 1966). Consequently, conclusions were hastily drawn that there was a direct correlation between thermostability of proteins and acidic isoelectric points. However after further investigation Amelunxen and Murdoch (1978) concluded that the correlation was based on insufficient data. This is borne out by the enzyme described here which exhibited a fairly high pI of 6.4. Unfortunately, time did not permit amino acid analysis of this cis-toluene dihydrodiol dehydrogenase which may have helped to reveal whether there is any correlation between the occurrence of particular amino acid sequences and the thermostability of proteins.

CHAPTER 7 : THE MUTAGENESIS OF STRAINS HTB16 AND AT50 IN
AN ATTEMPT TO PRODUCE CIS-TOLUENE DIHYDRODIOL

INTRODUCTION

Strains HTB16 and AT50 both oxidized toluene via cis-toluene dihydrodiol (Chapter 5). However, this compound could not be detected since it was rapidly degraded to 3-methylcatechol and, ultimately, tricarboxylic acid cycle intermediates. Since an initial aim of the project was to produce cis-toluene dihydrodiol using thermotolerant bacteria, it was necessary to obtain mutants of strains HTB16 and AT50. Unless a constitutive mutant was obtained, it would be necessary to induce the enzymes responsible for toluene metabolism. Consequently, when grown in the presence of toluene plus another carbon source (eg. pyruvate), a mutant strain lacking cis-toluene dihydrodiol dehydrogenase activity should accumulate cis-toluene dihydrodiol in the culture supernatant. In addition, the characteristics of mutants defective in various parts of the pathway would help to confirm the pathway of toluene metabolism in the bacteria (Chapter 5).

The conditions necessary for the isolation of a specific mutant type may vary considerably in different situations. Consequently, it was necessary to investigate the effectiveness of several different mutagenic procedures with each strain.

RESULTS AND DISCUSSION

7.1 Growth of strains HTB16 and AT50 on non-aromatic carbon sources.

To determine whether a mutant will produce cis-toluene dihydrodiol, it is necessary to grow the organism in the presence of toluene plus a fully metabolizable co-substrate and measure the accumulation of cis-toluene dihydrodiol in the culture supernatant using HPLC. Of course, the substrate used must not repress the synthesis of toluene dioxygenase.

The growth of strains HTB16 and AT50 on various non-aromatic carbon sources was investigated (Table 7.1). The poor growth observed with either strain on glucose may reflect an inability to transport glucose into the cell or simply a lack of the necessary enzymes for glucose metabolism. Pyruvate (0.5%w/v) appeared to be the best carbon source and a doubling time of 2.1h was observed with strain AT50. Growth of strain AT50 in continuous culture on pyruvate (0.5%w/v) was investigated to determine whether the toluene-metabolizing enzymes could be induced during growth on pyruvate. When the pyruvate-grown culture was either pyruvate-limited or ammonium-limited, no induction of toluene-metabolizing enzymes occurred on sparging the culture with toluene vapour. The growth-limiting pyruvate feed was discontinued and toluene was supplied as the sole

Table 7.1

Growth of strains HTB16 and AT50 on non-aromatic carbon sources

Carbon source	Concentration (%w/v)	Growth	
		HTB16	AT50
Pyruvate	0.5	++	++ ($t_d=2.1h$)
Acetate	0.5	++	++ ($t_d=3.5h$)
Gluconate	0.5	+	+
Glucose	0.5	-	-
Glucose	2	+	+

++ indicates growth

+ indicates poor growth

- indicates no growth after 7 days

t_d = doubling time

carbon and energy source. The enzymes were assayed after 12h and there had been co-ordinate induction of toluene dioxygenase, cis-toluene dihydrodiol dehydrogenase and catechol-2,3-oxygenase. This finding indicated that pyruvate caused catabolite repression of the toluene-metabolizing enzymes. Similarly, addition of pyruvate (0.5% w/v) to a continuous culture of strain AT50 grown on toluene (supplied in the vapour phase) caused repression of the toluene-metabolizing enzymes. However, the enzymes responsible for toluene metabolism were induced in overnight flasks containing 0.3%(w/v) pyruvate and 0.1%(v/v) toluene. 0.3%(w/v) Pyruvate appeared to limit growth since a lower OD was obtained than when cells were grown on 0.5%(w/v) pyruvate. Therefore, it appeared that growth on 0.3%(w/v) pyruvate in the presence of 0.1%(v/v) toluene allowed induction of the toluene-metabolizing enzymes after depletion of the pyruvate. Consequently, this concentration of pyruvate and toluene was used subsequently when characterizing mutants.

