The Metabolism of Aromatic Compounds by Thermophilic Bacilli

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ABSTRACT

Semi-defined media have been developed for a number of bacilli. Using these media, the microorganisms were screened for growth in the presence of a range of aromatic compounds. Five out of ten of the strains were able to utilize aromatic acids as sole carbon sources.

Other thermophilic bacilli were isolated after enrichment on aromatic compounds. One of these strains, Bacillus stearothermophilus IC3, degraded both phenol and benzoic acid. All further studies involve this organism.

The first step in phenol degradation involved the hydroxylation of phenol to catechol, a reaction that required NADH as a cofactor. Catechol then underwent meta fission to produce 2-hydroxymuconic semialdehyde. This compound was further metabolised, by both oxidative and hydrolytic pathways, to pyruvate and acetaldehyde via the following compounds: 4-oxalocrotonate; 2-oxopent-4-enoate; and 4-hydroxy-2-oxovalerate. All of the enzymes involved, with the exception of 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase and 4-hydroxy-2-oxovalerate aldolase, were induced in the presence of phenol but not glucose. A study was also made of the initial oxygenases, and the effects of substrates, temperature, inhibitors and metal ions upon their activity.

Experiments to produce mutants that gave constitutive production of the initial oxygenases, and/or had pathways that were glucose derepressed, are described, together with experiments to produce mutants defective in aromatic metabolism.

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CHAPTER 1

INTRODUCTION

Thermophilic bacteria have been found in several genera, and have been isolated from numerous sources, including; soil, mud, water (fresh and sea), sewage, decaying plant meterial, air and even freshly fallen snow. (Allen 1953).

Animals , higher plants and multicellular organisms are unable to grow above 50°C, and even lower eukaryotes (algae and fungi) above about 60°C. Photosynthetic prokaryotes show a well defined temperature maximum of 70-73°C, and above 90°C only members of the archaebacteria are able to grow (Brock 1985). It has been suggested that the upper limit for bacterial growth may be the presence of liquid water, thermophiles being able to grow even in boiling hot water springs. (Brock 1978).

These organisms have been of great interest in both biochemical and evolutionary studies. A study of *Clostridium* species (Tanaka *et al* 1971) suggested that thermophiles may be one of the older forms of life.

Woese (1987) has used complete rRNA sequences to study bacterial evolution. These studies indicated that archaebacteria were considerably older than eubacteria. The evidence derived from the analysis of rRNA sequences, together with evidence such as the widespread occurence of thermophiles amongst archaebacteria, led Woese to propose that the ancesteral archaebacteria were probably anaerobic,

and grew at temperatures near the boiling point of water.

1.1.Properties of Thermophiles.

There has been much research on thermophilic bacteria to deduce the mechanisms by which their subcellular components are stabilized at higher temperatures.

1.1.1.Proteins.

Three mechanisms for the maintenance of proteins at higher temperatures were proposed (Singleton and Ameluxen 1973):

1. The proteins were no more stable than mesophilic ones, but fast turnover enabled a constant supply.

2. An extra factor was produced that stabilized proteins and other macromolecules.

3. The proteins were inherently stable.

Studies of mutants in the subunit of tryptophan synthetase from Escherichia coli, with altered temperature stabilities (Yutani et al 1977), have shown that only a single amino acid substitution was required to produce more heat stable proteins. For example, in the mutant trpA33, an increase in hydrophobicity caused by substituting methionine for glutamic acid was sufficient to give the protein increased stability.

Thermophilic ferredoxins are stabilized by extra salt bridges between external polar groups and additional hydrogen bonds, but not, apparently, by more polar contacts (Perutz and Raidt 1975).

Thermal stability in glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase was shown to be caused by an additive series of very small improvements at many locations within the molecules (Argos et al 1979). The most significant changes were an increase in the internal hydrophobicity and the helix forming ability of residues in helices. To a lesser extent there was an increase in the tendancy of residues in sheets to form sheets and of internal residues to increase in bulk to obtain better packing organisation.

Klibanov (1983) indicated that arginine would produce increased hydrogen bonding at neutral and alkaline pH values compared to histidine or lysine due to the higher pK of the arginine side group.

Over 100 proteins from thermophiles (75 from Bacillus stearothermophilus) have been purified, and most of them are not stabilized by non-proteinaceous material, only one or two amino acid changes causing greater hydrogen bonding are required. (Ljungdahl 1979). However, the intrinsic thermal stability of proteins may be enhanced, in vivo, by one or more intracellular components which may include other proteins (Sundaram 1986).

1.1.2.Lipids.

It has been suggested that the lipid bilayer may be the most critical component when considering the thermal stability of an organism, since a single hole in the membrane could cause the death of the cell (Brock 1967).

Lipid bilayers in thermophilic microorganisms again appear to be inherently thermostable. Increasing the growth temperature of an organism, even within the same organism, increases the average fatty acid chain length, increases the amount of methyl fatty acids, and decreases the amount of unsaturated fatty acids. This leads to an increase in the average melting point of fatty acids in the cell. For example, in Bacillus stearothermophilus, 64% of the total acids are 15 and 17 carbon iso-branched chains, and considerable amounts of 15-17 carbon anteiso-chains are present. In the caldoactive bacilli, 15-17 carbon iso-branched fatty acids account for 80% of the total. (Lungdahl 1979, Langworthy and Pond 1986 and references therein).

However, studies with some branched fatty acid chains have indicated that they behave as fluidizers, rather than stabilizers, in the lipid bilayer. Bilayers of methyl iso-branched, and especially methyl anteiso-branched fatty acid containing phosphatidyl cholines, exhibited a reduced solid-fluid phase transition temperature compared to their straight chain equivalents (Kannenberg et al 1983). This suggests that other factors, such as the enrichment of lipid headgroups with carbohydrates, may be more important. The manner in which the sidechains are atta-ched to these headgroups may also be significant. Eubacteria cannot grow above 85-90°C, presumably because a normal bilayer will start to melt. However, in some thermophilic archaebacteria, the two halves of the lipid bilayer are covalently condensed. (Langworthy and Pond 1986, and references therein).

1.1.3.Other Intracellular Components.

It has been reported (Oshima 1979) that unique polyamines are produced that stabilize the protein synthesis apparatus and some macromolecules. In mesophilic organisms, only the diamine putrescine, and the triamine spermidine are found.

In addition to putrescine and spermidine, moderate thermophiles such as Bacillus stearothermophilus and Bacillus acidocaldarius produce spermine, a tetramine. The more extreme thermophile Thermus thermophilus produces twelve polyamines, which include tetramines (thermine and thermospermine) and hexamines (caldohexamine and homocaldohexamine). (Oshima 1986).

DNA can easily be stabilized by salt bridges, even at moderate ionic strength (Zeikus 1979), although thermophiles do possess DNA with a higher G:C ratio. This may be required to stabilize other nucleic acids produced from the DNA, such as tRNA, that is stabilized by a higher G:C content in its base paired region (Oshima 1979).

1.2.Industrial Aspects of Thermophilic Microorganisms.

Interest in thermophilic bacteria has increased recently due to their industrially important characteristics, (Zeikus 1979, Hartley and Payton 1983, Sonnleitner 1983), these include:

1. These organisms grow very quickly, and the higher metabolic rates should enable faster fermentations.

2. They tend to have broad temperature optima which may enable less stringent temperature controls.

3. The enzymes produced are usually more stable.

4. The high stability and low activity of the enzymes and cells at room temperature may lower the need for refri-geration during processing.

5. Although not eliminated, the potential number of pathogens and contaminants is reduced.

6. There are many physical advantages with working at higher temperatures. The amount of ionization in the broth is increased, as is the solubility of most organic compounds, including aromatics. The high temperature may also enhance the removal of volatile products such as ethanol. At the same time the viscosity of the broth is lowered, and efficient cooling will be possible with water at room temperature.

7. The cloning of thermophilic genes into mesophiles may enable the rapid purification of the thermophilic enzyme by a heat treatment technique. (Oshima 1986).

There are of course physical disadvantages, such as the lower solubility of gases, of which oxygen may be a particular problem.

1.2.1.The Production of Ethanol and Organic Acids.

Thermophiles have properties that make them especially attractive candidates for the production of ethanol. Firstly, the raised temperature and the low boiling point of ethanol may enable easy, or even continuous recovery of the product. Secondly, some thermophiles, unlike yeasts, are able to

produce ethanol from any hexoses, di and polysaccharides, and various pentoses, that they are able to ferment (e.g. Clostridium thermohydrosulfuricum and Thermoanaerobacter ethanolicus). Another organism, Clostridium thermocellum, is able to degrade cellulose, with ethanol as the major product. (Weigel 1980).

Weimer (1986) has suggested that the main use for microbially produced ethanol may eventually be ethylene, even though ethylene is currently used as a source of ethanol and used in the production of other important chemicals, for example polyethylene, ethylene glycol, vinyl chloride and acetate, and styrene. Such a proposition is likely to be dictated by oil prices.

Organic acids such as acetic and lactic acids may also be viable fermentation products. However, the high pH optima of the thermophilic organisms involved makes efficient recovery difficult (Weimer 1986).

1.2.2.Industrial Uses of Thermophilic Enzymes.

Enzyme characteristics, such as their high activity under mild conditions, their selectivity and their stereospecificity, enable their use as industrial catalysts. However, they do display very poor operational stability. Enzymes may be denatured by numerous factors, amongst which thermal inactivation is by far the most important (Klibanov 1983).

There are several approaches available to produce heat stable enzymes. Stabilizing compounds may be added, the

enzyme may be chemically modified or immobilized, and, of course, thermophilic enzymes may be utilized (Schmid 1979).

Of the 400 million dollars worth of enzymes sold in 1984, 90% were thermostable enzymes used by the detergent and starch industries (Ng and Kenealy 1986). These included carbohydrases, proteases and lipases. However, the majority of these enzymes were not derived from thermophilic microorganisms, mainly due to the prohibitive cost of research and development when the enzyme cost is compared to the overall process cost.

1.2.3.Thermophilic Biotransformations.

Enzymes have a clear superiority over chemical catalysts where stereospecific reactions are involved (Ng and Kenealy 1986).

Stereospecific reactions involving thermophilic microorganisms include the hydroxylation of cinerone to cinerolone, an important precursor in the synthesis of insecticidal pyrethrins, by thermophilic streptomycetes (Tabenkin et al 1969). The ratio of enantiomers formed varied from strain to strain.

Thermoactinomyces or Thermonospora strains can reduce 4-oxoisophorone to 3-R-dihydro-4-oxoisophorone, an intermediate in the synthesis of natural chiral xanthophylls with some value as a fragrance. Product yields (86mg/gram of cells) with Thermospora curvata were four times that with mesophilic organisms (Hori et al 1984).

A secondary alcohol dehydrogenase produced by

Thermoanaerobium brockii is both stereoselective and has a broad substrate specificity (Lamed and Zeikus 1981). Interestingly, the stereoselectivity was the opposite to that of Saccharomyces cerevisiae.

1.2.4.Other Applications of Thermophiles.

There are numerous other potential uses of thermophilic microorganisms that have not been covered above, for example, the production of amino acids, carbohydrates and vitamins. In addition, thermophilic Thermoactinomyces and Streptomyces strains appear to produce unique antibiotics. (Weimer 1986).

Thermophiles may also play an important role in "microbial mining" - the extraction of metals from minerals. (Brierly and Brierly 1986).

1.3.The Metabolism of Aromatic Compounds by Micro-organisms

The microbial metabolism of aromatic compounds has been studied for many years. These compounds are produced in vast quantities every year by plants, and if they were not degraded, large amounts of carbon would be lost from the carbon cycle (Dagley 1978). In more recent years, there have been many new man made aromatic compounds introduced into the biosphere such as insecticides and detergents (alkylbenzenesulphonates for example), as well as industrial waste.

1.3.1.Industrial Uses of Aromatic Degrading Organisms.

Aromatic degrading organisms may be of direct

industrial use, as some of the intermediates they produce are both difficult and expensive to produce chemically. The "dihydrodiols", for instance, appear particularly useful. Biologically produced benzene *cis*-dihydrodiol (*cis*-1,2-dihydroxycyclohexa-3,5-diene) was used as a starting material in the production of polyphenylene (Ballard *et al* 1983). This method enabled a soluble intermediate to be formed that could be used to cast films and fibres, these could be converted to polyphenylene by heating to 140-240°C. Chemically produced polyphenylene was insoluble and could only be processed by sintering and compression. Ley *et al* (1987) have used biologically produced *cis* dihydrodiol in a six step synthesis of (+)-pinitol from benzene with a 35% overall yield. The *cis* dihydrodiols may also be used in the production of azepines and chiral polymers.

Other intermediates in aromatic metabolism are potentially useful in chemical syntheses. The 2-hydroxymuconic semialdehydes may be converted to pyridines, 4-oxalocrotonate to pyrones, and keto acids of the *meta* cleavage pathway to amino acids.

As well as their uses in the production of chemicals, microorganisms that degrade aromatic compounds may be used in the treatment of industrial wastes that contain recalcitrant haloaromatics (Knackmuss 1983).

1.3.2. Initial Reactions in Aromatic Metabolism.

The general strategy in the metabolism of aromatic compounds is firstly to hydroxylate the compound until there

are at least two hydroxyl groups, two of which are positioned ortho or para with respect to each other on the aromatic nucleus. There are however a few exceptions to this, a 5-chlorosalicylate 1:2-dioxygenase (Crawford et al 1979a), and a 1-hydroxy-2-naphthoate 1:2-dioxygenase (Kiyohara and Nakao 1977) have been described.

These hydroxylations can be acheived by two mechanisms. Firstly, a dioxygenase can mediate a dihydroxylation. These dioxygenases are multicomponent enzymes requiring NAD(P)H, and in prokaryotes, the products are cis dihydrodiols. (Gibson et al 1968, Wiseman and King, 1981).

Alternatively, usually in the metabolism of phenolics, further hydroxyl groups can be added by mono-oxygenases (mixed function oxygenases), that carry out the general reaction;

$$S + O_2 + XH_2 \rightarrow SO + H_2O + X$$

where S represents the substrate and XH_2 is the reducing agent, usually $NAD(P)H_2$, FAD, or dihydrobiopterin. (Dagley 1971).

1.3.3. The Metabolism of Aromatic Intermediates

The first process in the metabolism of dihydroxy substituted intermediates, is a ring cleavage reaction by a dioxygenase. This can be performed either between or adjacent to the two hydroxyl groups, these reactions being called ortho and meta cleavage respectively. The ring fission products are then converted into intermediates that can enter normal cellular metabolism.

A summary of these reactions for simple monocyclic intermediates is given in Figures 1-3.

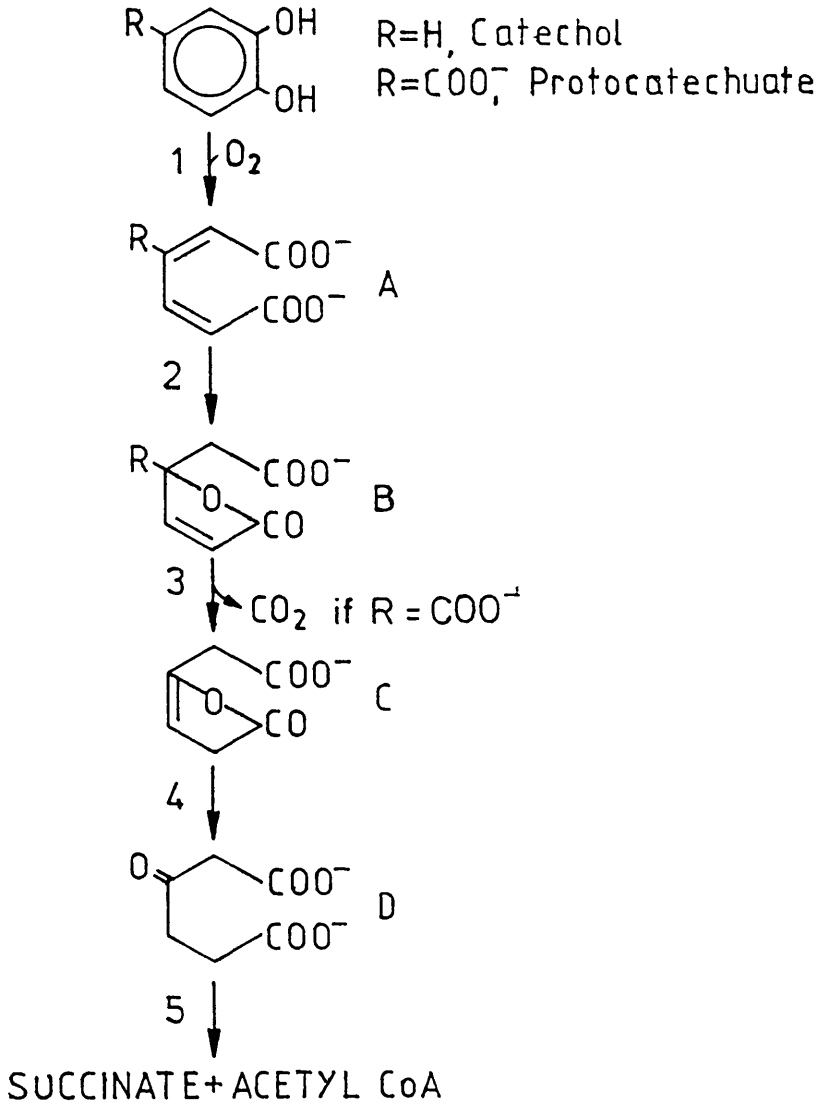
The ortho fission pathway is shown in Figure 1, and has been observed in Pseudomonas putida and Moraxella lwoffii with both catechol and protocatechuate (Ornston and Stanier 1966).

Meta fission (Figure 2), has also been observed with catechol (Dagley and Stopher 1959), which appeared to proceed by the hydrolytic route shown. However, an oxidative route for the further metabolism of 2-hydroxymuconic semialdehyde was found in both Pseudomonas (Nishizuka et al 1962) and Azotobacter (Sala-Trepat and Evans 1971) species.

Meta fission of protocatechuate may occur in the 2:3 position shown in Bacillus species (Crawford et al 1975, 1979). However, in Pseudomonas the 4:5-dioxygenase (Not shown in Fig. 2) appears to operate (Trippet et al 1960). Homoprotocatechuate 2:3-dioxygenase, followed by the oxidative route, has been reported in both Acinetobacter and Pseudomonas putida. (Sparnins et al 1974) and Escherichia coli (Cooper and Skinner 1980).