7.2 N-methyl-N'-nitro-N-nitrosoguanidine (NTB) autagenesis of strains HTB16 and AT50

NTB is a powerful and widely used mutagen (Carlton & Brown, 1981). It is an alkylating agent which reacts preferentially with guanine to form 7-ethyl or

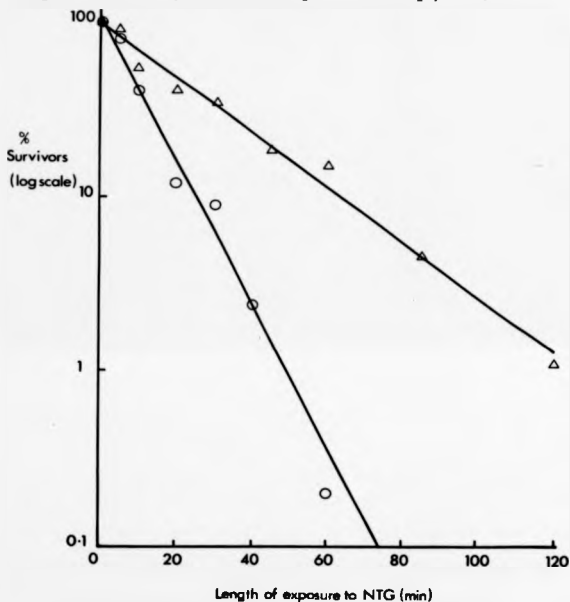
7-methylguanine, thereby destabilizing the nucleoside linkage between the purine base and the deoxyribose sugar moiety. Consequently, the guanine is lost from the DNA chain and guanine-cytosine to adenine-thymine transitions occur in double-stranded DNA.

7.2.1 NTB mutagenesis of strain HTB16

NTB was added to a culture of strain HTB16 growing exponentially on 0.1X(v/v) toluene, to a final concentration of 10 μg NTB/ml or 20 μg NTB/ml culture. The resulting dose-survival curves (Figure 7.1) indicated that NTB (20 μg /ml) induced approximately 50% killing after 10 min and 99.9% killing after approx. 70 min. Consequently, the mutagenic treatment of cells of strain HTB16 was carried out for 10 min, with 20 μg /ml NTB, to obtain approx. 50% killing in an attempt to obtain mutants with single mutations. After the treatment, the mutagenized culture was grown overnight on pyruvate (0.5Xw/v) to allow a period of outgrowth to permit the segregation of newly mutated DNA from non-mutated DNA in the same cell. Although bacteria are haploid, there are often 1.5-2 complete DNA copies in the cell, during cell division and DNA replication. Consequently, a newly induced mutation of a recessive phenotype (eg. an enzymic function) may be masked by the presence of unmutated DNA in the cell. Therefore, a period of outgrowth is necessary. After the mutagenic treatment of

Figure 7.1 Dose-survival curve of the effect of NTG
on strain HTR16

Counts were worked out as a % of the original count obtained after no mutagenic treatment, i.e. % survivors. The % survivors was plotted on a log scale against the length of time exposed to mutagen (semi-log plot).