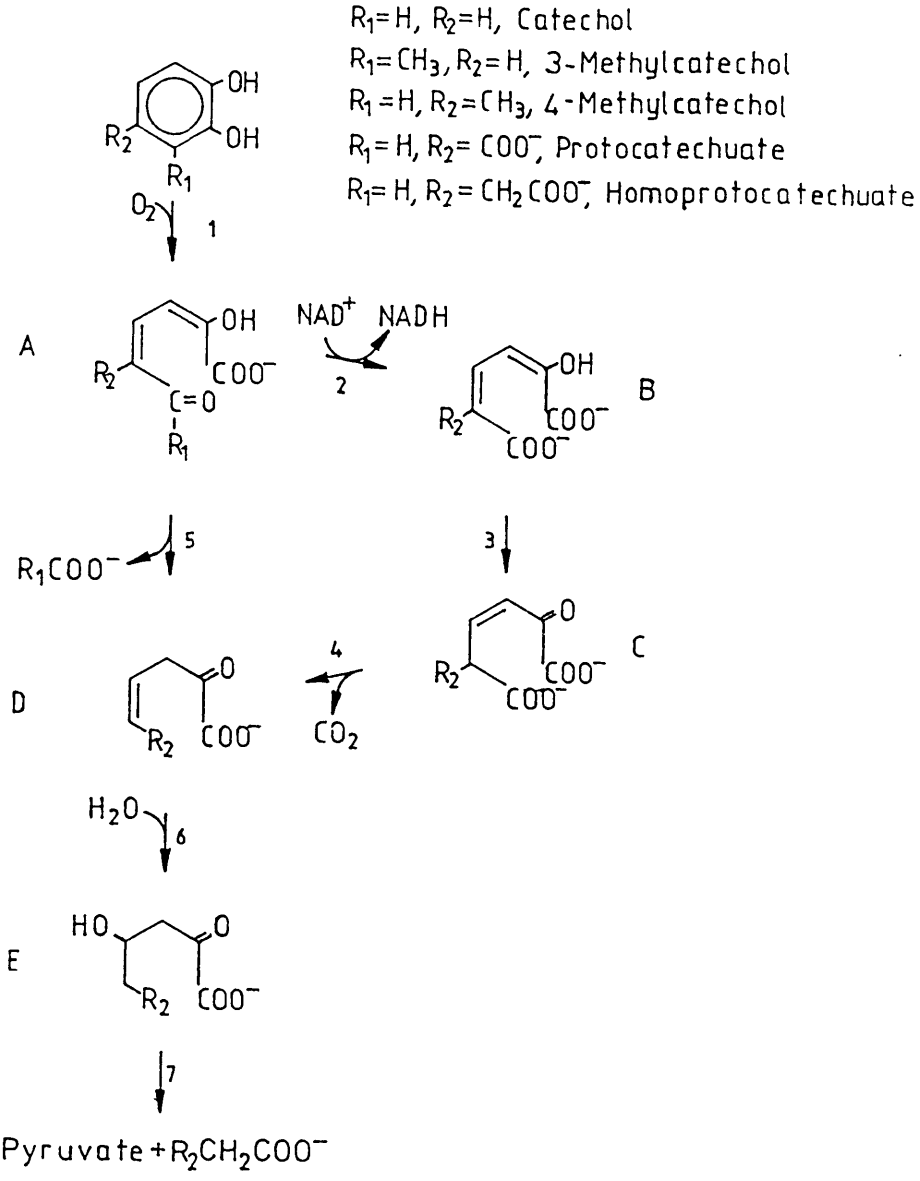
Methylcatechols may also undergo meta fission (Bayly et al 1966). Azotobacter species metabolise the ring fission products of catechol and 4-methylcatechol via an oxidative pathway, the hydrolytic route being of little physiological importance. The strains studied were not able to grow on o- or m-cresol (Sala-Trepat and Evans 1971). These authors also suggested that in Pseudomonas, both oxidative and hydrolytic routes were present, the latter required for the

Figure 1.Ortho Fission Pathways



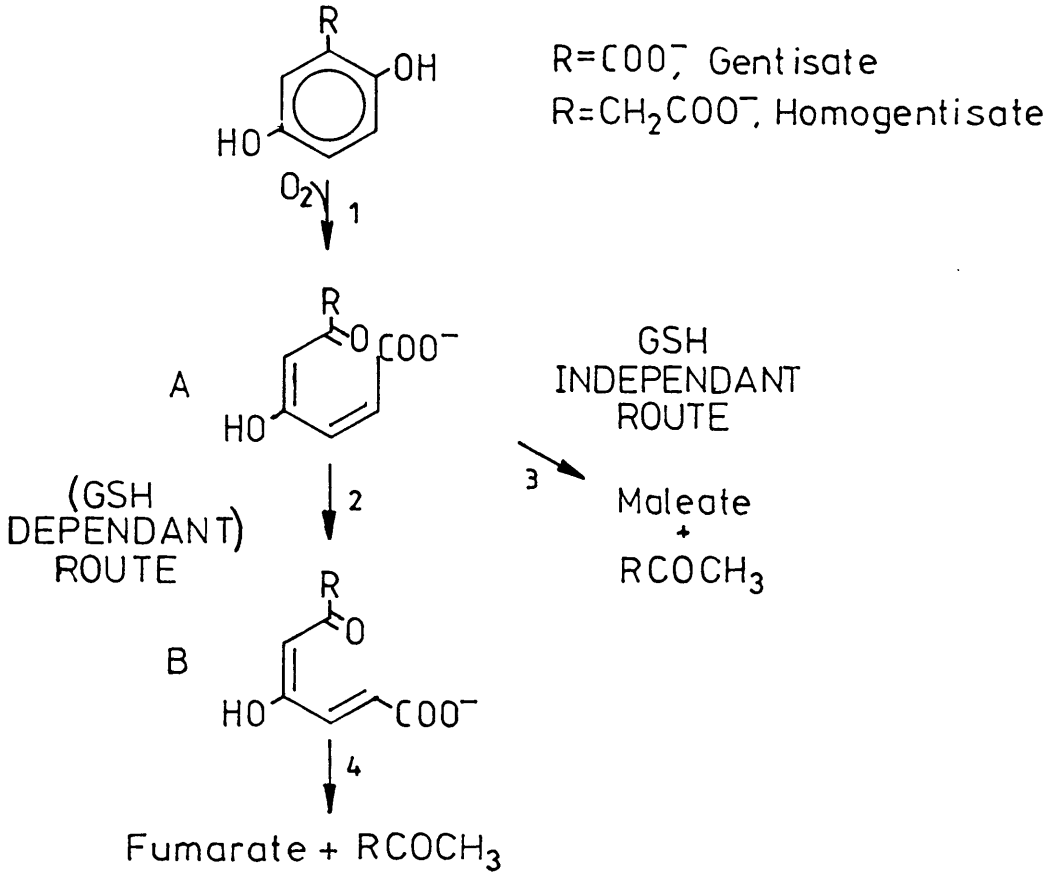
Chemical and enzyme names are shown for catechol metabolism only. Numerals indicate enzymes as follows: 1, catechol 2:3-dioxygenase; 2, muconate cycloisomerase; 3, muconolactone isomerase; 4, β-ketoadipate-enol-lactone hydrolase; 5, β-ketoadipate CoA transferase and β-ketoadipate thiolase. Letters indicate chemicals as follows: A, cis,cis-muconate; B, (±)-muconolactone; C, β-ketoadipate enol lactone; D, β-ketoadipate.

Figure 2.Meta Fission Pathways



Chemical and enzyme names are shown for catechol metabolism only. Numerals indicate enzymes as follows: 1, catechol 2:3-dioxygenase; 2, 2-hydroxymuconic semialdehyde hydrolase; 3, 4-oxalocrotonate tautomerase; 4, 4-oxalocrotonate decarboxylase; 5, 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase; 6, 2-oxopent-4-enoate hydratase; 7, 4-hydroxy-2-oxovalerate aldolase. Letters indicate chemicals as follows: A, 2-hydroxymuconic semialdehyde; B, 4-oxalocrotonate (enol); C, 4-oxalocrotonate (keto); D, 2-oxopent-4-enoate; E, 4-hydroxy-2-oxovalerate.

Figure 3. Gentisate Pathways



Chemicals and enzymes are shown for gentisate metabolism only. Numerals indicate enzymes as follows: 1, gentisate dioxygenase; 2, maleylpyruvate isomerase; 3, maleylpyruvate hydrolase; 4, fumarylpyruvate hydrolase. Letters indicate chemicals as follows: A, maleylpyruvate; B, fumarylpyruvate.

dissimilation of 3-methylcatechol and its precursors o- and m-cresol.

In the pathways for gentisate metabolism, (Fig. 3) the GSH dependant route has been observed in Pseudomonas for gentisate (Sugiyama et al 1958, Lack 1959, 1961), and homogentisate (Chapman and Dagley 1962). The alternative, GSH independant route, for gentisate metabolism, is known to occur in Pseudomonas (Hopper et al 1968), and Bacilli (Crawford 1975a).

1.3.4. Aromatic Metabolism By Bacillus Species

Aromatic metabolism in Bacillus species has only rarely been studied, mainly because the selective enrichment proce-dures used by most workers favour the faster growing Pseudomonads. However, Crawford (1975) showed that 10% of soil microbes were aerobic sporeformers, and that 1% of these could grow on a minimal medium with m-hydroxybenzoate as the sole carbon source.

Willets and Cain (1972, 1972a), and Willets (1974), isolated a Bacillus species capable of growing on alkylbenzenesulphonate detergents. This was achieved by firstly a desulphonation, releasing sulphite, which was converted to adenosine-5'-sulphatophosphate. Two carbon subunits of the alkyl sidechain were successively released by beta oxidation. This left, with an even numbered sidechain, p-hydroxyphenylacetic acid, which was hydroxylated to homoprotocatechuate, and with an odd numbered sidechain, p-hydroxybenzoate, which was hydroxylated to protocatechuate.

These two intermediates proceeded by ortho and meta cleavage routes respectively.

Crawford et al (1979a), isolated a B. brevis strain that would utilise 5-chlorosalicylate as a sole carbon source. This strain possessed a novel 5-chlorosalicylate 1:2-dioxygenase, which would also attack other 5-halogenated salicylate compounds, but not gentisate, protocatechuate or catechol. Crawford and Perkins-Olsen (1978) also isolated another Bacillus species that would grow on the lignin related compound vanillate. It was tentatively proposed that this was metabolised via guaiacol and catechol.

Ensign and Rittenburg (1964) deduced the pathway of nicotinic acid degradation in a Bacillus species which proceeded via 2,3,6,trihydroxypyridine to maleamic acid and fumarate.

The versatility of this genus was further demonstrated, again by Crawford (1976), who isolated three Bacillus strains that grew on p-hydroxybenzoate by three different pathways. B. brevis PHB2 utilised the ortho fission of protocatechuate, B. circulans the oxidative or hydrolytic meta fission of protocatechuate, and B. laterosporus PHB7a the non GSH dependant gentisate route.

Buswell (1975), and Buswell and Clark (1976), have isolated thermophilic Bacilli capable of growing on phenolics and p-hydroxybenzoates. Phenols and cresols being metabolised by meta fission (both hydrolytic and oxidative routes), and p-hydroxybenzoate via gentisate. It should be noted that in the cresol metabolising strain of B. stearothermophilus,

unlike *Pseudomonas*, 3-methylcatechol and not 4-methylcatechol, was the hydroxylation product of *m*-cresol.

Bacillus species may also perform their transformations using novel enzymes. Que *et al* (1981) studied a homoprotocatechuate 2:3-dioxygenase, and, unlike all well studied cleavage enzymes which contain iron, this enzyme contained tightly bound manganese. It was inhibited by iron, but not azide, cyanide or hydrogen peroxide. As the authors pointed out at the time, a protocatechate 2:3-dioxygenase from *B. stearothermophilus* (Jamaluddin 1977) showed similar inhibition characteristics, and a catechol 2:3 dioxygenase from another *B. stearothermophilus* strain (Buswell 1975), was also not inhibited by azide or cyanide. These results, although not conclusive, are suggestive that manganese enzymes may be more widespread.

The *Bacillus* species have therefore proved to be both ecologically important and versatile, and may be capable of producing useful chemicals and enzymes.

1.4.The Genetics and Regulation of the Dissimilation of Aromatic and Hydrocarbon Compounds.

Genetical studies of aromatic and hydrocarbon degradation have mainly centred on the plasmid encoded systems of *Pseudomonas* species. This, again, is probably due to the selective enrichment procedures used, and also, the availability of techniques for genetic manipulation in this genus.

Plasmids have been reported for the degradation of

salicylate (Chakrabarty 1972), naphthalene (Dunn and Gunsalus 1973), D- and L-camphor (Rheinwald and Chakrabarty 1973) and m- and p-toluate (Williams and Murray 1974) in Pseudomonas arvilla. Plasmids have also been described that metabolise n-octane and octanoic acid in Pseudomonas oleovorans (Chakrabarty et al 1973), nicotine and nicotinic acid in P. convexa, 3-chlorobenzoate in Pseudomonas strains (Dorn et al 1974, Chatterjee et al 1981) and Alcaligenes eutrophus (Ghosal et al 1985), and styrene in P. fluorescens (Bestetti et al 1984).

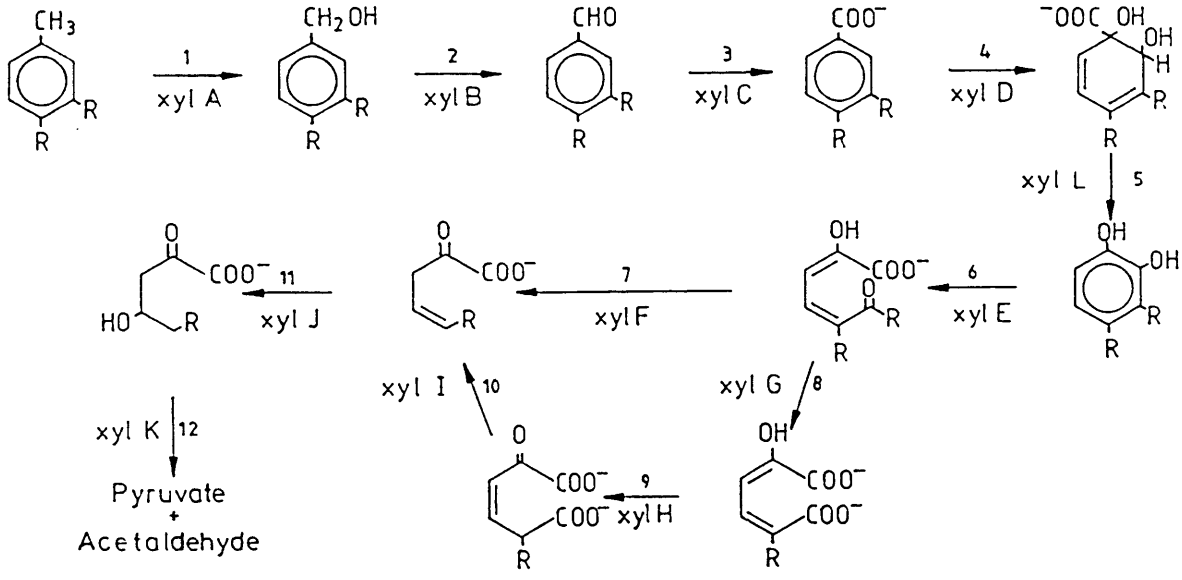
Of these the most extensively studied are the TOL, NAH, and OCT or CAM/OCT (Chakrabarty 1973) plasmids, which degrade the toluates, naphthalene, and alkanes respectively.

1.4.1.The TOL Plasmid

The archetypal TOL plasmid (117kb) encodes the genes for the degradation of m- and p-toluates and m- and p-xylenes in Pseudomonas putida mt-2 (Williams and Murray 1974, Worsey et al 1978) via a divergent meta cleavage pathway. (Fig. 4).

Tn5 and Tn1000 mutagenesis has shown that the genes for these enzymes are located in two operons, the first (the upper pathway) encodes the xylA, xylB and xylC genes responsible for the conversion of hydrocarbons to aromatic carboxylic acids. The lower pathway genes (xylD-xylL) are located in the second operon, which is 14kb downstream of the first, and transcribed in the same direction. The enzymes of this lower pathway are responsible for the breakdown of

Figure 4.Toluate Degradation



Numerals indicate the following enzymes: 1, xylene monooxygenase; 2, benzylalcohol dehydrogenase; 3, benzaldehyde dehydrogenase; 4, toluate dioxygenase; 5, benzoate diol dehydrogenase; 6, catechol 2:3-dioxygenase; 7, 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase; 8, 2-hydroxymuconic semialdehyde dehydrogenase; 9, 4-oxalocrotonate tautomerase; 10, 4-oxalocrotonate decarboxylase; 11, 2-oxopent-4-enoate hydratase; 12, 4-hydroxy-2-oxovalerate aldolase.

aromatic carboxylic acids into products metabolizable by the host organism. (Franklin *et al* 1981, Harayama *et al* 1984).

The mechanism for positive regulation shown in figure 5 was first proposed by Worsey *et al* (1978) and work since has supported this scheme (Franklin and Williams 1980, Nakazawa *et al* 1980). For example, in a partial diploid, *xylR*$^{+}$ was transdominant to *xylR*$^{-}$, suggesting that a diffusable protein product acts in conjunction with the appropriate hydrocarbon and alcohol inducers, eg. *m*-xylene or *m*-methylbenzyl alcohol, to induce the upper pathway enzymes, and, in conjunction with the *xylS* gene product (Inouye *et al* 1983), the lower pathway enzymes.

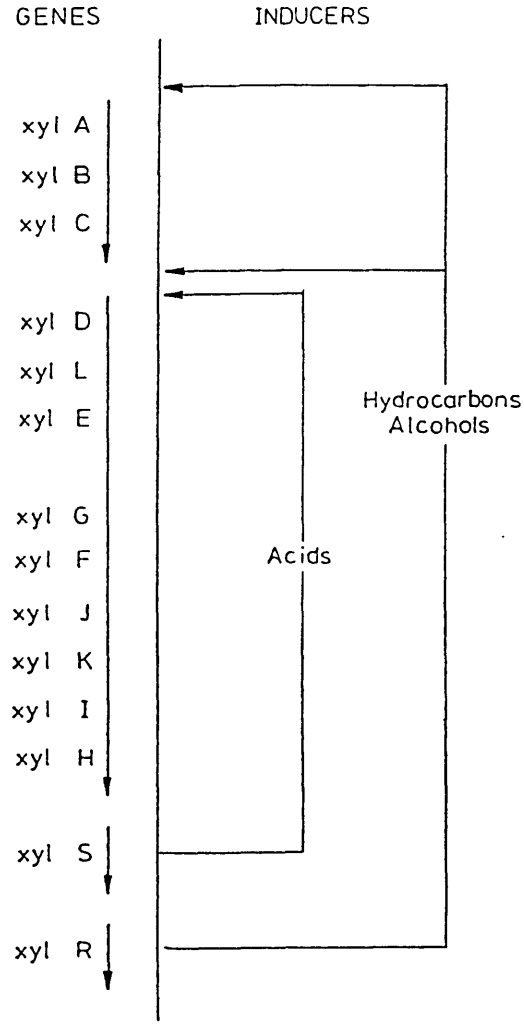
Aromatic carboxylic acids have been shown to be the co-inducers of the *xylS* product (Inouye *et al* 1981). Neither the *xylR* or *xylS* products have any repressor functions in the absence of their inducer (Inouye *et al* 1981,1983).

The *xylR* and *xylS* genes have been mapped by Tn5 mutagenesis, and lie close together, downstream of the lower operon. However, the two show no sequence homology. There is also some evidence that a third regulatory element may be involved (Franklin *et al* 1983).

1.4.2.The NAH Plasmids

Naphthalene degradation, or at least some of the genes involved, in a variety of *Pseudomonad* strains, are plasmid bo-rne (Dunn and Gunsalus 1973, Boronin *et al* 1980, Zuniga *et al* 1981, Cane and williams 1982, Connors and Barnsley 1982). The most extensively studied is the NAH7 plasmid (Yen and

Figure 5.Diagram Showing the Gene Organisation and Regulation of the TOL Plasmid



The product of the xylS gene, in the presence of acid inducers, positively regulates the lower operon. The product of the xylR gene, in the presence of hydrocarbon or alcohol inducers, positively regulates both operons.

Gunsalus 1982, 1985, Grund and Gunsalus 1983).

The 87kb NAH7 plasmid degrades naphthalene to salicylate, and then metabolizes this via the meta cleavage of catechol (Fig. 6). The genes for these enzymes are again organised in two operons, encoding firstly the genes for the conversion of naphthalene to salicylate, and secondly the salicylate hydroxylase and meta cleavage genes (Fig. 7). A regulatory gene, nahR, has also been mapped by Tn5 mutagenesis and lies between the two.

The nahR gene regulates the naphthalene pathway in a positive manner, and since nahR$^+$ mutants are transdominant to nahR$^-$ mutants, a protein product is presumed to be involved. The co-inducers for this product have not been extensively studied, but include salicylate.

1.4.3.The OCT and CAM/OCT Plasmids

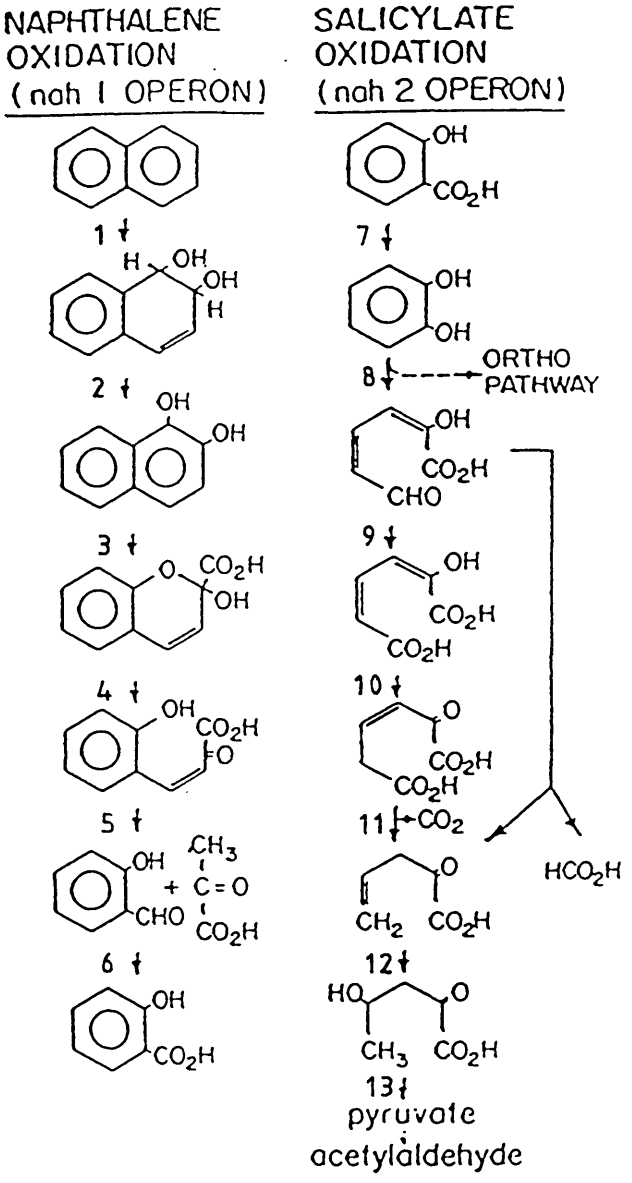
The alk genes on the OCT and CAM/OCT plasmids enable the host pseudomonads to utilize 6-10 carbon n-alkanes for growth. This system, which has been well researched, has proved to be much more complicated than originally envisaged. Industrial interest in the OCT plasmid has also been shown recently due to the potential production of long chain alcohols, dicarboxylic acids, and epoxides (Eggink et al 1984).

Alkanes are oxidized in the following sequence:

RCH_3, RCH_2OH, RCHO, RCOOH, beta oxidation

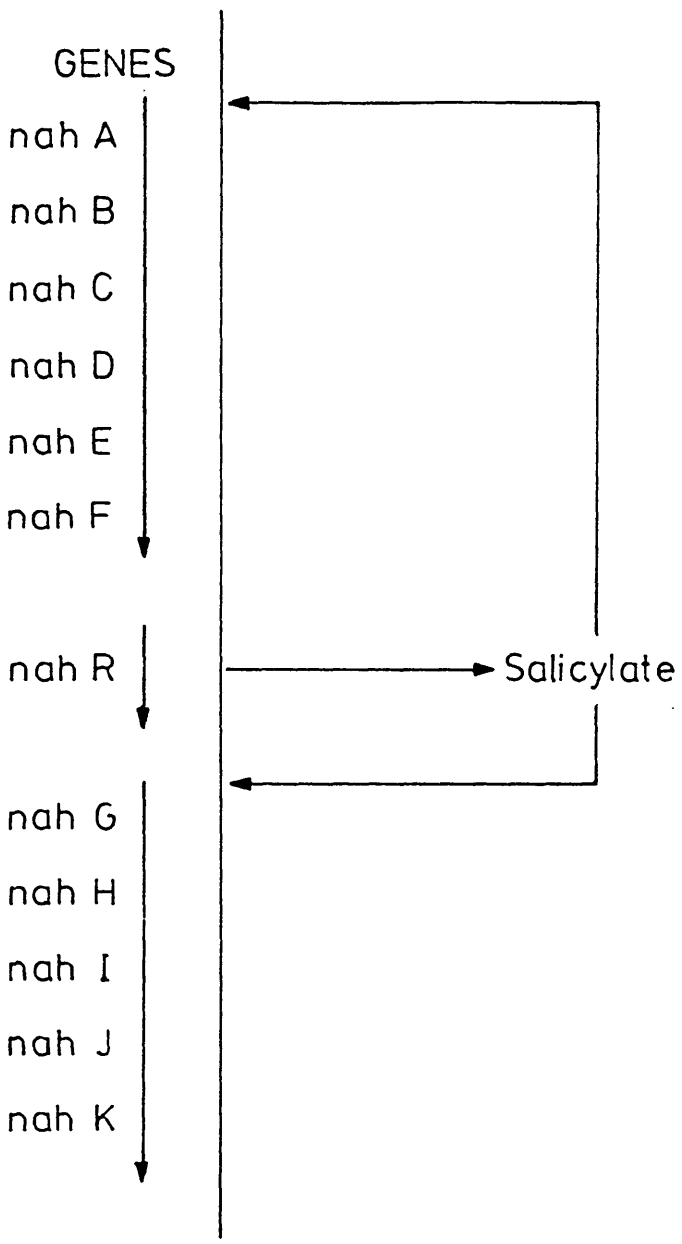
However, there were shown to be at least two chromosomally encoded alcohol and two chromosomally encoded aldehyde

Figure 6.Naphthalene Metabolism



Numerals indicate enzymes as follows: 1, naphthalene dioxygenase; 2, naphthalene dihydrodiol dehydrogenase; 3, 1,2-dihydroxynaphthalene dioxygenase; 4, 2-hydroxychromene-2-carboxylate isomerase; 5, 2-hydroxybenzalpyruvate aldolase; 6, salicaldehyde dehydrogenase; 7, salicylate hydroxylase; 8, catechol 2:3-dioxygenase; 9, 2-hydroxymuconic semialdehyde dehydrogenase; 10, 4-oxalocrotonate tautomerase; 11, 4-oxalocrotonate decarboxylase; 12, 2-oxopent-4-enoate hydratase; 13, 4-hydroxy-2-oxovalerate aldolase. Diagram taken from Yen and Gunsalus (1985).

Figure 7.Diagram Showing the Gene Organisation and Regulation of the NAH Plasmid



The product of the nahR gene, in the presence of salicylate, positively regulates both operons.

dehydrogenases. There was also a plasmid encoded alcohol dehydrogenase that had different specificities to the chromosomally encoded enzymes. (Grund *et al* 1975).

Tn7 Mutagenesis (Fennewald and Shapiro 1979) together with transduction and deletion mapping (Fennewald *et al* 1979), eventually lead to the scheme shown in Figure 8 to be proposed, where a combination of plasmid and chromosomal genes are required. The operon containing the *alkB*, *alkA*, and *alkC* genes is again under the control of a positive regulatory protein, the product of the *alkR* gene (Fennewald *et al* 1979, Kok *et al* 1984). The *alkD* gene was also considered to be regulatory in the same manner (Fennewald and Shapiro 1977), however, it later proved not to be involved in transcriptional control. It was postulated that it may act by posttranscriptional modification or may be a common subunit for the membrane hydroxylase and dehydrogenase (Fennewald *et al* 1979).

It is not known whether the *alkC*, *alkD*, and *alkR* are regulated or if they are constitutively produced.

1.4.4.Other Systems

The genetics of some systems have been studied in less detail than those above. For instance, in a *Pseudomonas putida* strain able to grow on phenol, it was proposed that the genes required for the *meta* fission of catechol were encoded in a single operon and separate to the phenol hydroxylase gene (Wigmore and Bayly 1977, Bayly *et al* 1977, Wigmore *et al* 1977). There was also a suggestion of a

Figure 8.Diagram Showing the Genetic Control of Alkane Regulation

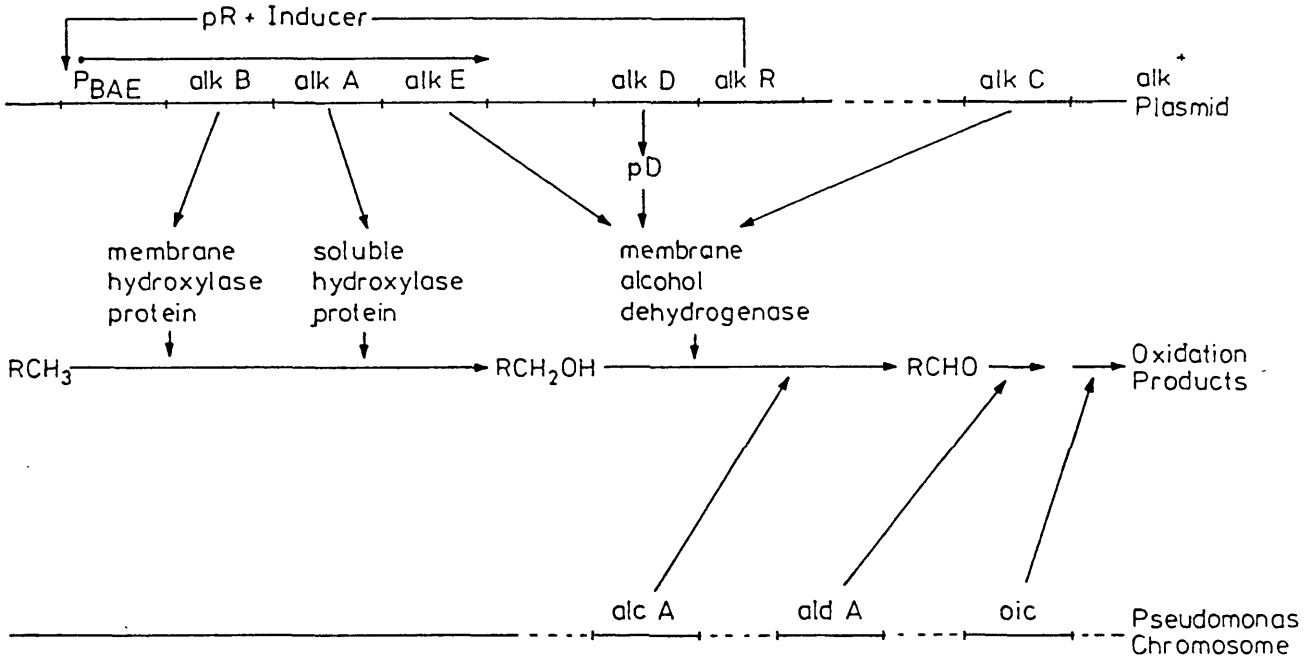


Diagram showing products of chromosomal and plasmid genes, and their function. Diagram taken from Fennewald et al (1979).

positive regulatory protein acting as a repressor in the absence of an inducer (Wigmore et al 1977).

The virtually identical (Chatterjee and Chakrabarty 1983) plasmids pB13 and pAC25 have been isolated from pseudomonads (Dorn et al 1974, Chatterjee et al 1981) as well as the plasmid pJP4 from Alcaligenes eutrophus (Ghosal et al 1985). All these organisms grew on 3-chlorobenzoate.

Although the genes responsible for 3-chlorobenzoate degradation on these plasmids have not been shown to be arranged in an operon, they are closely linked. Three genes required: pyrocatechase II, cycloisomerase II and hydrolase II, were located on a 4.2kb fragment of pAC27 (a pAC25 derivative (Chatterjee and Chakrabarty 1984)), and a 10kb fragment of pJP4. The sizes of these plasmids are 110 and 85kb respectively.

Interestingly a lack of positive regulatory elements was also reported (Ghosal et al 1985).

In all of the above examples, except phenol metabolism, where it was not investigated, the genes involved in aromatic/hydrocarbon degradation were plasmid encoded. Although the presence of dissimilatory pathway genes on plasmids is common, it is not the rule. For example, benzoate metabolism via an ortho pathway has been reported in Pseudomonas putida which was chromosomally encoded (Williams and Murray 1974). The disproportionate amount of work that has been carried out on plasmids is probably a result of their easier and more numerous systems for genetic analysis.

The genes for the genetic systems studied were largely arranged in "operons". This form of gene structure was first described by Jacob and Monod (1961) as a segment of DNA consisting of two or more contiguous genes subject to joint control through one region, the promotor-operator complex, located at the "upstream" end of the unit. The promotor site determining where and how transcription is initiated, and the operator being the site of repressor action. (Jacob and Monod 1961, Pastan and Adhya 1976).

It is not known whether the arrangement of the genes of dissimilatory pathways as similar operons reflects a close relatedness, or is the result of common physical constraints, theoretical argu-ments for the phenomenon of operons having been made previously (Riedl 1978).

Positive regulation by a regulatory protein and metabolite co-inducer was often observed. However no glucose repression was reported. Four other well characterized operon systems, *lac*, *gal*, *hut* and *ara*, all displayed a cAMP mediated glucose repression system. (Pastan and Adhya 1976, Reznikov and Abelson 1978, deCrombrugge and Pastan 1978, Magasanik 1978, Lee 1978).

CHAPTER 2

METHODS

2.1.Materials

2.1.1.Bacterial Strains

The bacterial strains used, and their sources, are shown in Table 1.

2.1.2.Chemicals

Chemicals not mentioned below were obtained from major manufacturers and phenolics were purified twice by resublimation if solid, or a double vacuum distillation if liquid.

Bio-Rad Protein Assay reagent was obtainted from Bio-Rad Laboratories LTD, Watford, Herts.

The acridine mutagen ICR-191 was obtained from Fluorochem LTD, Glossop, Derbyshire.

Authentic 4-oxalocrotonic acid and 4-methyl-2-oxobutyrolactone were synthesised by Dr John Rossiter of this department.

2-Hydroxymuconic semialdehyde, and its methyl substituted analogues, were generated *in situ* by the action of catechol 2:3-dioxygenase upon catechol and methyl catechols.

2.1.2.1.Preparation of Benzoate Hydrodiol.

Benzoate hydrodiol (BHD) was accumulated using a mutant, *Pseudomonas putida* PpU103, which is known to accumulate the compound from benzoate (Cass *et al* 1987). The compound was isolated by a method based on that of Reiner and

Table 1. Sources of Bacterial Strains

STRAIN	SOURCE
Bacillus Strains	
RS 5	1
RS 20	1
RS 53	1
RS 93	1
RS 173	1
RS 240	1
IC2	5
B. stearothermophilus ATCC 8005	1
B. stearothermophilus ATCC 10149	1
B. stearothermophilus NCA 1503	2
B. stearothermophilus IC3	5
B. coagulans ATCC 8038	1
B. coagulans ATCC 12245	1
B. thermodenitrificans DSM 465	1
B. thermodenitrificans DSM 466	1
B. caldotenax	1
B. licheniformis	1
Thermus Strains	
YT-1	1
T. thermophilus	1
T. flavus	1
Pseudomonas Strains	
P. putida mt-2	3
P. putida PpU103	4
Unidentified Strains	
IC1	5

Note:

(1) Sources are as follows: 1, Dr Richard Sharp, PHLS Centre For Applied Microbiology Research, Porton Down, Salisbury, Wiltshire; 2, Mr Sam Armartey of this department; 3, Dr Marc Woodland of this department; 4, Dr John Rossiter of this department; 5, isolation described in this thesis.

Hegeman (1971).

P. putida PpU103 (6 litres) was grown at 30°C on R medium, supplemented with (final concentrations): 0.1% peptone; 10mM succinate; 5mM benzoate. The cells were harvested, in the late log growth phase, washed in 100mM phosphate buffer (pH7.2, 0°C), and resuspended, in 200ml of the same buffer, awaiting use.

Benzoate was added to the cells, at 30°C, in 200mg quantities, and the accumulation of BHD followed by HPLC analysis (Table 3, conditions 2). When transformation of benzoate by the cells ceased, presumably due to NADH depletion, they were left overnight, at 30°C, in phosphate buffer (100mM, pH7.2) containing 10mM succinate before being reharvested and used again.

The pooled supernatants containing BHD were reduced to 20ml, at less than 50°C, with a rotary evapo-rator. The supernatant was chilled to 0°C, and acidified to pH2.0, with HCl. The BHD was then extracted ten times with 100ml of ethyl acetate. The ethyl acetate was reduced to 2ml and the BHD extracted into water (partition coefficient in water to ethyl acetate, 7:1). The BHD was then extracted into 150ml of ether which was dried with anhydrous $MgSO_4$. An oily residue was formed on removal of the ether. The BHD was crystalized from ethanol.

2.1.2.2.Preparation of 2-Oxopent-4-enoic Acid

The preparation of 2-oxopent-4-enoate was based upon methods previously described for the production of this

compound (Collinsworth et al 1973, Marcotte and Walsh 1978) and a methyl substituted analogue (Coulter and Talalay 1968).

A 10ml sample containing 25mM Tris buffer (pH8.2), 50mM DL-allyl glycine, 25 units of D-amino acid oxidase and 1250 units of catalase, was incubated at 25°C until the maximum concentration of 2-oxopent-4-enoate (as determined by HPLC analysis) had appeared (approximately 30 minutes). The solution was then acidified to pH1.5 with HCl, and the compound extracted twice with 25ml of ether. The ether was dried down and the precipitate resuspended in 5ml of dilute HCl (pH1.5). These solutions were stored at -20°C and used within one week.

This crude preparation of the compound had absorbance maxima at the following wavelengths: pH2.0, 268nm; pH7.5, 265nm; pH12.0, 303nm. These values are in agreement to those previously obtained for 2-oxopent-4-enoate: in acid, 270nm; in aqueous solution, 265nm; in alkali, 305nm (Collinsworth et al 1973).

2.1.2.3.Preparation of 4-Hydroxy-2-oxovalerate

This compound was prepared by mild alkaline hydrolysis of 4-methyl-2-oxobutyrolactone using the method of Dagley and Gibson (1965). A solution containing a final concentration of 10mM 4-methyl-2-oxobutyrolactone and 10mM NaOH was allowed to stand at room temperature. After 3 hours the lactone had completely disappeared, and a compound with the characteristics of 4-hydroxy-2-oxovalerate had been produced. When the product was boiled in the presence of 1M HCl (Section 2.5.4.), a compound with the same retention time as

the lactone (by HPLC analysis) was produced with a yield of 70%.

2.1.3.Enzymes

The following enzymes were obtained from the Sigma Chemical Company LTD, Poole, Dorset: aldehyde dehydrogenase from bakers yeast; D-amino acid oxidase from porcine kidney; catalase from bovine liver; formate dehydrogenase from Pseudomonas oxalacticus; NADase from Neurospora crassa; and pyruvate dehydrogenase from bovine heart.

2.2.Microbiological Techniques

2.2.1.Enrichment and Isolation of Strains Utilizing Aromatic Compounds

The following strains were isolated from soil samples (1g of soil in 4ml distilled water), that had been pasteurized for 10 minutes at 80°C. Strains IC1 and IC2 were enriched and isolated on the media shown below with benzoate (5mM) as the main carbon and energy sources. Strain IC3 was enriched on m-cresol (2mM) but finally isolated using phenol (2mM). Strain IC2 was identified as a thermophilic Bacillus, and IC3 as Bacillus stearothermophilus atypical in growing at pH5.7, by the National Collections of Industrial and Marine Bacteria Ltd., Torry Research Station, PO Box 31, 135 Abbey Road, Aberdeen, Scotland. (Appendix).

2.2.2.Storage Conditions

All thermophilic strains were stored at -20°C after

adding glycerol, to a concentration of 12%, to an overnight culture. Pseudomonads were stored at 4°C on nutrient agar plates.

2.2.3.Media

The medium of Buswell (1974) was used for the growth of Bacillus stearothermophilus ATCC 10149 and B. thermodenitrificans DSM 465. The same medium, without the casamino acids, supported the growth of Bacillus stearothermophilus NCTC 10003, B. coagulans ATCC 8038 and the Bacilli strains RS 20, RS 173, RS 53 and IC2. Another variation on the medium of Buswell was developed for B. stearothermophilus IC3, 40mM tris buffer (pH7.5) was added, the yeast extract concentration was lowered to 0.0375 g/l, the casamino acids omitted, and 0.0375 g/l brain heart infusion was added. These media have been summarized in Table 2.

The medium of Jamaluddin (1977) was employed for growth of B. stearothermophilus NCA 1503 and the unidentified strain IC1. No medium supported growth without an added carbon source.

Following the report of Rowe et al (1975) that growth of B. stearothermophilus NCA 1503 on solid media was inhibited by phosphate concentrations greater than 5mM, when any of the above media were required in solid form, the phosphate content was lowered to 5mM, and 40mM Tris buffer (pH7.5) added. In addition, with the medium for B. stearothermophilus IC3, the yeast extract and brain heart

Table 2.Media for Thermophilic Bacilli

Strain	Addition To Media
B. stearothermophilus NCTC 10003, *B. coagulans* ATCC 8038, *Bacillus* strains RS 20, RS 173, RS 53, IC2.	Yeast Extract (0.01g/l)
B. stearothermophilus ATCC 10149, *B. thermodenitrificans* DSM 465.	Yeast Extract (0.01g/l) Casamino Acids (0.01g/l)
B. stearothermophilus IC3.	Yeast Extract (3.75mg/l) Brain Heart Infusion (3.75mg/l) Tris Buffer pH7.5 (40mM)

NOTES:

(1) The above ingredients were added to the following salts (g/l): K_2HPO_4, 0.5; NH_4Cl, 1.0; $MgSO_4.7H_2O$, 0.2.

(2) Also added, at a dilution of 1ml/l, was the following trace metal solution (g/l): NaCl, 10.0; $CaCl_2.2H_2O$, 6.5; $MnSO_4.4H_2O$, 0.4; $ZnSO_4.7H_2O$, 0.1; $CuSO_4.5H_2O$, 0.04; $CoCl_2.6H_2O$, 0.04; $NaMoO_4.2H_2O$, 0.05; $FeSO_4.4H_2O$, 0.4.

(3) The salts and trace metal solutions were autoclaved separately.

infusion concentrations were halved. All media were solidified with 0.75% w/v Oxoid Bacterial Agar No. 1.

Carbon sources, unless indicated otherwise, were used at the following concentrations; aromatics and glucose 5mM, glycerol 10mM and ethanol 25mM.

Pseudomonas putida mt-2 was grown in the "R medium" described by Wigmore and Ribbons (1980), with 2.5mM p-toluate as the sole carbon source. P. putida PpU103 was grown in "R medium" supplemented with 0.1% (final concentration) of peptone with the carbon sources as indicated in the text.

2.2.4.Growth Conditions

Liquid cultures were incubated in a shaking incubator, rotating at 200rpm. Media, in quantities of 40, 200 and 800 mls, were used in 100ml, 200ml and 2l conical flasks respectively.

Where solid media were employed for thermophiles, the Petri dishes were wrapped in plastic bags to reduce evaporation.

Bacillus strains were grown at 55°C, unless otherwise indicated. Thermus strains were grown at 70°C, and pseudomonads at 30°C.

2.2.5.Growth Measurements

Growth was measured by following the absorbance of the culture at 600nm, and, where necessary, the dry weight of the cells was calculated by reference to a calibration curve of absorbance (600nm) against dry weight. The dry weights were

determined by the method of Coultate and Sundaram (1975). Growth yields were also measured according to the methods of Coultate and Sundaram (1975).

Doubling times were determined from plots of the natural log of growth against time. Only the linear sections of these plots were used in the calculations.