Length of exposure to NTG (min)
Plot obtained after exposure to 10 µg/ml NTG

Plot obtained after exposure to 20 µg/ml NTG

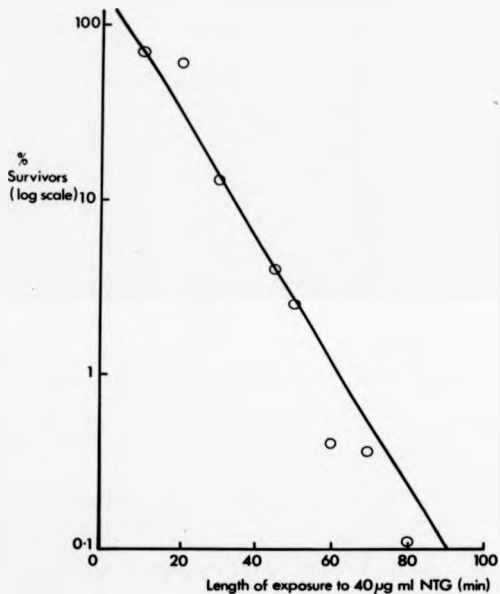
strain HTB16, the survivors were plated out onto nutrient agar. Of the 1000 colonies screened, no mutants unable to metabolize toluene were detected. The procedure was repeated with 20 $\mu\text{g}/\text{ml}$ NTB for 70 min to give approx 99.9% kill. 2 000 colonies were screened and only 6 mutants unable to grow on toluene, but capable of growth on pyruvate, were detected. The mutants were screened for the various enzyme activities and all lacked toluene dioxygenase, as measured by the ability to produce indigo from indole (in the presence of 0.03% (w/v) pyruvate). None of the mutants produced cis-toluene dihydrodiol when grown in liquid medium in the presence of 0.1% (v/v) toluene and 0.3% (w/v) pyruvate. All the mutants possessed functional catechol-2,3-oxygenase activity since when the colonies were sprayed with 1M catechol they turned yellow due to the production of a hydroxymuconic semialdehyde. After the mutagenic treatment, approx. 2% auxotrophs were detected.

7.2.2 NTB mutagenesis of strain AT50

The dose-survival curve of strain AT50 revealed that approx. 99.9% killing occurred after exposure to 40 $\mu\text{g}/\text{ml}$ NTB for 80 min (Figure 7.2), and these conditions were adopted for the mutagenesis. The survivors were plated out and 2 500 colonies screened for growth on toluene. Eight mutants were detected which were unable to utilize toluene as the sole

Figure 7.2 Dose-survival curve of the effect
of 40 $\mu\text{g/ml}$ NTG on strain AT50

40 $\mu\text{g/ml}$ NTG was added to a culture of strain AT50 and the % survivors were determined after specified periods of time.



source of carbon and energy. These mutants were not auxotrophs since they grew on a minimal medium containing pyruvate (0.5%w/v). All the eight mutants were found to lack toluene dioxygenase activity as judged by their inability to form indigo from indole in plate tests. This mutagenic treatment was repeated on a separate occasion and 5 mutants, which were unable to grow on toluene, were obtained after screening 2 000 colonies. Unfortunately, all these mutants also lacked toluene dioxygenase activity and did not accumulate cis-toluene dihydrodiol when grown on toluene (0.1%v/v) and pyruvate (0.3%w/v). The mutants all possessed catechol-2,3-oxygenase activity, as detected by yellow colouration of the colonies on spraying with 0.1M catechol.

7.3 Mutagenesis of strain HTB16 with ultraviolet (UV) light.

Mutagenesis with UV light is a relatively simple procedure. In addition to causing base transitions and transversions, it also induces deletions (Hars, 1980). Since many bacterial cells possess efficient photoreactivating mechanisms (which are promoted by visible light) for repairing the damage, the cells were incubated in the dark, immediately after treatment.

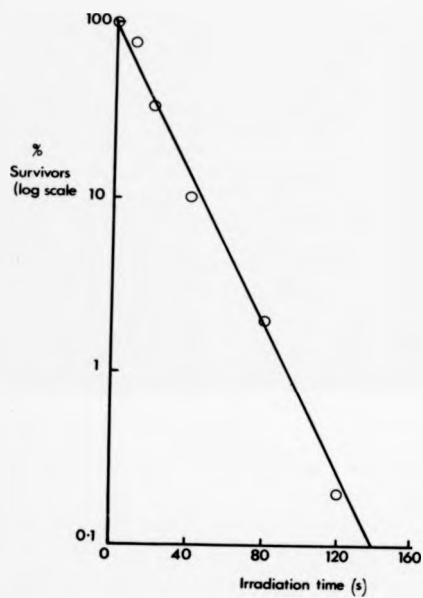
UV mutagenesis was carried out on strain HTB16 (as described

in Materials and Methods subsection 2.5.3.2) and a dose-survival curve plotted (Figure 7.3). An irradiation time of 2 min was used to give approx. 99.9% killing. The survivors were plated out and 1 000 colonies were replica plated onto nutrient agar and minimal medium plates incubated in the presence of toluene vapour. The frequency of auxotrophs amongst survivors was approx. 1% but no mutants unable to grow on toluene, as the sole carbon and energy source, were isolated.