2.2.6.Whole Cell Experiments

Washed samples of whole cells were prepared by harvesting at the mid-log growth phase and washing twice in 100mM Tris buffer, pH7.5 (at room temperature), preheated to 55°C. Cells were spun down at 20,000g for two minutes, resuspended, and held at 55°C until used. A suitable dilution of cells were then added to a vessel heated to 55°C, into which was inserted a Clark oxygen electrode (Yellow Springs Instruments). All whole cell uptakes shown are the average of three determinations that have been corrected for an endogenous oxygen uptake of 120nmols of oxygen per mg of cells per minute.

2.3.Biochemical Techniques

2.3.1.Preparation of Cell Free Extracts

An overnight culture (200mls) was used to inoculate 800mls of media, the cells were grown and harvested at the mid-log growth phase. These were washed twice in 50mM phosphate buffer, pH7.0, at room temperature. For extracts where catechol 2:3-dioxygenase activity was required, the cells were resuspended in 50mM, pH7.0, phosphate buffer at

4°C containing 10mg bovine serum albumin/ml, 10% acetone and 100μM phenylmethylsulphonyl fluoride. When phenol hydroxylase activity was required, the cells were resuspended in the above, minus the acetone, and with 1mM dithiothreitol added.

The resuspended cells were then sonicated for 15 seconds, with intermittent cooling to 4°C, for a total of 3 minutes. The extracts were subsequently clarified by centrifugation at 35,000g for 40 minutes at 4°C. The cleared extracts were stored at 4°C.

2.3.2.Measurement of Protein Concentrations

Protein concentrations were measured by the method of Bradford (1976), using Bio-Rad Protein Assay reagent. Bovine serum albumin was used as a standard.

2.3.3.Incubation of Cell Free Extracts with Metal Salts

In enzyme assays to determine the effect of metal salts on the activity, the cold cell free extract was incubated with the salt (1mM) for 5 minutes before use.

2.3.4.Calculation of Michaelis Constants

Apparent Michaelis constants for aromatic compounds were obtained by measuring the initial reaction rates at various aromatic concentrations. The constants for oxygen were measured by adding 1mM aromatic to an extract sample in an oxygen electrode. The tangent of the curve obtained at various oxygen concentrations was then used for the appropriate plot. For phenol hydroxylase, a control was performed with NADH only, and the reaction rates at different

oxygen concentrations subtracted from the rates in the presence of phenol. The constants for oxygen were an average of three plots: a Lineweaver-Burke plot (1/v against 1/S), a Haynes plot (S/v against S), and an Eadie-Hofstee plot (v against v/S). The catechol constants were derived from plots of S/v against S.

2.4.Assays for Enzyme Activities

All enzyme rates shown are the average of at least two determinations. When an oxygen electrode was not used, enzymes were assayed in the following manner using a Shimadzu UV240 recording spectrophotometer at 55°C. Accurate temperature measurements, when required, were obtained using a thermocouple wire. Unless otherwise indicated, cell free extracts were diluted in 50mM phosphate buffer (pH7.0).

2.4.1. Phenol Hydroxylase

Phenol hydroxylase was assayed by following the rate of oxidation of 1mM NADH at 340nm in the presence of 1mM phenol, and subtracting the rate of the same mixture before the addition of phenol.

2.4.2.Catechol 2:3-Dioxygenase

Catechol 2:3-dioxygenase was assayed by following the production of its ring fission products 2-hydroxymuconic semialdehyde (2HMS), 2-hydroxy-6-oxohepta-2,4-dienoic acid and 2-hydroxy-5-methylmuconic semialdehyde at 375, 388 and 382 nm respectively. Experiments with heat treated cell free

extracts of Pseudomonas putida mt-2, produced by the methods of Bayly et al (1966), were used to deduce spectrophotometric data for the ring fission products at pH7.0. With these extracts the following molar extinction coefficients were calculated: 2HMS, 27,900; 2-hydroxy-6-oxohepta-2,4-dienoic acid, 7,600; and 2-hydroxy-5-methylmuconic semialdehyde, 20,200. The wavelengths of maximum absorbance were unchanged. The rates at which these peaks disappeared were used as a measure of the activity of the degrading enzymes.

2.4.3. 2-Hydroxy-6-oxohepta-2,4-dienoate Hydrolase

2-Hydroxy-6-oxohepta-2,4-dienoate hydrolase was assayed by following the disappearance of 2HMS, or a methyl substituted analogue, at a suitable wavelength (Section 2.4.2.).

2.4.4. 2-Hydroxymuconic Semialdehyde Dehydrogenase

2-Hydroxymuconic semialdehyde dehydrogenase was assayed in the same manner as the 2HMS hydrolase, except that 1mM NAD was added to the reaction mixture. The rate of degradation in the absence of NAD was subtracted from the value obtained.

2.4.5. 4-Oxalocrotonate Tautomerase

4-Oxalocrotonate tautomerase was assayed by measuring the initial rate of decrease in absorbance at 292nm on addition of 4OC dissolved in ethanol. Rates are expressed as the rate of decrease of absorbance at 292nm per mg of protein per minute, corrected for a spontaneous tautomerization of 3.3 units/minute.

2.4.6. 4-Oxalocrotonate Decarboxylase (4OC Decarboxylase)

This enzyme was measured by following the decrease in concentration of 4OC. The total 4OC concentration was obtained by adding a one tenth volume of 10M NaOH to a sample, and measuring its absorbance at 350nm. Authentic 4OC was found to have a molar extinction coefficient of 18,900.

2.4.7. 2-Oxopent-4-enoate Hydratase

2-Oxopent-4-enoate hydratase activity was calculated by following the disappearance of 2-oxopent-4-enoate. Two hundred microlitres of a cell free extract solution were added to 20µl of concentrated HCl. The protein precipitate was removed by centrifugation, and the concentration of 2-oxopent-4-enoate determined by HPLC analysis (Table 3, conditions 4).

In the absence of cell free extracts, at 55°c, a spontaneous disappearance of 2-oxopent-4-enoate was observed. The enzyme activities have been corrected for this rate (0.0071 units/min).

Due to the instability of the substrate, previous workers have expressed the hydratase activity as the decrease in absorbance at 265nm per mg per minute. The hydratase activity of the enzyme from strain IC3 has therefore been converted into the same units.

2.4.8. 4-Hydroxy-2-oxovalerate Aldolase

The activity of this enzyme was also measured by following the disappearance of its substrate. The calculation of concentration of the substrate is described

later.

Activities were again corrected by subtraction of the spontaneous disappearance of the substrate.

2.5.Assays for Chemical Compounds

Simple, spectrophotometric assays of chemicals are described in the text. Other methods used are described below.

2.5.1.HPLC Analysis

HPLC analysis was used to determine the presence and/or concentration of a range of compounds. Experiments were performed on a Spectra Physics 8700 pump, 8440 XR detector and 4200 computing integrator. A Spherisorb 10 C8 or C18 column was utilized. The different experimental conditions, and the retention times of various chemicals under these conditions, are shown in Table 3.

2.5.2.Glucose

Glucose concentrations, of cell free media, were measured using a Beckman glucose analyser.

2.5.3.Formate

Formate was measured chemically by the method of Grant (1948).

The enzymic determination of formate production, from the hydrolysis of 2HMS, is described below.

NADH, produced by the action of formate dehydrogenase upon formate and NAD, was oxidised by a *B. stearothermophilus*

Table 3. HPLC Techniques and Retention Times

	Mobile Phase	Flow Rate (ml/min)	Monitoring Wavelength (nm)	Column Type	Chemicals And Retention Times (Mins)
1.	Acetonitrile 50% Orthophospho-ric Acid 10mM	1	210	C18	NAD and NADH 2.70 Catechol 3.98 Phenol 4.95
2.	Propan-2-ol 20% Orthophosho-ric Acid 8mM	1	270	C8	Phenol 4.35 Benzoate 5.20 o-Cresol 4.92 m-Cresol 5.14 p-Cresol 5.18 o-Nitrophenol 5.07 o-Chlorophenol 5.14 m-Chlorophenol 6.25 p-Chlorophenol 6.16 Benzoate hydro-diol 3.48
3.	Propan-2-ol 5% Orthophospho-ric acid 9.5mM	2	270	C8	Phenol 4.35 o-Methoxyphenol 2.69 m-Methoxyphenol 2.74 p-Methoxyphenol 2.58 m-Nitrophenol 3.01 p-Nitrophenol 2.89
4.	Propan-2-ol 20% Orthophospho-ric Acid 8mM	2.5	270	C18	2-Oxopent-4-enoic Acid 3.45
5.	Propan-2-ol 20% Orthophospho-ric Acid 8mM	1	230	C18	4-Methyl-2-oxo-butyrolactone 4.18

NADH oxidase. One mole of NADH, and hence one mole of formate, consumed 0.5 moles of oxygen. The reaction was followed with an oxygen electrode.

The reaction mixture, incubated at 37°C, contained in 3 ml of phosphate buffer: CFE, 0.3-0.5mg/ml; and 50uM catechol. The exact concentration of catechol was determined by the ensuing oxygen uptake (1 mole of oxygen = 1 mole of catechol). After a period of about 1 hour, NAD was added to a final concentration of 1mM, and some oxygen uptake was observed as a result of the oxidative degradation of 2HMS. This value was used to calculate the actual amount of 2HMS metabolised. The formate concentration was then determined by the oxygen uptake on addition of 0.3 units of formate dehydrogenase.

2.5.4. 4-Hydroxy-2-oxovalerate

The concentration of this compound was based upon the method of Dagley and Gibson (1965).

A sample (180µl) of the cell free extract solution to be assayed was added to 20µl of concentrated HCl. The sample was then boiled for 5 minutes, during which time 70% of the 4-hydroxy-2-oxovalerate present recyclised, producing, after HPLC analysis, a peak that corresponded to 4-methyl-2-oxobutyrolactone. Protein precipitate was removed by centrifugation.

2.5.5.Pyruvate

Pyruvate concentrations were determined enzymatically

by a method based upon that of Brown and Perham (1976).

Cell free extract solutions (1ml) were boiled for 30 minutes to destroy the thermophilic NADH oxidase activity present. The samples were clarified by centrifugation and 0.5ml removed and added to a cuvette. To the sample was added: NAD, 1µmole; thiamine pyrophosphate, 0.2µmoles; $MgSO_4$, 4µmoles; Coenzyme A, 0.5µmoles; L-cysteine 2.6µmoles; and pyruvate dehydrogenase, 0.25 units. The sample volume was made up to 1ml with 50mM, pH7.0, phosphate buffer. The samples were incubated at 30°C and the pyruvate concentration determined by following the production of NADH at an absorbance of 340nm.

2.5.6.Acetaldehyde

Acetaldehyde was determined enzymically by a method based upon that of Lundquist (1958).

The NADH oxidase activity of a cell free extract sample was removed in the manner described above for the determination of pyruvate. To a cuvette was added: Clarified sample, 0.5mls; NAD 1µmole; L-cysteine, 1µmole; aldehyde dehydrogenase 0.25 units. The sample volume was made up to 1ml with 50mM, pH7.0, phosphate buffer.

The samples were incubated at 25°C, and the aldehyde concentration determined by following the production of NADH at an absorbance of 340nm.

2.6.Genetic Techniques

2.6.1.Mutagenesis

The methods used for mutagenesis were based on the procedures of Miller (1972).

2.6.1.1.Nitrosoguanidine Mutagenesis.

N-Methyl-N'-Nitro-N-Nitrosoguanidine (NG) was added to 40mls of cells, in the mid log growth phase, to a final concentration of 50ug/ml. The cells were then incubated, under normal growth conditions, for 8 minutes. A death curve performed previously had shown that this was the time required for 50% survival. The cells were then harvested and washed in media, preheated to 50°C.

2.6.1.2.Ultra-violet Light Mutagenesis.

Ultra-violet (UV) light mutagenesis was used due to the high frequency of highly stable deletion mutants produced (Schwartz and Beckwith 1969).

Washed cells were resuspended to a final concentration of 400-600µg/ml in 5mls of media, without the brain heart infusion, yeast extract or trace metals. These cells were irradiated in a glass Petri dish for 5.5 minutes (1% survival from a previously performed death curve). Short wave UV light, from a hand held Mineralight UVGL-25 lamp, at 15cm, was used in all experiments. (Wavelength used = 254nm)

2.6.1.3.ICR-191 Mutagenesis.

The acridine half-mustard ICR-191, is known to produce predominantly frameshift mutations (Oeschger and Hartman 1970).

Cultures (40ml) were inoculated (1%), and incubated for 2 days with growth limiting concentrations (5µg/ml) of ICR-191. After this time, the cells were harvested and washed with media, preheated to 55°C.

2.6.2.Enrichment Techniques

Simple enrichment techniques are described in the text.

The enrichment procedure for mutants defective in phenol metabolism, using D-cycloserine and penicillin G, was based on those previously described (Ornston et al 1969, Carhart and Hegeman 1975).

Following mutagenesis, cells were washed and resuspended in medium with 5mM phenol, and incubated at 55°C. After 3 hours, D-cycloserine (10µg/ml) and penicillin G (50units/ml) were added. Four hours later the cells were harvested, washed twice, and incubated overnight in medium containing 25mM ethanol as a carbon source. The cycle was repeated a total of three times, before the cells were diluted, and plated onto a solid medium with a non-aromatic carbon source.

2.6.3.Plasmid Curing

Heat curing was attempted by growing strain IC3 on, nutrient agar, at 70°C. The strain was subcultured three times, at the same temperature, before colonies were screened for growth on phenol at 55°C.

Attempted curing with acridine orange was performed by growing a culture (1% inoculum) in the presence of a growth limiting concentration (100µg/ml) of the reagent. After 2

days, the cells were harvested and washed twice, before plating onto solid media.

2.6.4.Reversion Frequencies

Some strains would revert to the parental phenotype in the presence of NG (Miller 1972).

The spontaneous reversion frequency, of mutants deficient in phenol metabolism, was calculated by plating out suitable dilutions of cells onto phenol containing solid media.

CHAPTER 3

Aromatic Carbon Sources for Thermophilic Microorganisms

3.1.Media Development

To allow quick screening of the ability of thermophilic microorganisms to grow on various carbon sources, it was firstly necessary to develop media on which the organisms would not grow unless an additional carbon source was present. To this end, growth on a variety of defined and semi-defined media was attempted. With the semi-defined media used, the concentration of complex ingredients was insufficient to support growth in the absence of a carbon source.

3.1.1.Media for Thermus Strains

Defined and semi-defined media could not be developed for the *Thermus* species; *T. thermophilus*, *T. flavus* or YT-1. These species would grow on the complex medium of Brock and Freeze (1969), but lowering the concentration of tryptone and yeast extract, while adding a carbon source, failed to give growth. No strain grew on the medium of Sonnleitner *et al* (1982), designed for the growth of *Thermus* species.

3.1.2.Media for Bacillus Strains

No *Bacillus* strain would grow on the defined media of Atkinson *et al* (1975), Kuhn *et al* (1979) or in R medium (Wigmore and Ribbons 1980). It was also not possible to develop semi-defined media for the following bacilli: *Bacillus* strains RS 5, RS 93 and RS 240; *B. caldotenax*; *B.*

thermodenitrificans DSM 466; B. coagulans ATCC 12245; and B. licheniformis. With the above strains the semi-defined medium of Heinen (1971) would not support growth, nor would the medium of Buswell and Clark (1976), even where the latter medium was supplemented with 0.1% brain heart infusion, tryptone and tryptone soya broth.

The bacilli that would grow in semi-defined media, together with the appropriate medium, are listed in Chapter 2.

3.2 Growth of Thermophilic Bacilli on Aromatic Carbon Sources

The results of experiments to determine the ability of thermophilic bacilli to grow in semi-defined media, with aromatic compounds (2 and 5 mM) as the main carbon and energy sources, together with results previously published, are summarized in Table 4. Additional carbon sources on which no strains grew were; o-, m- and p-toluate, o-, m- and p-cresol, o-, and m-hydroxybenzoate, orcinol, resorcinol, p-cumate, D-phenylalanine, D- or L-tyrosine and D- or L-tryptophan.

Catechol, its mono-methyl and mono-carboxy substituted derivatives, and gentisate were all unstable at 55°C, and were not used in the above experiments.

No growth was observed with any of the following strains in the presence of any aromatic compound; Bacillus stearothermophilus strains NCA 1503, NCTC 10003, ATCC 10149, Bacillus coagulans ATCC 8038 and Bacillus thermodenitrificans DSM 465.

Although Bacillus stearothermophilus IC3 failed to grow

Table 4. Aromatic Carbon Sources for Thermophilic Bacteria

Strain and Source		Carbon Source								
		Benz	PHB	PA	PHPA	LPA	Phen	OCr	MCr	PCr
Bacillus strains										
RS 20	1	-	-	+	+	-	-	-	-	-
RS 53	1	+	+	-	+	-	-	-	-	-
RS 173	1	-	-	+	-	+	-	-	-	-
IC2	1	+	+	+	+	+	-	-	-	-
Bacillus stearothermophilus										
ATCC 8005	1	-	-	+	+	-	-	-	-	-
PH 24	2	NT	NT	NT	NT	NT	+	+	+	+
IC3	1	+	-	-	-	-	+	-	-	-
Numerous strains	3	NT	NT	NT	NT	NT	+	NT	NT	NT
Bacillus caldovelox	1	-	-	+	+	-	-	-	-	
Thermus strains										
ZA	4	-	-	+	NT	NT	NT	NT	NT	NT
Z05	4	-	-	+	NT	NT	NT	NT	NT	NT
YT-1	4	-	-	+	NT	NT	NT	NT	NT	NT
Unidentified Strains										
One strain	5	+	NT	NT	NT	NT	NT	NT	NT	NT
One strain	6	NT	NT	NT	NT	NT	+	NT	NT	NT
IC1	1	+	+	+	+	+	-	-	-	-

Notes:

(1) + indicates growth, - indicates no growth and NT indicates Not Tested.

(2) Chemical abbreviations are as follows: Benz, benzoate; PHB, *p*-hydroxybenzoate; PA, phenylacetate; PHPA, *p*-hydroxyphenylacetate; LPA, L-phenylalanine; Phen, phenol; OCr, *o*-cresol; MCr, *m*-cresol; PCr, *p*-cresol.

(3) Sources are as follows: 1, this thesis; 2, Buswell (1974); 3, Golovacheva and Oreshkin (1975); 4, Degryse *et al* (1978); 5, Buswell and Twomey (1974); 6, Egorova (1942).

on any of the cresol isomers in initial experiments, further studies have shown that all three isomers were completely metabolised when 25mM ethanol was added to the media. The growth of strain IC3 on cresols is discussed later.

3.3.Discussion

Thermophilic Bacilli have been previously isolated that were able to grow in semi-defined media, utilizing phenols (Egorova 1942, Golovacheva and Oreshkin 1975), Phenols and cresols (Buswell 1974, 1975), benzoate (Buswell and Twomey 1974) and p-hydroxybenzoate (Buswell and Clark 1976) as main carbon sources. The Thermus strains Z05, ZA and YT-1 were also able to use phenylacetate, but not benzoate or p-hydroxybenzoate, as a carbon source (Degryse et al 1978).

In the above investigation, thermophilic Bacillus species that grow on aromatic compounds appear to be numerous. Of those organisms screened that were not specifically enriched on aromatic media, five out of ten would utilize aromatic acids as main carbon and energy souces. Strains metabolizing aromatic compounds were also readily isolated from soil samples. One of these isolates, B. stearothermophilus IC3 was the only organism screened that was able to use phenolics.

CHAPTER 4

THE GROWTH OF BACILLUS STEAROTHERMOPHILUS IC3 UPON AROMATIC COMPOUNDS

One of the thermophilic strains, *Bacillus stearothermophilus* IC3, that had been isolated from soil after enrichment on *m*-cresol and phenol, was chosen for further study.

In this chapter, some of the basic growth characteristics of this organism are described.

4.1.The Effect of Aromatic Substrate Concentration upon Growth

The growth of strain IC3, with different concentrations of phenol and benzoate as carbon sources, are shown in Figures 9 and 10. Liquid cultures (40 ml), inoculated with a 1% volume of cells cultured overnight, were employed in these experiments.

4.2.Growth Yields and Doubling Times

The growth yields and doubling times of strain IC3, grown on glucose, phenol and benzoate, are shown in Table 5. These results were obtained using 200ml liquid cultures. The concentration of aromatic compounds was measured by HPLC analysis (Table 3, conditions 2).

4.3.Discussion

4.3.1.The Effect of Aromatic Substrate Concentration upon Growth

The results in Figure 9 show that strain IC3 will

Figure 9.Effect of Phenol Concentration upon Growth

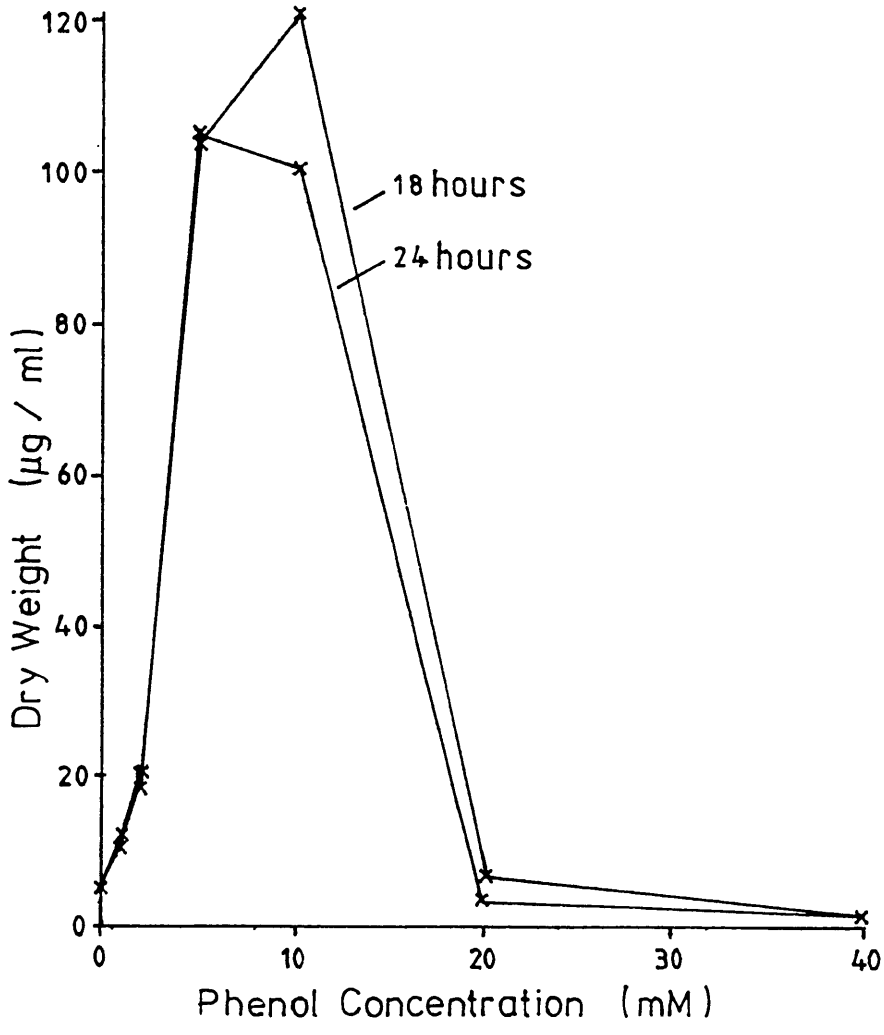


Figure 10. Effect of Benzoate Concentration upon Growth

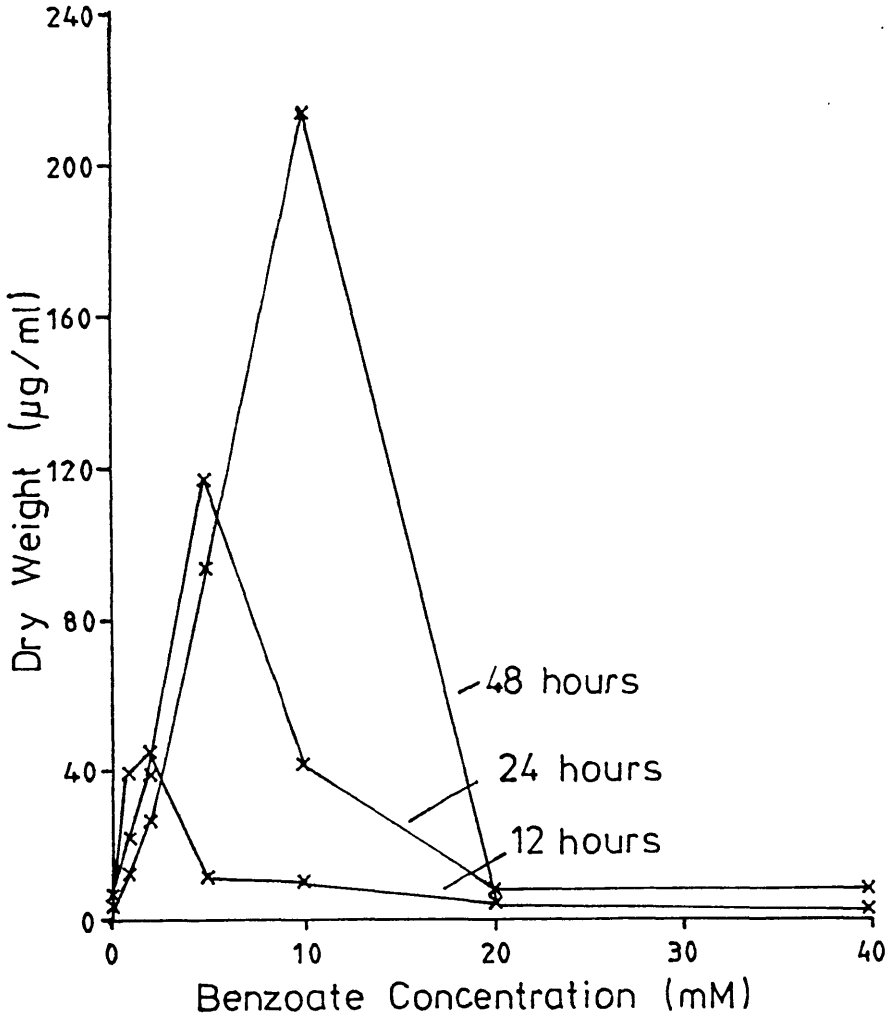


Table 5.Growth Yields and Doubling Times of Strain IC3

Carbon Source	Growth Yields g/mol	g/g	Doubling Times (hrs)
Glucose	43	0.24	1.1
Phenol	29	0.30	2.4
Benzoate	31	0.26	6.8

NOTES:

(1) Growth yields are measured as grams of cells produced per mole or per gram of substrate utilized.

(2) All carbon sources were added to a final concentration of 5mM.

utilize phenol at a concentration of 5mM. Growth also occurs with 10mM phenol, but only gives slightly higher cell yields. No significant growth occurs in the presence of 20mM phenol.

Phenol is therefore toxic to the thermophile at low concentrations. This is also the case in the pseudomonads, in a taxonomic study of this genera (Stanier et al 1966), phenol was supplied at concentrations of 0.025% (2.7mM), due to inhibition observed with some strains growing on 0.05% (5.3mM) concentrations of the aromatic.

Growth of B. stearothermophilus strain PH24 was also inhibited by phenol concentrations above 0.05% (5.3mM) (Buswell and Twomey 1975). Other thermophiles have been reported to grow in the presence of considerably higher amounts of phenol, a thermophilic bacterium would grow at 60°C in 0.1% (10.6mM) phenol. The aromatic at 0.2% (21.3mM) hindered development, and only at phenol concentrations of 4g/l (42.5mM) was growth inhibited (Egorova 1942). Other strains of B. stearothermophilus were able to grow on phenol at concentrations of 0.3% (31.9mM) (Golovacheva and Oreshkin 1975).

Jones et al (1973), showed that phenol, even at low concentrations, inhibited the growth of "Bacterium NCIB 8250", now classified as Acinetobacter calcoaceticus (Bousfield and Graham 1975). This resulted in decreased yields and slower growth. This behaviour was not observed with the same organism grown on benzoate (Kennedy and Fewson 1968). This effect of phenol upon growth, which was also

shown by benzoate, occured with B. stearothermophilus IC3 (Figures 9 and 10).

Benzoate was not as toxic as phenol when used as a carbon source for pseudomonads, 0.1% (8.2mM) benzoate being used to support growth by Stanier et al (1966). 10mM benzoate also suppoted growth of strain IC3, inhibition being observed at concentrations of 20mM.

4.3.2.Growth Yields and Doubling Times

The growth yields (Table 5) of strain IC3 grown on glucose, 43.1g/mol (23.9g/g), was significantly lower than those of 64g/mol (36g/g) observed at the same temperature with B. stearothermophilus var. nondiastaticus (Coultate and Sundaram 1975). However, cell yields with the latter organism, unlike mesophiles, were highly temperature dependant. Yields over 90g/mol (50g/g) were found at 41°C, but at 59.3°C, a yield of only 54g/mol (30g/g) was observed. It was suggested that the drop in growth yield was due to less efficient coupling of respiration and energy production, with acetate as a waste product. Glucose grown cultures of strain IC3 also showed acidification of the media, with pH values as low as 4.8 being observed. This was the initial reason for the addition of Tris buffer to the medium in which this organism was cultured.

The growth yield of strain IC3 grown on phenol and benzoate, expressed as grams of cells produced per gram of substrate, were higher than those observed on glucose (Table 5). The growth of the mesophilic organism, "Bacterium NCIB

8250", has also been studied on a variety of carbon compounds at 30°C, including benzoate (Kennedy and Fewson 1968) and phenol (Jones et al 1973). This organism showed higher growth yields on these compounds than strain IC3; 55.1g/mol (0.45g/g) on benzoate, and 67.7g/mol (0.72g/g) on phenol. Even higher growth yields have been reported for mesophiles growing on benzoate, for example 86.8g/mol (0.71g/g) by Pseudomonas C12B (Mayberry et al 1967).

The growth yields of the thermophile, with aromatic compounds, were therefore lower than those displayed by mesophiles, although no attempt was made to optimize the growth conditions. The high maintenance requirements of some thermophiles (Sonnleitner 1983, Sundaram 1986) could have contributed to the low growth yields. It was also suggested (Jones et al 1973), that the high maintenance requirement of phenol grown Bacterium NCIB 8250, was in part due to the expenditure of energy involved in the cleaving of the aromatic nucleus. This process may also have contributed to the low growth yields of strain IC3 on phenol.

The growth rates of the thermophilic strain IC3 were also lower than those previously reported. Doubling times of 36 minutes for B. stearothermophilus on glucose (Epstein and Grossowicz 1969), and 50 minutes for B. stearothermophilus strain PH24 (Buswell and Twomey 1975), have been observed. No doubling times for a thermophile on benzoate are known, but the figure of 6.8 hours for strain IC3 is very poor.

CHAPTER 5

INITIAL OXYGENASE REACTIONS IN THE METABOLISM OF PHENOL

5.1.Whole Cell Experiments

The results of experiments showing the stimulation of oxygen uptake by washed whole cells of *B. stearothermophilus* IC3 are summarized in Table 6. No stimulation of oxygen uptake was detected subsequent to the addition of any of the following compounds; *o*-, *m*- or *p*-hydroxybenzoate, phenylacetate, *p*-hydroxyphenylacetate, orcinol, resorcinol, *p*-cumate, D- or L-phenylalanine, D- or L-tyrosine, D- or L-tryptophan, protocatechuate, 2,3-dihydroxybenzoate and gentisate. These results indicate that phenol is metabolised via catechol, the uptake of oxygen in the presence of cresols probably being a result of relaxed substrate specificity of the initial hydroxylase.

Phenol grown cells used 2.02 moles of oxygen per mole of phenol added, and 1.03 moles of oxygen per mole of catechol added.

5.2.Phenol Metabolism by Cell Free Extracts

Further evidence for the conversion of phenol to catechol in cell free extracts was provided by HPLC analysis. (Table 3, Conditions 1). Cell free extracts incubated with 1mM phenol and 1mM NADH, at 55°C, showed the appearance of a new peak that had the same retention time as catechol.(15% yield)

The phenol hydroxylase enzyme is specific for NADH as a cofactor, no phenol induced oxygen uptake was observed in its absence, nor in the presence of NADPH. The background NADH

Table 6.Oxygen Uptake by Whole Cells of Strain IC3

AROMATIC ADDED	GROWTH SUBSTRATE		
	PHENOL	BENZOATE	GLUCOSE
Phenol	391	0	0
Benzoate	0	296	0
o-Cresol	368	219	0
m-Cresol	327	204	0
p-Cresol	273	170	0
o-Toluate	0	323	ND
m-Toluate	0	155	ND
p-Toluate	0	137	ND
p-Hydroxybenzoate	0	82	ND
p-Cumate	0	35	ND
Catechol	964	804	0
3-Methylcatechol	784	799	0
4-Methylcatechol	1029	865	0

NOTES:

(1) ND=Not Determined

(2) Oxygen uptakes are expressed as nmols of oxygen per mg of cells per minute.

(3) Aromatics added to a final concentration of 1mM.

oxidase activity was too high to measure the stoicheiometry of NADH utilization. However, from the whole cell results, oxygen was used in a ratio of 2:1 with phenol, the results shown below would indicate that half of this uptake was due to catechol metabolism. The phenol is therefore converted to catechol by a phenol hydroxylase (phenol 2-monooxygenase) enzyme.

5.3.Catechol Metabolism in Cell Free Extracts

The results in Table 7 show the rate of production of ring fission products from catechol and 3- and 4-methylcatechol. The ratio of oxygen:catechol used by this enzyme was 0.95:1, with 99% recovery of 2-hydroxymuconic semialdehyde from catechol.

From the spectrophotometric data shown in Table 7, it would appear that the ring fission products of catechol, 3-methylcatechol and 4-methylcatechol are 2-hydroxymuconic semialdehyde, 2-hydroxy-6-oxohepta-2,4-dienoic acid and 2-hydroxy-5-methylmuconic semialdehyde respectively. These results are consistent with a catechol 2:3-dioxygenase enzyme.

5.4.The Metabolism of Benzoate

The metabolism of benzoate by strain IC3 was not extensively studied, mainly due to the inability to demonstrate any benzoate metabolism in cell free extracts. In attempts to obtain cell free extracts displaying a benzoate

metabolising activity, various methods were employed, incuding the use of a sonicator and a French press for cell disruption, and resuspending cells in, firstly 40mM Tris buffer (pH7.5) containing 10mg/ml bovine serum albumin (BSA) and 1mM dithiothreitol (DTT). All other buffers were made in 50mM phosphate buffer (pH7.0), and contained the following ingredients: 1, 10mg/ml BSA; 2, 1mM DTT and 0.1mM phenylmethylsulphonylfluoride (PMSF); 3, as for 2 with 4mM $FeSO_4$, 10% ethanol and 10% glycerol; 4, as for 2 with 4mM $FeSO_4$ and 10mg/ml BSA. The particulate fraction of the cell free extract was also assayed for benzoate metabolising activity.

The extracts were assayed with an oxygen electrode and by HPLC (Table 3, conditions 2), after adding 1mM benzoate, 1mM benzoate and 1mM NADH, or 1mM benzoate, 1mM NADH and 1mM FAD.

Benzoate grown cells of strain IC3 also failed to utilize benzoate hydrodiol when it was added to the media, and cell free extracts produced by the methods used to display phenol hydroxylase and catechol 2:3-dioxygenase activity showed no ability to degrade the diol. However, extracts produced by the method used to display catechol 2:3-dioxygenase activity in phenol grown cells, showed a similar activity when benzoate grown cells were used. With these extracts, 100% yields of 2-hydroxymuconic semialdehyde were obtained from catechol.

Table 7. Production of Ring Fission Products in Cell Free Extracts

	SUBSTRATE		
	CATECHOL	3-METHYL CATECHOL	4-METHYL CATECHOL
MAXIMUM WAVELENGTH OF PRODUCT ABSORBANCE (nm)	375	388	382
RATE OF FORMATION OF PRODUCT (nmols/mg/min)	865	675	761
RELATIVE RATE OF PRODUCT FORMATION (%)	100	78	88
YIELD (%)	99	113	101

NOTE:

(1) Yields represent % recovery of ring fission product from substrate.

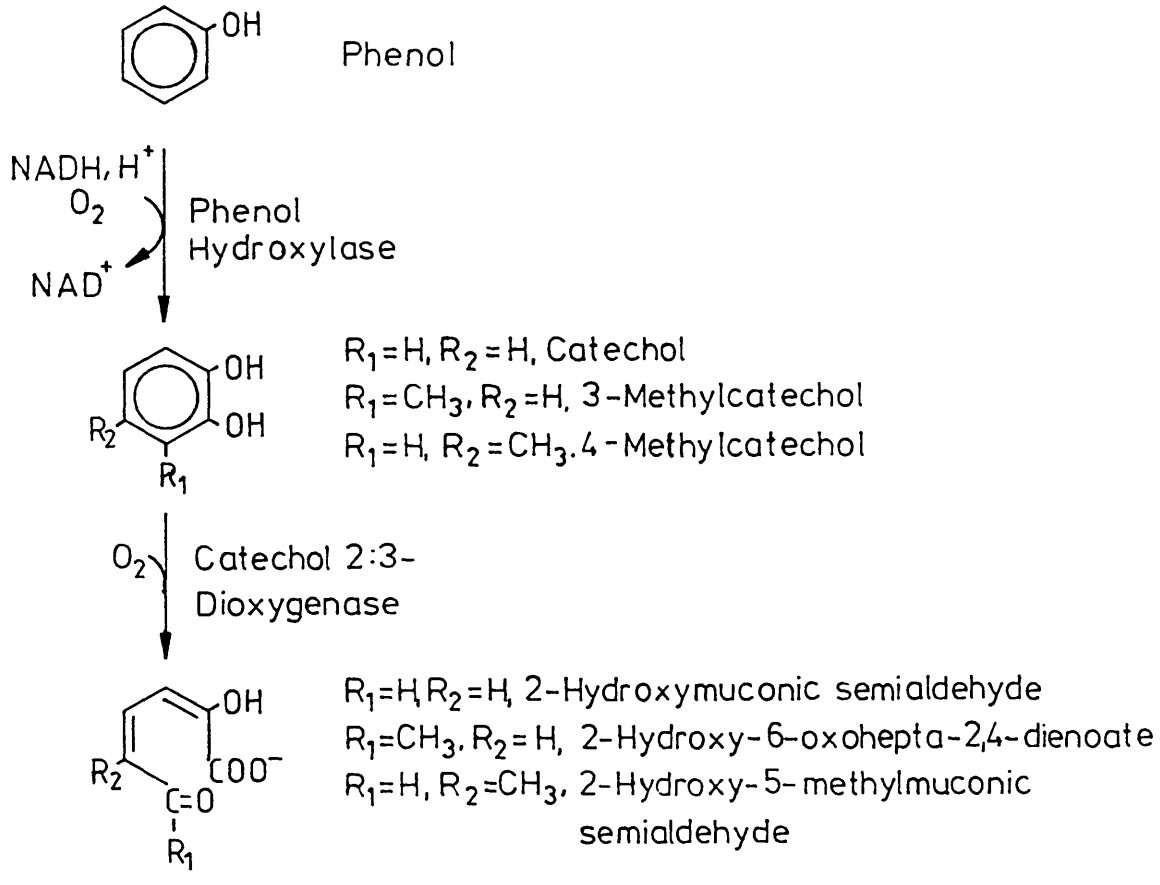
5.5.Discussion

5.5.1.Phenol Metabolism

The initial reactions in the metabolism of phenol by B. stearothermophilus IC3 (Figure 11) are performed by oxygenases. Phenol is firstly hydroxylated in the ortho position to produce catechol. This reaction has been observed in the degradation of phenol by Brevibacterium fuscum (Nakagawa and Takeda 1962), Pseudomonas putida (Bayly et al 1966), Bacillus stearothermophilus (Buswell 1975), and the fungi Trichosporon cutaneum (Neujahr and Varga 1970), Candida tropicalis (Neujahr et al 1974, Krug et al 1985, Krug and Straube 1986) and Streptomyces setonii (Antai and Crawford 1983). The fungal hydroxylase systems were strictly dependant on NADPH as a cofactor, whereas B. stearothermophilus strains IC3 and PH24 require NADH. No Enzyme Commision Number has been allocated to phenol hydroxylases requiring NADH as a cofactor (Enzyme Nomenclature 1984). The description of the Trichosporon enzyme (EC 1.14.13.7), i.e. Phenol, NADPH: Oxygen Oxidoreductase (2-Hydroxylating), is specific in the requirement for NADPH.

The fungal systems also differed from the bacterial systems, including strain IC3, in the further metabolism of catechol. The fungi metabolised this intermediate via ortho cleavage, unlike the bacilli and pseudomonads where meta cleavage was observed. The meta cleavage observed in strain IC3 was performed by a catechol 2:3-dioxygenase (EC 1.13.11.2. Catechol: Oxygen 2,3-oxidoreductase (Decyclizing))

Figure 11. Initial Oxygenase Reactions in the Metabolism of Phenol



5.5.2. Benzoate Metabolism

The results above suggest that benzoate is metabolised via catechol, a pathway that is well established (Dagley *et al* 1960). This route involves the intermediate benzoate hydrodiol ((-)-3,5-cyclohexadiene-1,2-diol-1-carboxylate) in *Alcaligenes eutrophus* (Reiner and Hegeman 1971) and *P. putida* (Yamaguchi and Fujisawa 1980, Cass *et al* 1987). However, no benzoate metabolising activities were observed in cell free extracts of strain IC3, the reason for which is unclear. Nor was there any metabolism of benzoate hydrodiol by benzoate grown whole cells or cell free extracts.

The benzoate dioxygenase of *P. putida* has been extensively investigated (Yamaguchi *et al* 1975, Yamaguchi and Fujisawa 1978, 1980, 1981, 1982). This enzyme consists of two main components, the first of which, component A, is an NADH-cytochrome *c* reductase, containing one FAD, two iron and three labile sulphide molecules per molecule of enzyme. Component B has two unequal subunits, three of each being present in the active component. This component has three active sites, each containing a [2Fe2s] iron-sulphur group and an additional iron molecule.