7.4 Transposon mutagenesis of strain AT50

Transposons are mobile genetic elements which can insert at random into the bacterial chromosome (or plasmids) independently of the host cell recombination system. In addition to genes necessary for maintenance and transposition, they may encode resistance to antibiotics. Consequently, transposons can generate stable mutations by insertional inactivation of a gene, which causes complete loss of gene function and simultaneously provides a selectable antibiotic resistance (e.g. Kleckner et al., 1977). In order to achieve transposon mutagenesis, a vector is required which, upon introduction to the recipient cell, fails to replicate e.g. a heat-sensitive plasmid (Youngman et al., 1983). Selection of the transposable antibiotic resistance marker then ensures that all survivors carry transposon insertions.

Figure 7.3 Survival of strain HTB16 after UV irradiation



Attempts were made to carry out transposon mutagenesis of strain AT50 with the Streptococcus faecalis transposon, Tn917, which encodes erythromycin resistance. Bacillus subtilis PY143 contains a heat-sensitive plasmid, pTV1, which encodes chloramphenicol resistance and contains Tn917 (Youngman et al., 1983). Therefore, plasmid pTV1 was isolated from B. subtilis PY143 and used as the vector for Tn917 transposition. This vector was suitable for use with AT50 since preliminary experiments indicated that strain AT50 was not resistant to 20 µg/ml erythromycin or 5 µg/ml chloramphenicol. The plasmid pTV1 was isolated from B. subtilis PY143, as described in Materials and Methods subsection 2.5.4. Since Bacillus species cannot undergo conjugation, it was necessary to introduce pTV1 into the cells by the process of transformation. Recipient cells must be in a competent state for transformation to occur. Spizizen (1958) developed a medium in which certain strains of Bacillus became competent. This medium was used to obtain cells of strain AT50 in a competent state. Transformation with pTV1 was then carried out at 37°C and the resulting cells were grown at a temperature which was non-permissive for plasmid replication (48°C). The strain was then plated to select for erythromycin resistance. The resulting colonies were screened for loss of chloramphenicol resistance (indicating loss of the plasmid) and erythromycin

resistance (indicating Tn917 transposition). 1 000 erythromycin resistant colonies were screened to identify any mutants which could no longer grow on toluene as the sole carbon and energy source. No such mutants were obtained. Unfortunately, time did not permit further experiments to be performed which would confirm whether the cells had been transformed. Firstly, it is necessary to carry out experiments to determine the rate of spontaneous erythromycin resistance of strain AT50. This would provide an indication of the number of cells in which Tn917 transposition had occurred compared to the number which had simply become resistant to erythromycin. Also, it is necessary to confirm whether the cells had developed competence and subsequently been transformed. This could be confirmed by transforming the cells with any plasmid encoding antibiotic resistance (to which the cells exhibited low spontaneous resistance). The transformed cells could then be identified by screening for resistance to the antibiotic.

7.5 Isolation of native plasmids from strain AT50

Since mutagenesis of strain AT50 (and strain HTB16) produced a very low yield of mutants unable to grow on toluene as the sole carbon and energy source, experiments were carried out to screen strain AT50 for the presence of a plasmid involved in toluene metabolism. If the enzymes responsible for

toluene metabolism are encoded on a plasmid fewer mutants may be detected. This would occur because multiple copies of plasmids are often present in cells and mutation of one plasmid would be masked by the presence of other plasmids.

A large-scale isolation of plasmid DNA was attempted, as described in Materials and Methods subsection 2.5.4.2, using cells of strain AT50 grown on toluene in continuous culture. The preparation of DNA was run on an agarose gel and a plasmid band was detected after observation under UV light (Figure 7.4). The plasmid appeared to be fairly small (<20Kb) when run next to DNA markers. It is noteworthy that a plasmid preparation from an NTG mutant (of strain AT50), which could no longer grow on toluene as the sole carbon and energy source, did not reveal any plasmids. Further experiments are necessary to determine whether the plasmid encodes the enzymes responsible for toluene metabolism in strain AT50. The function of the plasmid may be determined by transforming an NTG mutant of strain AT50 with the plasmid. If the cells transformed with the DNA simultaneously regain the ability to grow on toluene (as the sole carbon source) then the plasmid must encode the enzymes for toluene metabolism. Alternatively, it may simply be a cryptic plasmid to which no function can be assigned.

Figure 7.4 Detection of a native plasmid in strain AT50

The plasmid was isolated as described in Materials and Methods subsection 2.5.4, before being subjected to agarose gel electrophoresis.