The metabolism of benzoate in strain IC3 however remains unclear, but would appear to proceed via catechol. This is in contrast to a previous report of benzoate metabolism by a thermophile, where evidence suggested that benzoate was degraded via gentisate (Buswell and Twomey 1974).

CHAPTER 6

PROPERTIES OF PHENOL HYDROXYLASE

Bacterial phenol hydroxylases have not been extensively studied. This is not the case for the fungal equivalent, the phenol hydroxylase of Trichosporon cutaneum being a pertinent example (Neujahr and Varga 1970; Neujahr and Gaal 1973, 1975; Neujahr et al 1974; Neujahr 1976; Neujahr and Kjellen 1978, 1980, Gaal and Neujahr 1979). This enzyme has a total molecular weight of 148,000 Daltons, and contains 2 subunits of 74 kD. Each of these subunits contains one FAD molecule and 8 sulphydryl groups, some of which are involved in the binding of the flavin nucleotide.

The detailed reaction mechanism of this enzyme has also been well studied (Detmer et al 1984, Detmer and Massey 1984, 1985).

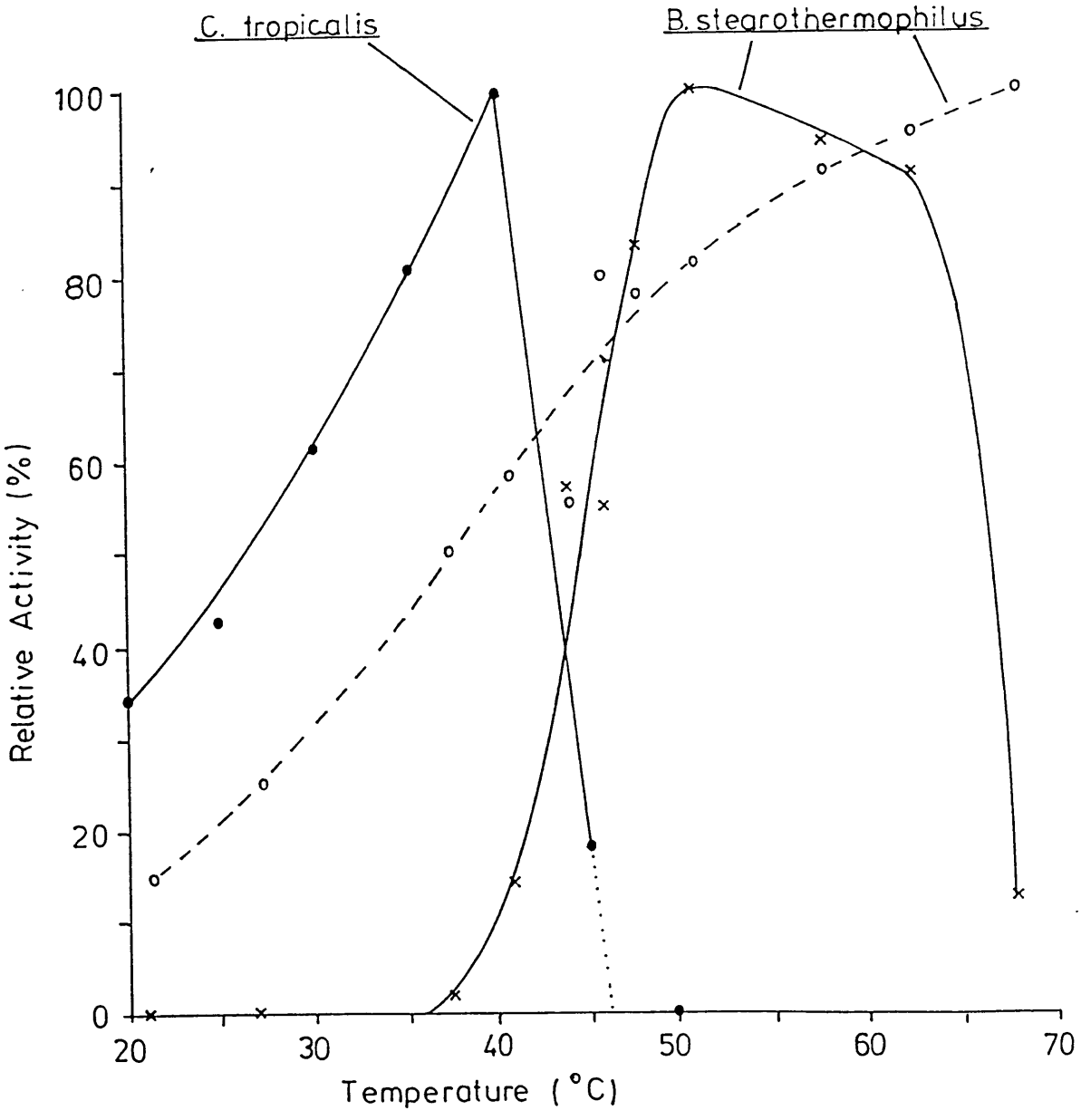
6.1.Stability of Phenol Hydroxylase Activity in Cell Free Extracts

Cell free extracts, prepared in the manner described in Chapter 2, displayed phenol hydroxylase activity having a half life of 19 days when stored at $4^{0}C$.

6.2.Effects of Temperature upon the Activity of Phenol Hydroxylase

The results of experiments where the phenol hydroxylase activity of cell free extracts was assayed spectrophotometrically, at different temperatures, are shown in Figure 12. Also shown are results for similar experiments with Candida tropicalis (Krug and Straube 1986).

Figure 12. Effects of Temperature upon Phenol Hydroxylase and NADH Oxidase Activity



closed circles,

Crosses and solid lines indicate results for phenol hydroxylase. Open circles and dashed line indicates results for the NADH oxidase of B. stearothermophilus.

Experiments were also performed to calculate the half life of phenol hydroxylase, after incubating at various temperatures, and reassaying at 55°C (Table 8a). However, the results were not consistant with an exponential decay type curve. This behaviour was not observed with the NADH oxidase activity that was present (Table 8b).

6.3.Michaelis Constants for Phenol Hydroxylase

The apparent Michaelis constants of phenol hydroxylase for phenol and oxygen were determined by the procedures described in Chapter 2. The results for oxygen are shown in Figure 13 as Lineweaver-Burke, Haynes and Eadie-Hofstee plots. Only the Haynes plot (Figure 14) was used to calculate the apparent Michaelis constant for phenol, as this had the most even distribution of points. For the oxygen constant, the Lineweaver-Burke, Haynes and Eadie-Hofstee plots gave apparent values of 37.8, 41.0 and 39.3 μM respectively, giving an average value of 39μM. The apparent Michaelis constant for phenol was 2μM.

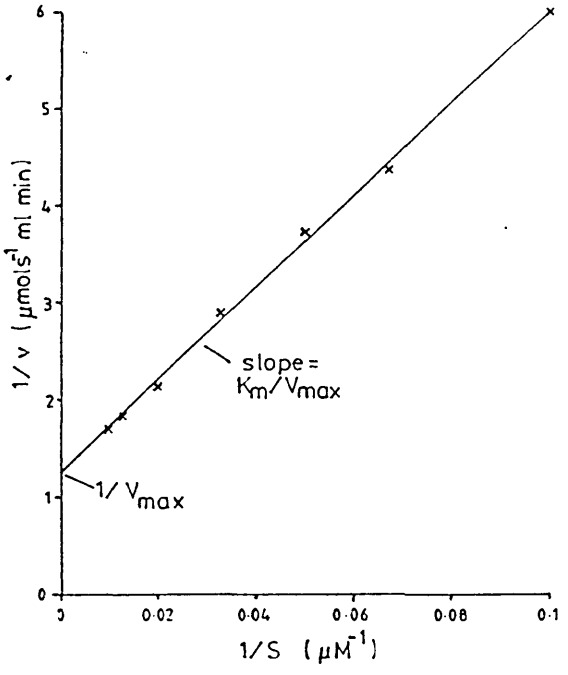
6.4.The Effects of Inhibitors and Metal Ions upon the Activity of Phenol Hydroxylase

The activity of phenol hydroxylase in the presence of inhibitors is shown in Table 9.

In view of the effect of various metal chelators on the phenol hydroxylase enzyme, a range of metal salts were incubated with the crude extracts, and the enzyme reassayed. The results of this experiment are shown in Table 10.

Figure 13. Plots Used to Determine the Michaelis Constant of Phenol Hydroxylase for Oxygen

a) Lineweaver-Burke Plot.



b) Haynes Plot

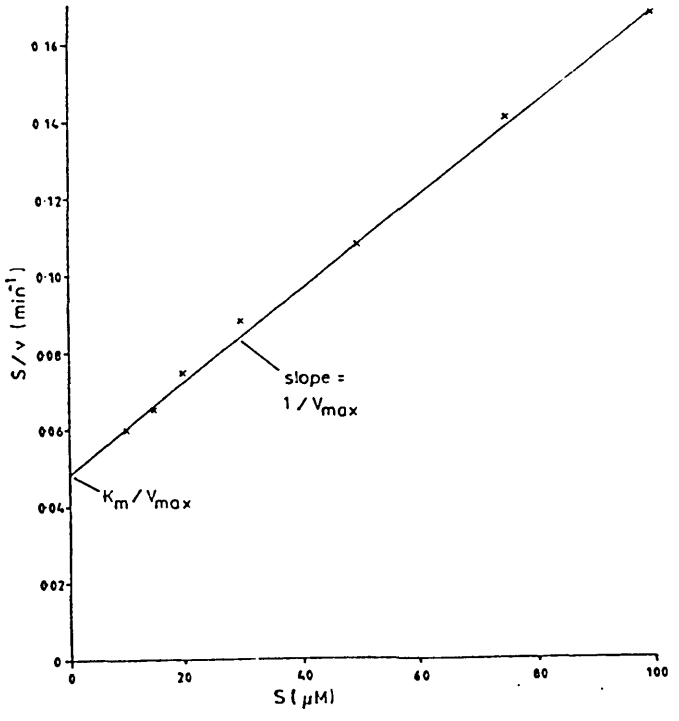


Figure 13.Continued

c) Eadie-Hofstee Plot

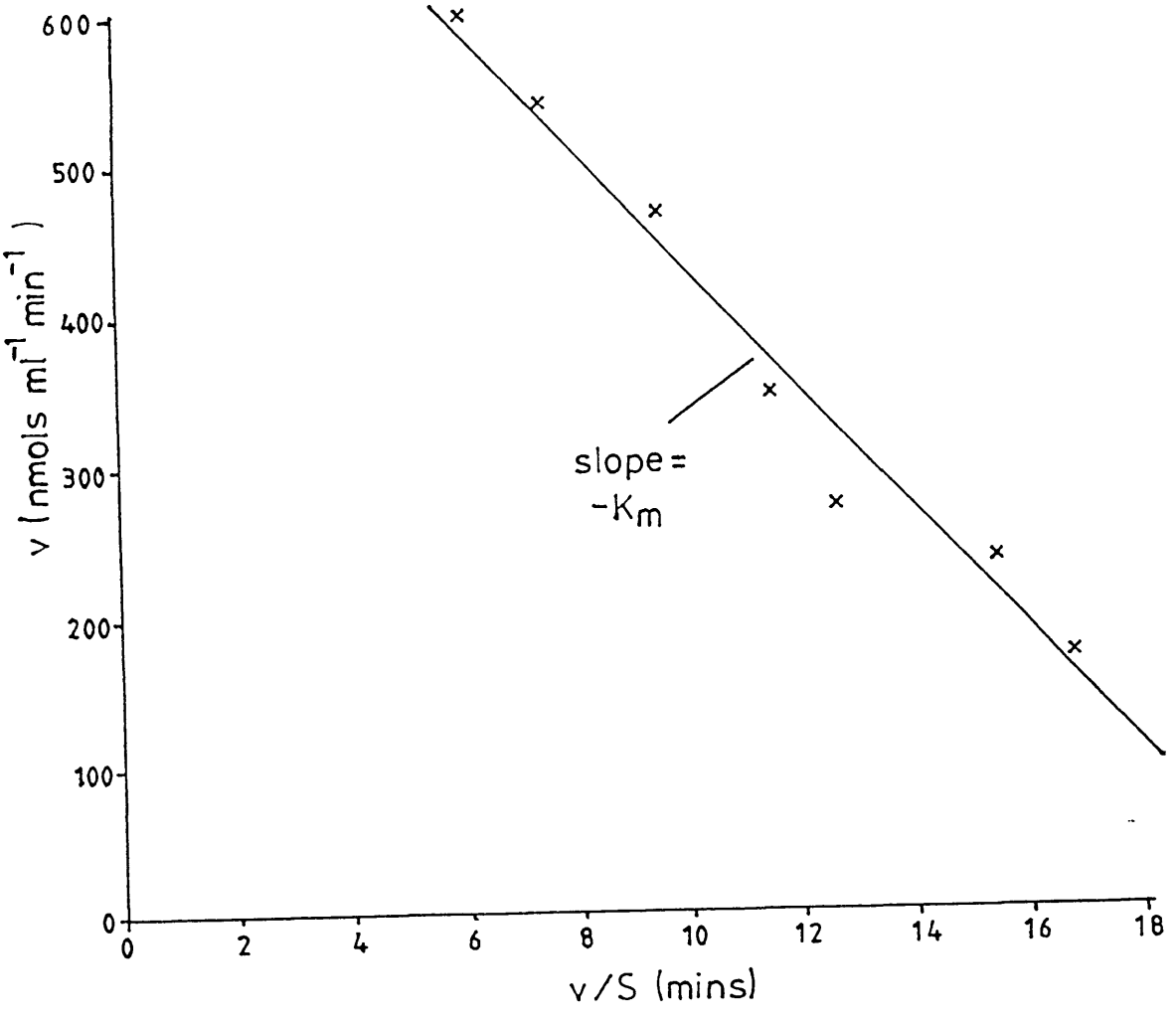


Figure 14.Plot Used to Determine the Michaelis Constant of Phenol Hydroxylase for Phenol

Haynes Plot

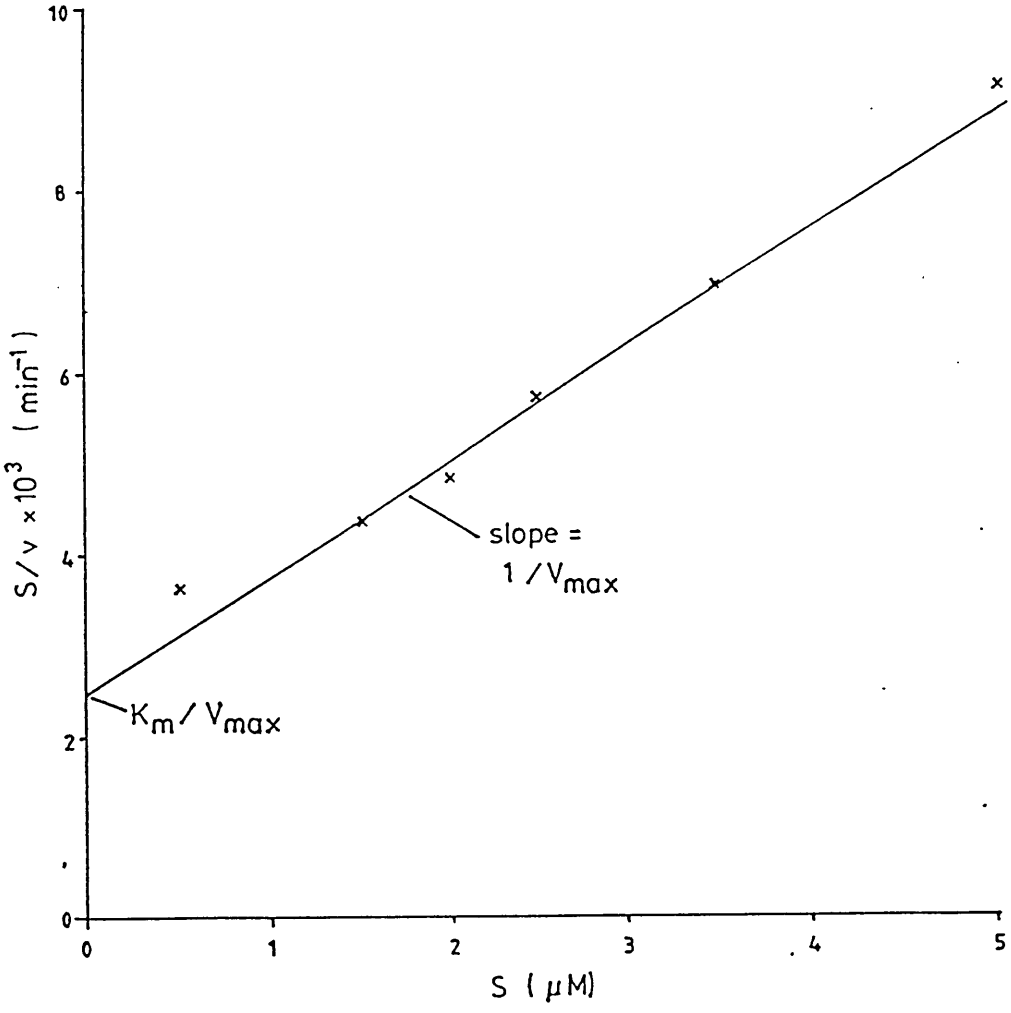


Table 8.Effects of Incubating Cell Free Extracts at Various Temperatures

a)Phenol Hydroxylase Activity.

Incubation Temperature	Incubation Time (Mins)			Half Life (Mins)
	0	5	10	
55	100	53	52	-
60	100	76	84	-
65	100	54	56	-
70	100	0	0	-
75	100	0	0	-

b) NADH Oxidase Activity.

Incubation Temperature	Incubation Time (Mins)			Half Life (Mins)
	0	5	10	
55	100	94	90	68
60	100	92	82	36
65	100	82	78	27
70	100	77	64	16
75	100	33	20	4.3

NOTE:

(1) All temperatures are expressed in ^{o}C.

(2) Activities are expressed as a percentage of that at zero time.

(3) After incubations the enzymes were assayed at $55^{o}C$.

Table 9.Effect of Inhibitors on Phenol Hydroxylase and NADH Oxidase

Inhibitor	Concentration	NADH Oxidase Activity	Phenol Hydroxylase Activity
	(mM)	(%)	(%)
None	-	100	100
PCMB	0.01	91	57
o-Phenanthroline	1	146	15
DDCA	1	123	0
EDTA	1	135	87
Na azide	1	88	73
KCN	1	48	119
Rotenone	1	5	210
2,2'Bipyridine	1	119	52

NOTE:

(1) Abbre-viations:

PCMB=p-chloromercuribenzoate.

DDCA=diethyldiaminocarbamic acid.

(2) The background NADH activity was three times that of the phenol hydroxylase activity.

(3) Reaction rates were measured spectrophotometrically.

Table 10. Reaction Rates of Phenol Hydroxylase and NADH Oxidase in the Presence of Metal Ions (1mM)

SALT ADDED	NADH OXIDASE ACTIVITY (%)	PHENOL HYDROXYLASE ACTIVITY (%)
None	100	100
$CuSO_4$	169	0
$FeSO_4$	69	119
$Fe(NO_3)_3$	79	93
$MnSO_4$	105	181
Na_2SO_4	88	114
$NaNO_3$	104	71

NOTE:

(1) Reaction rates were measured spectrophotometrically.

6.5.The Metabolism of Substituted Phenols

The results in table 11 show that the disappearance of NADH is stimulated by a wide range of substituted phenols. The simultaneous disappearance of the aromatic substrate was established by HPLC analysis (Table 3, conditions 2 and 3). Therefore, a complete uncoupling reaction, as observed in other flavoprotein hydroxylases (White-Stevens and Kamin 1970, Howell and Massey 1970, Ribbons and Ohta 1970), was not occurring. However, partial uncoupling cannot be ruled out. Also shown in Table 11 are results for similar experiments involving Trichosporon cutaneum (Neujahr and Gaal 1973) and Candida tropicalis (Krug and Straube 1986).

Attempts to isolate the products of hydroxylation, with the exception of catechol, have not been successful.

6.6.Discussion

6.6.1.The Stability of Phenol Hydroxylase

Previous workers have found that the stability of phenol hydroxylases is highly dependant on the buffering system used. Thus, purified enzyme from Trichosporon cutaneum in phosphate buffer containing protective agents (mercaptoethanol, FAD, EDTA), lost no activity over a period of seven days at 5°C. However, in Tris buffer, under identical conditions, 75% of activity was lost in three days. (Neujahr and Gaal 1973). A phenol hydroxylase preparation of Brevibacterium fuscum, in Tris buffer, lost over half its activity when stored overnight at -15°C (Nakagawa and Takeda 1962), and a crude extract from Candida tropicalis, again in

Table 11. Reaction Rates of Phenol Hydroxylase with Substituted Phenols

Aromatic Added	Phenol Hydroxylase Activity		
	Bacillus stearothermophilus	*Trichosporon cutaneum*	*Candida tropicalis*
Phenol	100	100	100
o-Cresol	136	7	39.9
m-Cresol	143	10	57.1
p-Cresol	169	16	78.6
o-Fluorophenol	76	29	ND
m-Fluorophenol	65	66	ND
p-Fluorophenol	72	93	ND
o-Chlorophenol	84	10	13.0
m-Chlorophenol	78	26	54.3
p-Chlorophenol	74	29	65.4
o-Methoxyphenol	211	ND	ND
m-Methoxyphenol	160	ND	ND
p-Methoxyphenol	175	ND	ND

NOTES:

(1) Substrates for *B. stearothermophilus* were added at 1mM concentrations.

(2) Reaction rates were measured spectrophotometrically.

(3) ND=Not Determined.

(4) Results for *Trichosporon cutaneum* are for purified protein and are taken from Neujahr and Gaal (1973). The results for *Candida tropicalis* (Crude extracts) are from Krug and Straube (1986).

Tris buffer, was only stable for some hours at 4°C, or weeks at -20°C (Krug and Straube 1986).

It is difficult to compare the stability of the B. stearothermophilus IC3 preparation due to the different conditions used (phosphate buffer, DTT and PMSF). However, a half life of 19 days at 4°C is in the same order as those above. It would of course be expected that the thermophilic enzyme would be at least as stable as its mesophilic counterpart for reasons discussed in the introduction (1.1.1.), and no efforts were made to stabilise the enzyme further.

6.6.2. Effects of Temperature upon Hydroxylase Activity

The results in Figure 12 show that the phenol hydroxylase from strain IC3 showed maximum activity over a temperature range of 50-62°C. Either side of this range, the activity dropped sharply, and none was observed below 35°C or above 70°C. As expected, maximum activity was seen at a higher temperature than in the mesophile Candida tropicalis, which had a maximum activity at 40°C. The thermophilic enzyme also displayed close to maximum activity over a broader range of temperature.

The unusual behaviour of the thermophilic hydroxylase at higher temperatures, when after being held at 60 and 65°c for 10 minutes, greater activity was seen than after 5 minutes, was not further investigated, but may be due to the protein resuming a more active conformation, or subunits reassociating. It is unlikely that this phenomenon would

greatly effect the results in Figure 12, as in this experiment, the temperature in the cuvettes usually stabilized within 60 seconds.

6.6.3.Michaelis Constants of Phenol Hydroxylase

The phenol hydroxylase from strain IC3 had an apparent Michaelis constant for phenol of 2μM at 55°C. This is an order of magnitude below that of 18μM for T. cutaneum (Neujahr and Gaal 1973), and lower than that of 5μM for C. tropicalis (Krug and Straube 1986).

The apparent constant for oxygen in the thermophile (39μM) is also lower than the figure of 53μM reported for T. cutaneum. However, the figure for B. stearothermophilus IC3 should be treated with some caution, due to the experimental procedure, which results in a small amount of catechol product being present during the assay. Catechol was utilized as a substrate by phenol hydroxylases from Trichosporon cutaneum, Candida tropicalis and strain IC3. Nevertheless, a reaction sequence that has been postulated for the hydroxylase from T. cutaneum accounts for the fact that catechol is not hydroxylated in vivo (Detmer et al 1984, Detmer and Massey 1984, 1985). In the mechanism proposed, a concerted substitution type IIb (Bi Uni Uni Bi ping-pong), the substrate is firstly bound by the oxidized enzyme, this enhances the binding of the NADPH cofactor (Neujahr and Kjellen 1978). Electrons are transfered to the flavin and the oxidised pyridine nucleotide dissociates. Oxygen is then bound, and if the substrate dissociates, the reaction becomes

uncoupled, and oxygen reduced to hydrogen peroxide. Otherwise, the product is oxidized and must leave before the flavin ring can close, enabling completion of the enzymes cycle. This mechanism requires that the catechol dissociates, and high levels of catechol oxygenase *in vivo* makes it unlikely that a second hydroxylation would occur.

The uptake of oxygen by whole cells of *Bacillus stearothermophilus* IC3; 2.02 moles of oxygen per mole of phenol, and 1.03 per mole of catechol, suggest that in this organism also, no hydroxylation of catechol, or for that matter any uncoupling of phenol hydroxylase, takes place *in vivo*.

6.6.4. The Effects of Inhibitors and Metal Ions

The results in Table 9 indicate that the phenol hydroxylase of *B. stearothermophilus* IC3 is sensitive to a number of inhibitors. *p*-Mercuribenzoate, which attacks sulphydryl groups, inhibited the enzyme despite equal amounts of DTT in the reaction mixture. Inhibition by this reagent was also observed in *T. cutaneum* (Neujahr and Gaal 1973) and *B. stearothermophilus* PH24 (Buswell 1975). Some sulphydryl groups are necessary for the binding of FAD in the yeast enzyme (Neujahr and Gaal 1975). The only other significant inhibition of the hydroxylase from strain IC3 was by metal chelators. This was also observed with the phenol hydroxylase of *B. stearothermophilus* PH24, but not in the *Trichosporon cutaneum* enzyme.

The results in Table 10 show that, surprisingly, cupric

ions, as well as the copper chelator diethyldiaminocarbamic acid, completely inhibited the enzyme. This may have been due to non specific binding by the chelator with other divalent ions. To suggest that the stimulation of activity by manganese ions implies that they may be involved in the hydroxylase reaction centre would be premature. However, a manganese (II) oxygenase has been described in the Bacillus genus, a 3,4-dihydroxyphenylacetate 2:3-dioxygenase containing manganese (II) ions having been purified and characterised from Bacillus brevis (Que et al 1981). This enzyme had a similar inhibition pattern to that above, with the exception of inhibition by the copper chelator.

6.6.5.The Metabolism of Substituted Phenols

It is apparent from the results in Table 11, that the phenol hydroxylase from B. stearothermophilus IC3 shows a greater stimulation of NADH uptake, in the presence of substituted phenols, than the fungal hydroxylases. It is also apparent that sidegroups that withdraw electrons from the aromatic ring give reduced rates in the assay, and vice versa. This inductive effect has been observed in aromatic chemical reactions, and can be described by the Hammett equation:

$$\log (K/K_o) = \rho \sigma$$

Where K is the reaction rate of the substituted aromatic, K_o is the reaction rate of the unsubstituted aromatic, ρ is a reaction constant, and σ is a Hammett substitution constant (Hammett 1937, Jaffe 1953, McDaniel and

Brown 1957). Therefore, for any particular reaction, log K/K_0 is proportional to σ, or an adjusted value, σ^+, if resonance interactions are involved (Okamoto and Brown 1957). Data are not available for ortho substituents as any inductive effects are masked by steric effects.

Studies on benzoate 1:2-dioxygenase have revealed a close correlation between log K/K_0 and σ in whole cells of Pseudomonas putida mt-2, but not Pseudomonas B13 or Alcaligenes eutrophus B9, presumably due to the masking of electronic effects by steric effects (Reineke and Knackmuss 1978). A similar correlation was also seen with a 2,3-dihydroxybenzoate 3:4-dioxygenase from Pseudomonas putida (Essenger et al 1987). Interestingly, pyrocatechase II from Pseudomonas B13 showed a greater correlation to σ^+ than σ, suggesting a charged transition state (Dorn and Knackmuss 1978).

The phenol hydroxylase of Trichosporon cutaneum did show a correlation with the Hammett constant when the rate of production of an oxygenated intermediate was followed by stopped-flow spectroscopy (Detmer and Massey 1985).

Figure 15 shows the data in Table 11 plotted against σ and σ^+ (Dean 1987). The following correlationcoefficients were obtained with B. stearothermophilus: σ_m, -0.74; σ_m^+, -0.81; σ_p, -0.90; σ_p^+, -0.87. The slopes of the best fitting lines were -0.60, -0.62, -0.87 and -0.43 respectively.

For the meta substituted values calculated with the thermophilic enzyme, the slopes are greater than that of -0.5 obtained with the enzyme from T. cutaneum (Detmer and Massey

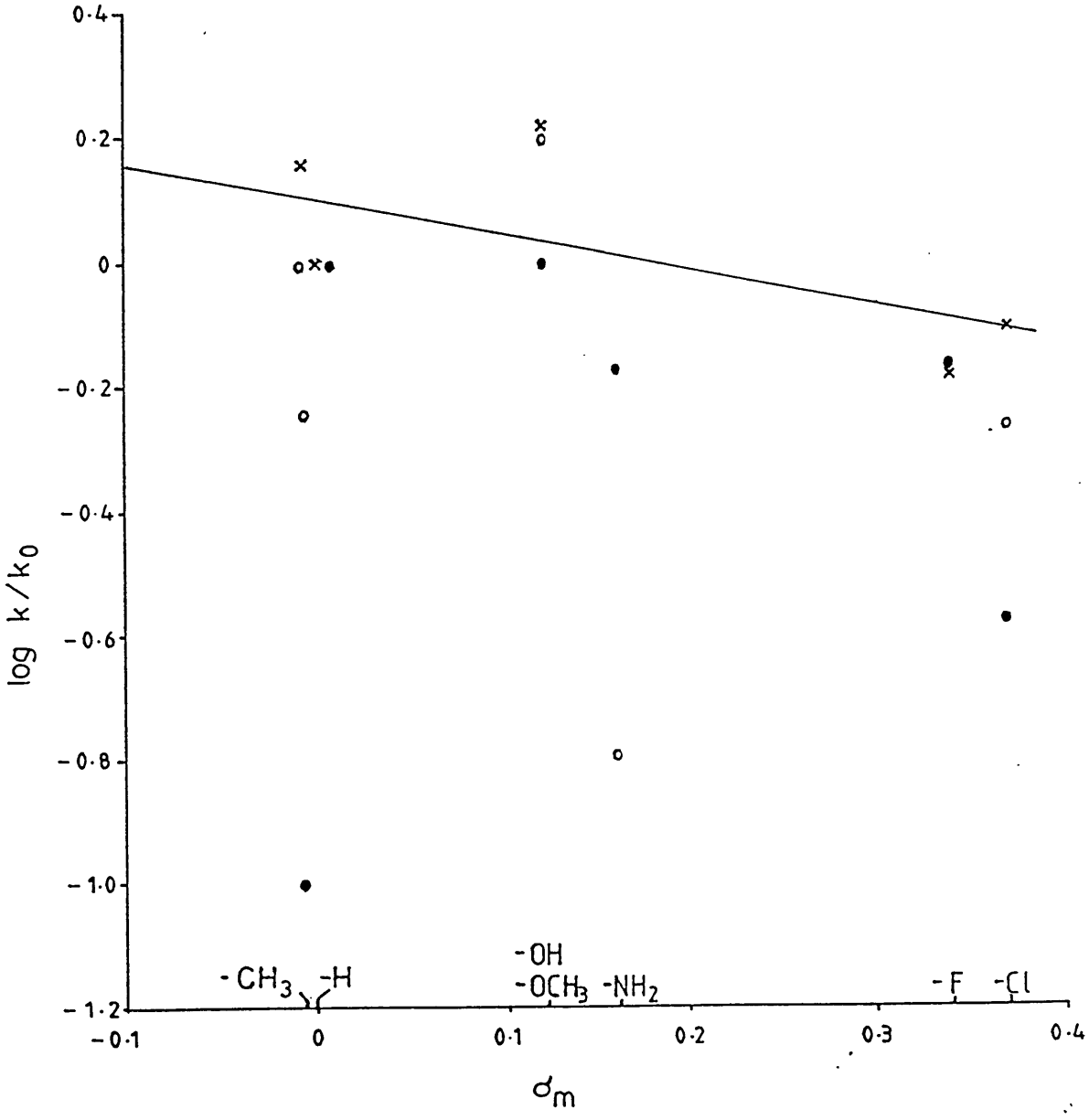
1985), but lower than the figure of -1.4 for benzoate 1:2-dioxygenase of *P. putida* mt-2 (Reineke and Knackmuss 1978). The latter authors suggested that their reaction constant, still low when compared to many chemical reaction constants, was due to an uncharged transition state (a biradical mechanism was proposed). Detmer and Massey (1985), suggested that the low gradient was a result of no fully positive charge being generated in the transition state. This may also be the case in the thermophilic hydroxylase, no (fully) positively charged intermediates would also explain the better fit of the data with σ , and not σ^+.

Overall, the phenol hydroxylase of *B. stearothermophilus* IC3 showed many differences to the well studied fungal enzyme. Firstly, as with the hydroxylase of *B. stearothermophilus* PH24, NADH was used as a cofactor. The enzyme was also very stable, and showed maximum activity at a high temperature. The Michaelis constant for phenol was lower than, and the constant for oxygen comparable to, the figures reported for the fungal enzyme. The oxygen constant may have been expected to be lower, considering the reduced solubility of oxygen at higher temperatures. This may be due to oxygen not being a limiting factor in the normal growth of the organism.

One of the most significant observations with the thermophilic phenol hydroxylase was the inhibition by metal chelators, and the stimulation of activity by metal ions. No

Figure 15.Hammett Plots for Meta and Para Substituted Phenols

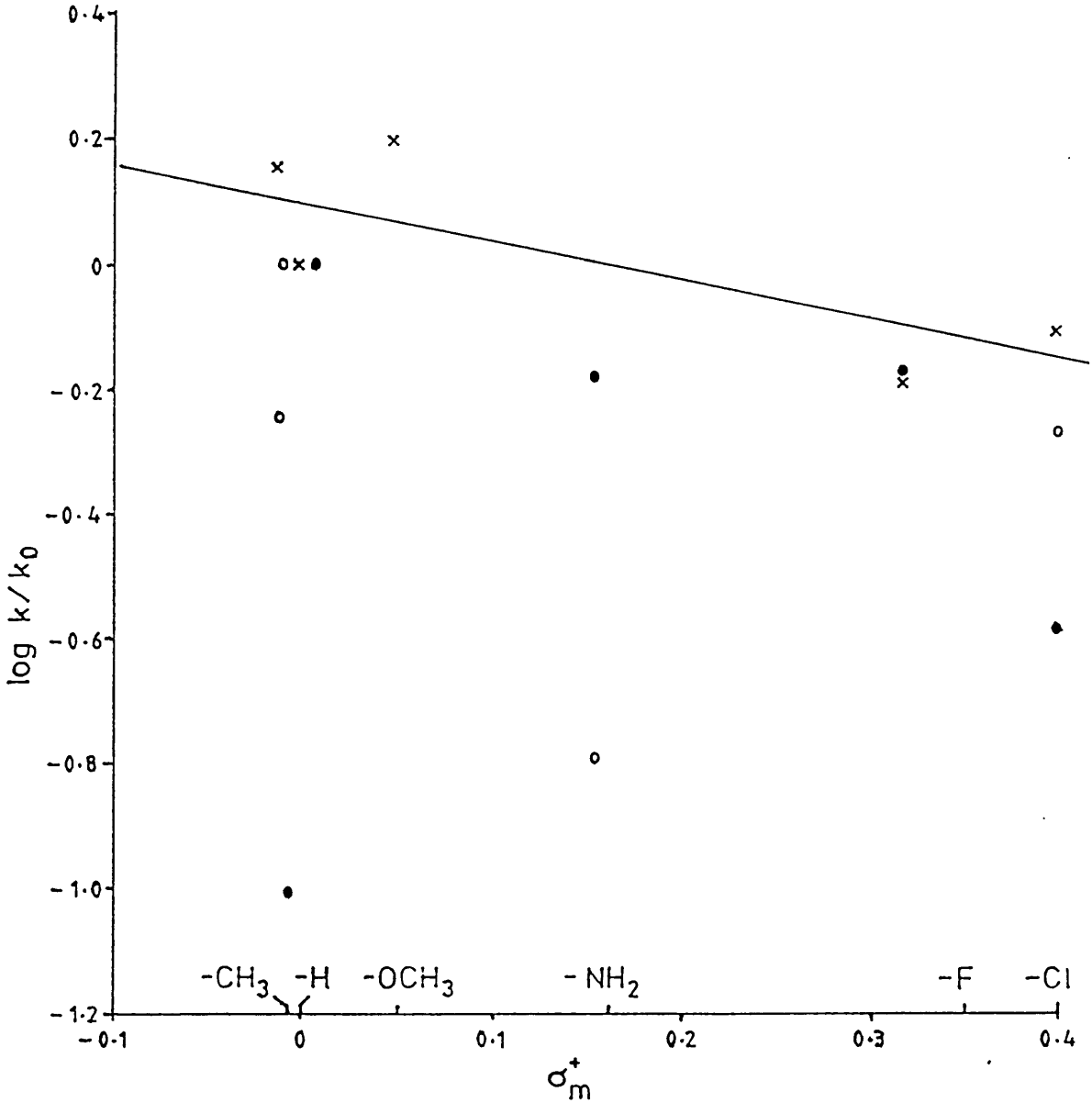
a) Meta $\log K/K_o$ against σ_m



Crosses represent results for B. stearothermophilus, closed circles results for T. cutaneum and open circles results for C. tropicalis. The line shows the line of best fit for the B. stearothermophilus results.

Figure 15.Continued

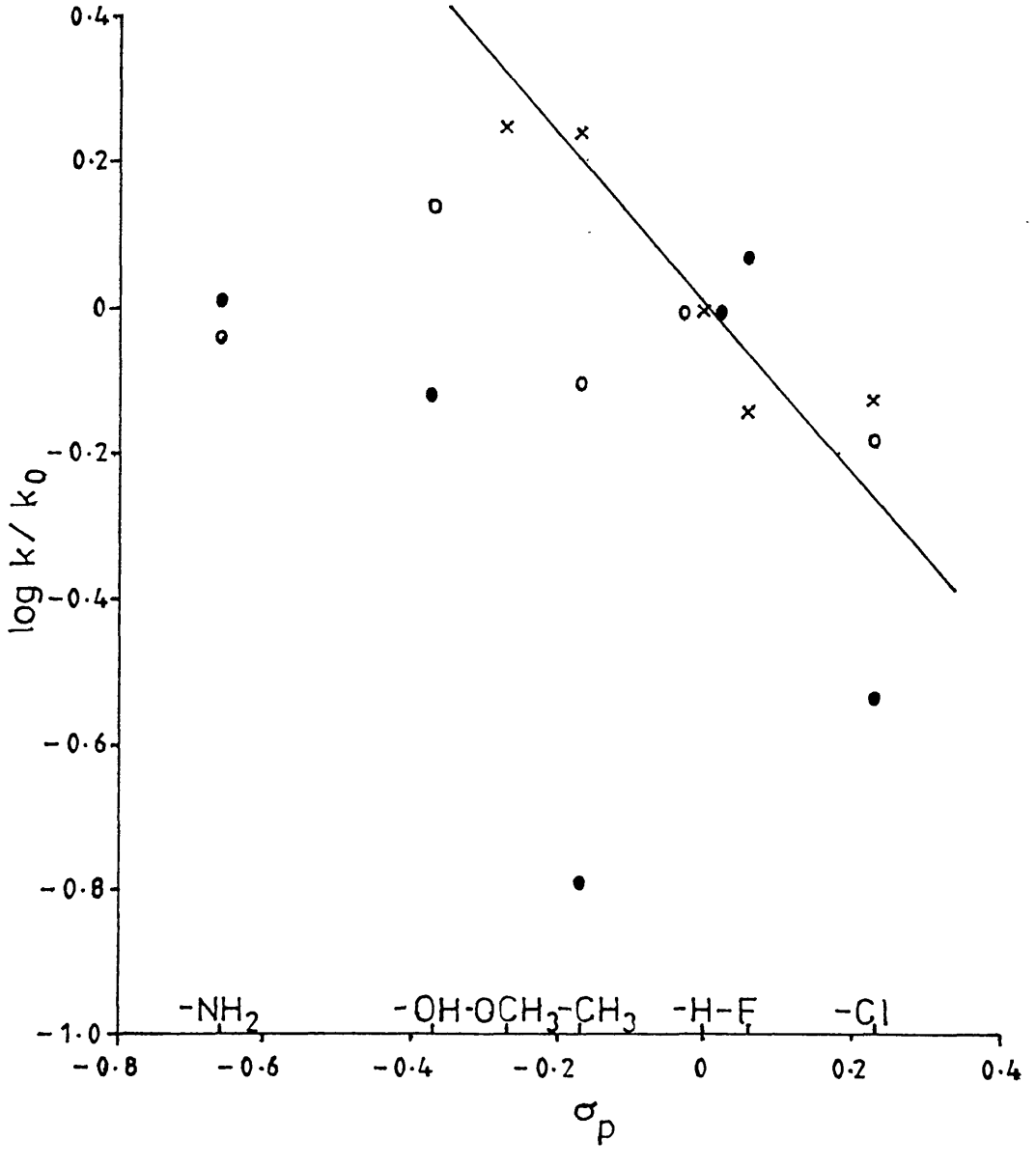
b) Meta log K/K_0 against σ_m^+



Crosses represent results for B. stearothermophilus, closed circles results for T. cutaneum and open circles results for C. tropicalis. The line shows the line of best fit for the B. stearothermophilus results.