Lane 1 : Hind III-EcoRI - digested λ DNA

Lane 2 : Plasmid DNA from strain AT50

7.6 DISCUSSION

Strains of HTB16 and AT50 were subjected to several different mutagenic procedures in an attempt to obtain mutants deficient in cis-toluene dihydrodiol dehydrogenase activity. Unfortunately, no such mutants were obtained. This could be due to several reasons. Firstly, the screening of insufficient presumptive mutants would decrease the probability of obtaining a mutant lacking functional cis-toluene dihydrodiol dehydrogenase. Secondly, the probability of obtaining a suitable mutant would be lower if the enzymes responsible for toluene metabolism were encoded on a plasmid. Usually more than one copy of a plasmid is present in a cell. Therefore, mutants would not be detected since a mutation in one plasmid would be masked by the presence of other functional plasmids. Thirdly, mutants would not be detected if there was duplication of the genes which encoded the enzymes for toluene metabolism. Jenkins et al. (1987) reported that a number of mutants, which were unable to fully metabolize toluene, were isolated after transposon mutagenesis of Pseudomonas putida NCIB11767. Since all these mutants lacked toluene dioxygenase, the authors proposed that this may be due to the presence of two forms of cis-toluene dihydrodiol dehydrogenase in strain 11767, which were the products of two different genes. This could explain why no mutants of strain 11767 deficient in

cis-toluene dihydrodiol dehydrogenase activity were obtained. However, strain AT50 did not appear to possess two forms of cis-toluene dihydrodiol dehydrogenase as only one form was detected when the enzyme was purified (Chapter 6). Furthermore, only one band possessing cis-toluene dihydrodiol dehydrogenase activity was demonstrated (using an activity stain) after non-denaturing gel electrophoresis of crude extract of strain AT50. However, there is the possibility that two forms of cis-toluene dihydrodiol dehydrogenase exist in strain HTB16. After the various mutagenic treatments, very low mutant yields were obtained. This may be due to the fact that most mutagens, eg. NTG, are most effective on cultures growing exponentially i.e. on cells in which the DNA is replicating (Cárdena-Olmedo *et al.*, 1968). The mutagenesis of strains HTB16 and AT50 was routinely carried out on cultures growing on toluene (0.1%v/v). Since relatively slow growth occurs on this substrate, it may explain why few mutants were obtained. However, when the procedures were repeated on cells grown on pyruvate (0.5%w/v) similar yields were obtained. Also, it was noticeable that the yields of auxotrophs were relatively high. The fact that mutants deficient in the enzymes of toluene metabolism were obtained in very low yields may be due to the presence of a plasmid, gene duplication, or simply bad luck.

Of the various mutagenic procedures investigated, NTG

mutagenesis appeared to be the most successful method for obtaining a relatively high percentage of mutants amongst the survivors. NTG is a powerful mutagen which is relatively easy to use and can be applied to a wide variety of organisms. One disadvantage is that, on obtaining high mutant yields, multiple mutations can occur within a single organism (Carlton & Brown, 1981). However, this is only a problem when the mutants are required for complete genetic analysis of a strain (see Hopwood, 1970). Plate UV mutagenesis is useful for the rapid isolation of mutants of some Gram-positive bacteria (Dijkhuizen et al., 1981). It is a simple method (which for some reason does not require a period of outgrowth) but it is not applicable to all bacterial species. Transposon mutagenesis is a more complex procedure which necessitates a fairly detailed knowledge of the genetics of the strain to be mutagenized. In order to achieve transposon mutagenesis of bacteria, it is necessary to have a vector which can deliver the transposon into the cell. Although several indigenous Bacillus plasmids have been developed as vectors for molecular cloning (eg. Kraft et al., 1978), the use of antibiotic resistance plasmids from the Gram-positive bacteria Staphylococcus aureus (eg. Ehrlich, 1977) and Streptococcus faecalis (eg. Youngman et al., 1983) have proved more successful. For bacteria which do not possess well-developed transduction systems, heterologous DNA must be introduced into the cell by the

process of transformation. One disadvantage of this method is that plasmid transformation is a very inefficient process, in contrast to chromosomal DNA transformation (Contente & Dubnau, 1979). Also, for transformation to occur the recipient cell must exhibit competence i.e. have the ability to transport DNA from the culture medium into the cell. Spizizen (1958) found that several species of Bacillus can develop a natural physiological state in which they are capable of absorbing DNA from the surrounding medium. However, this phenomenon does not apply to all bacilli.

Although its function is not known, preliminary experiments revealed that strain AT50 contains a plasmid. Since the initial discovery of plasmid DNA in Bacillus megaterium (Carlton & Melinski, 1969), many plasmids indigenous to the bacilli have subsequently been discovered (eg. Bingham et al., 1979). Furthermore, TOL plasmids have been reported to be present in several Pseudomonas species (eg. Williams, 1981). Further experiments are necessary to confirm whether strains AT50 and HTB16 contain plasmids, which encode the enzymes responsible for toluene metabolism, since their presence/absence could influence which is the most suitable method for mutagenizing the strains. There is the possibility that certain enzymes responsible for toluene metabolism are encoded on a plasmid whereas others may be encoded on the chromosome.

CHAPTER 8 : FINAL CONCLUSIONS AND OUTLOOK

FINAL CONCLUSIONS AND OUTLOOK

Two therotolerant Bacillus species which could grow on toluene as the sole carbon and energy source, were isolated. It was previously believed that the bacteria responsible for the degradation of aromatic compounds were predominantly pseudomonads (eg. Timmis et al., 1985). However, many different bacterial species can degrade aromatic compounds and the isolate obtained depends on the enrichment conditions employed eg. medium composition, temperature, etc.

Strains HTB16 and AT50 were both potential cis-toluene dihydrodiol producers as this compound was an intermediate of toluene metabolism in both organisms. Strain AT50 appeared to be more suitable for an industrial process since it had a higher optimum growth temperature (50°C) than strain HTB16 (45°C). However, investigation with continuous cultures of strain AT50 suggested that one possible disadvantage of growing toluene-utilizing bacteria at 50°C is the low solubility of toluene in aqueous medium at this temperature. One advantage is that the higher growth temperature of strain AT50 was reflected by a greater stability of the enzymes. cis-Toluene dihydrodiol dehydrogenase from strain AT50 was particularly stable and therefore, relatively easy to purify. The purified enzyme exhibited a half-life of 100 min at 80°C, the temperature

optimum. Further work is necessary to develop a new assay for toluene dioxygenase before purification of this enzyme is possible. The purification and subsequent characterization of the dioxygenase from strain AT50 may provide further evidence that enzymes from thermotolerant organisms are inherently thermostable. In addition to great heat-stability, the cis-toluene dihydrodiol dehydrogenase also appeared to be relatively resistant to chemical inhibitors. Consequently, it may not be feasible to produce cis-toluene dihydrodiol by specifically inhibiting the dehydrogenase in cultures of strain AT50.

The most promising approach to produce cis-toluene dihydrodiol is to produce mutants of strain AT50 which lack functional cis-toluene dihydrodiol dehydrogenase. However, the genetics of the bacteria need to be studied in more detail before a suitable mutagenesis protocol can be devised. If the enzymes responsible for toluene metabolism are encoded on a plasmid, of which greater than one copy is present in the cell, then many mutations would not be detected. Mutations in one plasmid would be masked by the presence of other functional plasmids. Also, certain mutagens are known to 'cure' plasmids from the cell (Novick, 1969). Consequently, a strain in which the enzymes responsible for toluene metabolism are not encoded on a plasmid is more desirable. Further experiments are also needed before transposon mutagenesis of strain AT50 can be

evaluated. It is necessary to confirm that the cells developed competence i.e. the ability to take up DNA into the cell (Anagnostopoulos & Spizizen, 1960; Bott & Wilson, 1967). An alternative approach which could be attempted to transform cells of strain AT50 is to induce DNA uptake in protoplasts and subsequently regenerate the bacterial cell wall (Chang & Cohen, 1979).

Once a mutant lacking functional cis-toluene dihydrodiol dehydrogenase is obtained, further mutagenesis steps could be carried out to produce further mutants. A constitutive mutant could be isolated thereby obviating the need for the induction step during the bioconversion. Also, mutants which are not susceptible to catabolite repression could be obtained. Investigation of the bioconversion could then be carried out to determine whether thermotolerant organisms are indeed more suitable for industrial processes. The yields of aromatic cis-dihydrodiols could then be compared with those already reported for mutant strains of Pseudomonas putida (eg. Taylor et al., 1983).

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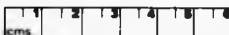
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