Figure 15.Continued

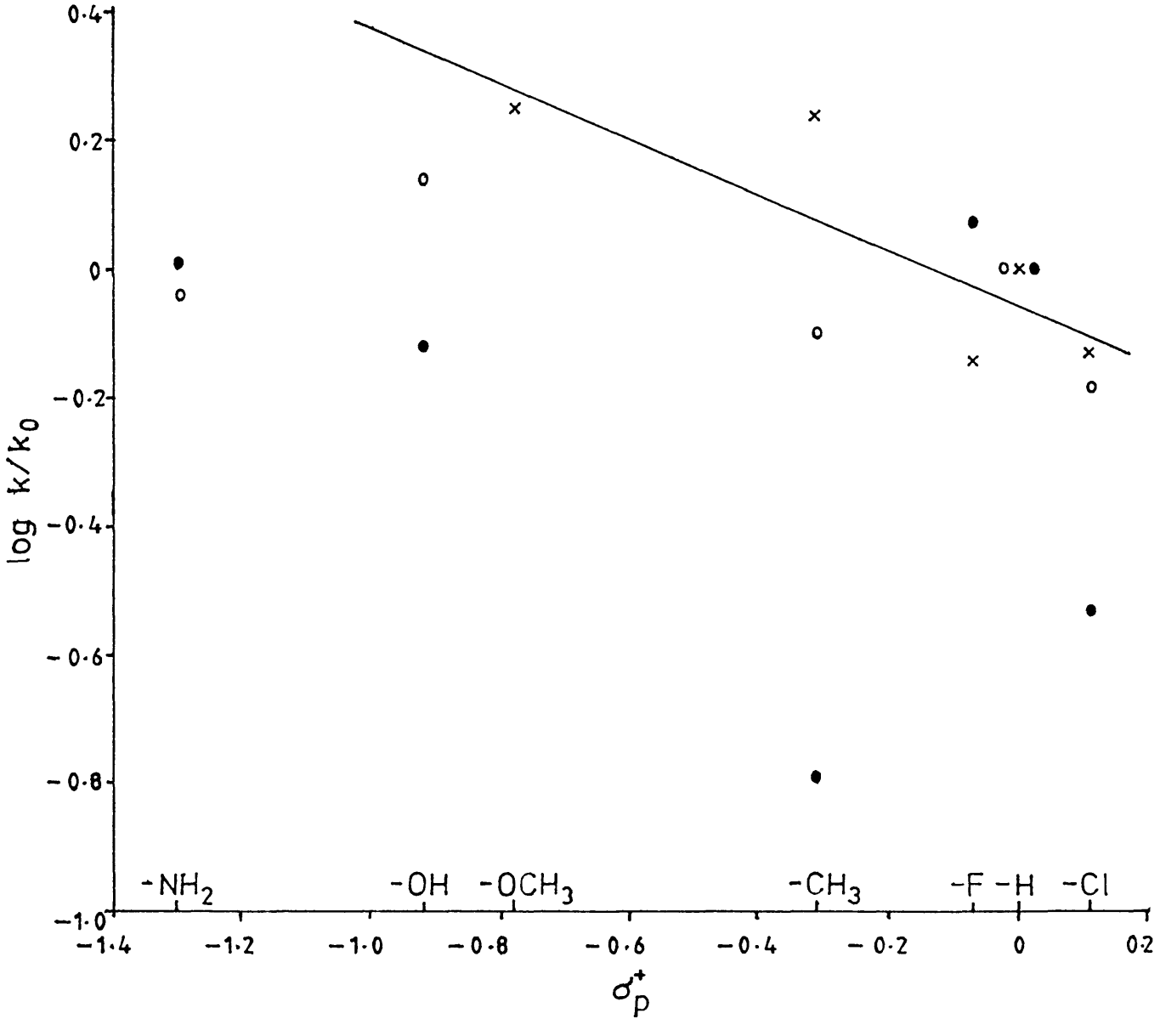
c) Para log K/K_o against σ_p



Crosses represent results for B. stearothermophilus, closed circles results for T. cutaneum and open circles results for C. tropicalis. The line shows the line of best fit for the B. stearothermophilus results.

Figure 15.Continued

d) Para $\log K/K_0$ against σ_p^+



Crosses represent results for B. stearothermophilus, closed circles results for T. cutaneum and open circles results for C. tropicalis. The line shows the line of best fit for the B. stearothermophilus results.

role was proposed for metal ions in the reaction mechanism for the phenol hydroxylase of T. cutaneum.

Finally, substituted phenols affected the reaction rate in a manner that was consistent with inductive effects, little steric hinderance being observed. The electronic effect suggested an electrophilic type reaction.

CHAPTER 7

Properties of Catechol 2:3-Dioxygenase in Cell Free Extracts

Unlike the bacterial phenol hydroxylases, catechol 2:3-dioxygenase (metapyrocatechase) from Pseudomonas putida (formerly arvilla), has been studied in some detail. Observations that the enzyme activity was strongly stabilized in the presence of 10% acetone, enabled the enzyme to be purified and crystallized (Nozaki et al 1963, 1963a). This enzyme was subsequently shown to consist of four identical subunits, giving a total molecular weight of 140,000 Daltons. Each subunit contains one ferrous iron molecule that is directly involved in the reaction centre (Nozaki et al 1968, Nozaki 1979, Nakai et al 1983).

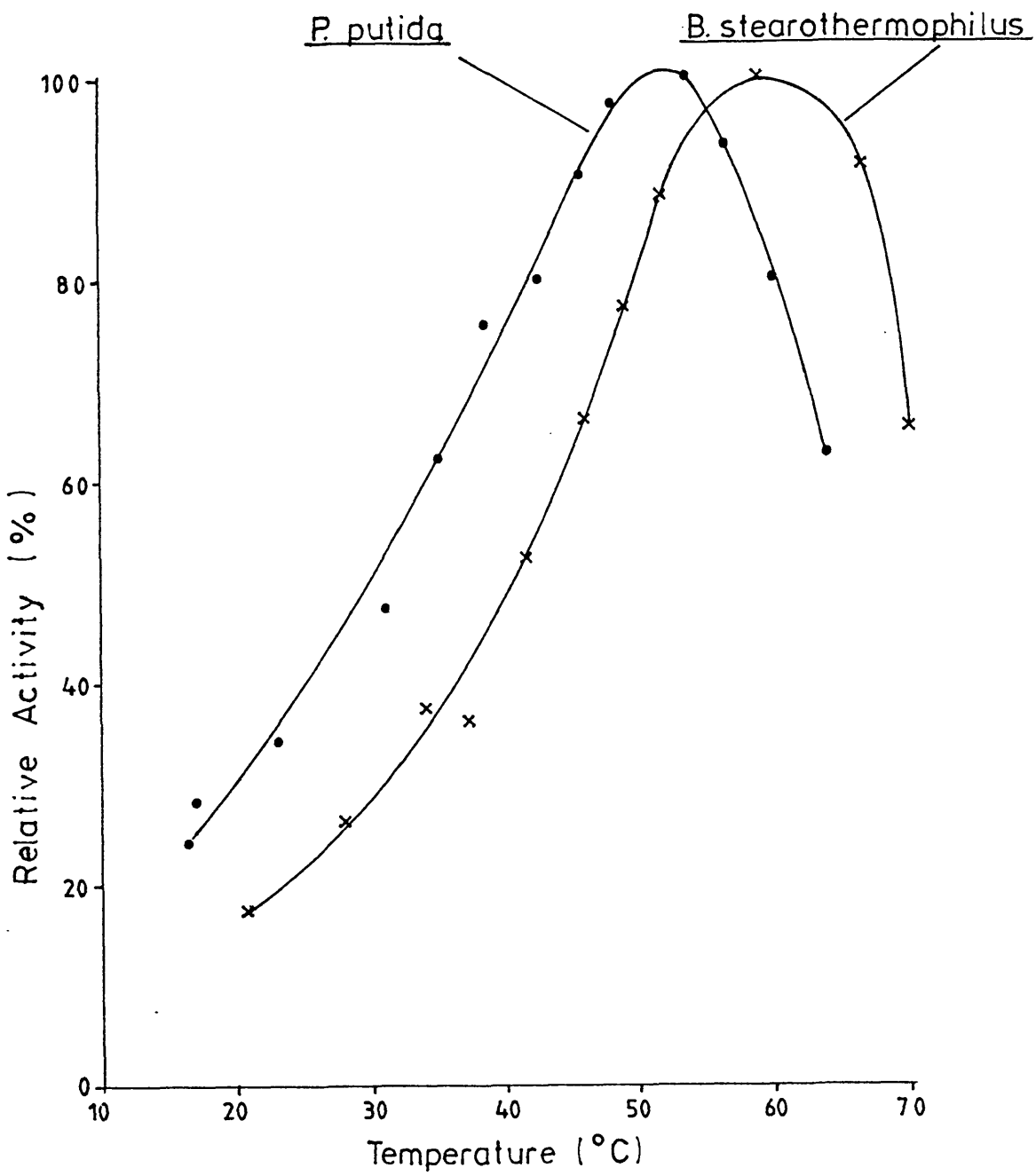
7.1.Stability of Catechol 2:3-Dioxygenase Activity in Cell Free Extracts

Cell free extracts, prepared in the manner described in Chapter 2, displayed catechol 2:3-dioxygenase activity having a half-life of 15 days, when stored at 4°C. No activity was detected if the acetone was omitted from the extracts.

7.2.Effects of Temperature upon the Activity of Catechol 2:3-Dioxygenase

The results of experiments to determine the activity of catechol 2:3-dioxygenase, from B. stearothermophilus IC3 and P. putida mt-2, at different temperatures, are shown in Figure 16. The results in Table 12 show the half-lives of the dioxygenase at different temperatures. Also shown in Table 12 are results for the same enzyme from Pseudomonas putida mt-2

Figure 16. Effects of Temperature upon Catechol 2:3-Dioxygenase Activity



Crosses represent results for B. stearothermophilus IC3 and closed circles results for P. putida mt-2.

The maximum specific activities for the dioxygenases of B. stearothermophilus and P. putida were 1.8 and 7.9 μmols per mg of protein per minute.

Table 12. Half-lives of Catechol 2:3-Dioxygenases

Strain	Temperature (°C)	Half-Life (Mins)
B. stearothermophilus IC3	55	16.0
	60	9.5
	65	6.0
	70	2.3
	75	2.2
P. putida	55	73.8
	63	21.1
	65	18.0
	68	14.7
	71	6.1

(Hill 1987).

7.3.Michaelis Constants for Catechol 2:3-Dioxygenase

The apparent Michaelis constants of catechol 2:3-dioxygenase for oxygen was calculated from Lineweaver-Burke, Haynes and Eadie-Hofstee plots (Figure 17). The constant for the aromatic was again obtained from a Haynes plot (Figure 18). With regard to the Michaelis constant for oxygen, the Lineweaver-Burke, Haynes and Eadie-Hofstee plots gave values of 34.8, 35.1 and 33.8μM respectively. The average value of these plots was 35μM. The apparent Michaelis constant for catechol was 0.32μM.

7.4.The Effects of Inhibitors upon the Catechol 2:3-Dioxygenase Activity

The inhibitors p-chloromercuribenzoate, at a concentration of 0.01mM, and o-phenanthroline, EDTA, azide, cyanide and 2,2'bipyridine at a concentration of 1mM, had no significant effect on catechol 2:3-dioxygenase activity. A slight increase in activity (13%) was observed with the copper chelator diethyldiaminocarbamic acid (1mM), which may have been due to the chelating of interfering metal ions.

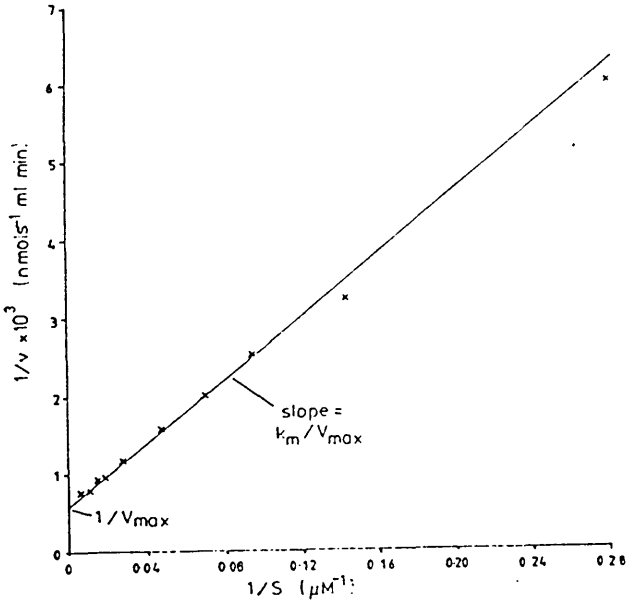
7.5.Discussion

7.5.1.The Stability of Catechol 2:3-Dioxygenase

The thermophilic catechol 2:3-dioxygenase was strongly stabilized by the presence of 10% acetone. This behavior was also observed in the enzyme from P. putida. No dioxygenase

Figure 17.Plots Used to Determine the Michaelis Constant of Catechol 2:3-Dioxygenase for Oxygen

a) Lineweaver-Burke Plot



b) Haynes Plot

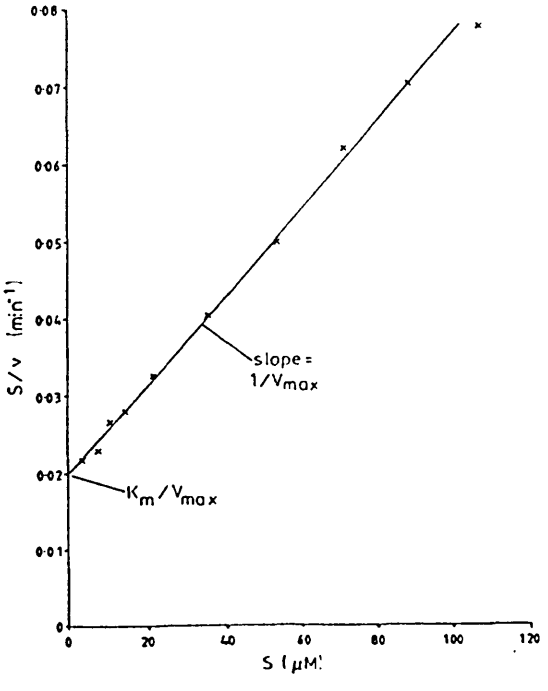


Figure 17.Continued

c) Eadie-Hofstee Plot

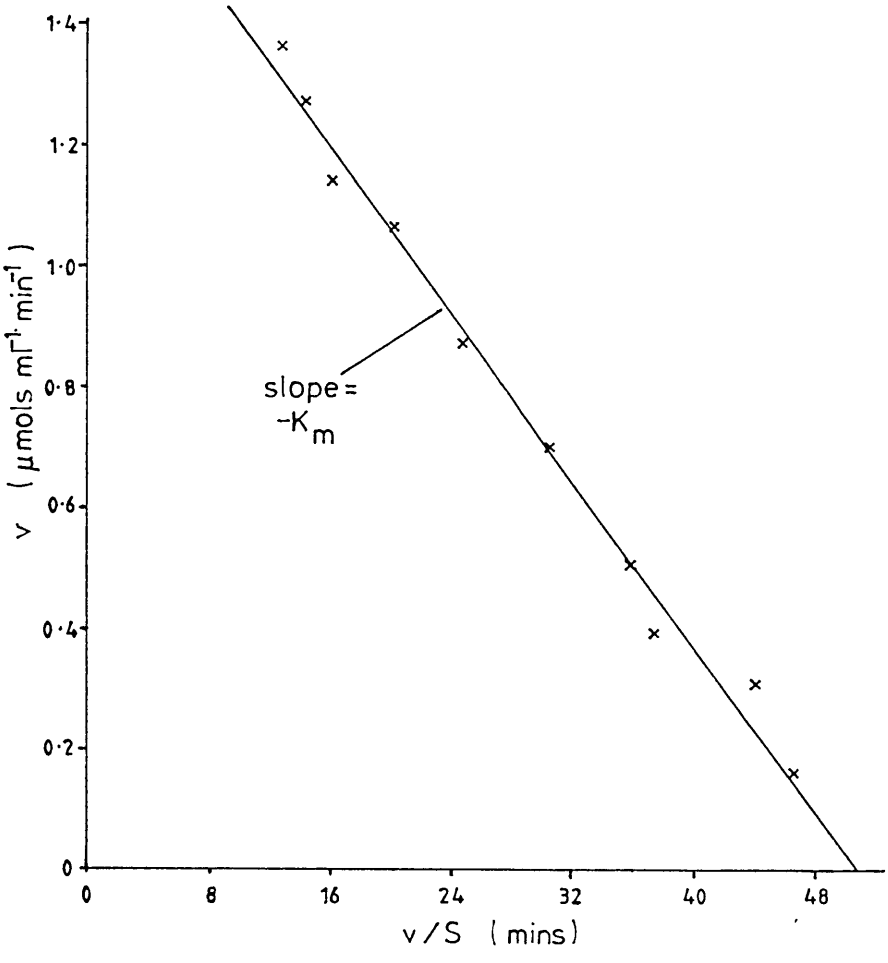
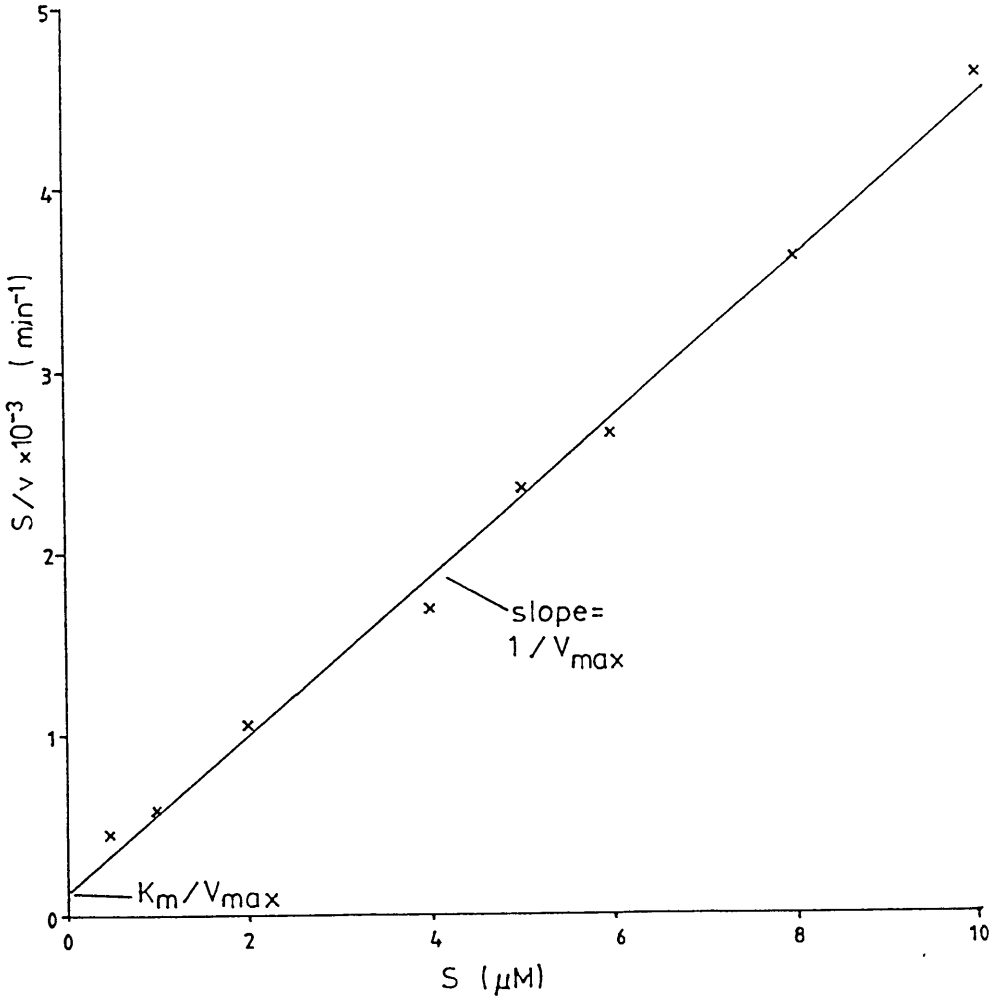


Figure 18.Plot Used to Determine the Michaelis Constant of Catechol 2:3-Dioxygenase for Catechol

Haynes Plot



activity could be detected in cell free extracts prepared without acetone. Whereas crude extracts of P. putida prepared without acetone displayed a catechol 2:3-dioxygenase activity that was highly unstable, over 80% being lost in 20 hours. (Nozaki et al 1963a).

7.5.2.Effects of Temperature upon Catechol 2:3-Dioxygenase Activity

The results in Figure 16 show that the thermophilic catechol 2:3-dioxygenase is active at the growth temperature of 55°C. In fact, maximum activity was observed at the slightly higher temperature of 60°C. The temperature optimum of the catechol 2:3-dioxygenase of P. putida mt-2, 52°C, was only slightly lower than that of the thermophile.

Interestingly, the stability of the thermophilic dioxygenase, at high temperatures, was lower than that of the mesophilic enzyme. The heat resistance of the mesophilic catechol 2:3-dioxygenase has been previously noted (Dagley et al 1960). The presence of any thermophilic proteases, not inhibited by the serine protease inhibitor (PMSF) present in the extracts, may have contributed to the loss of activity in the incubations of the thermophilic dioxygenase.

7.5.3.Michaelis Constants of Catechol 2:3-Dioxygenase

The catechol 2:3-dioxygenase from strain IC3 has apparent Michaelis constants of 0.32uM for catechol, and 35uM for oxygen. As with the thermophilic phenol hydroxylase, the constant for the aromatic was an order of magnitude lower than in the mesophilic enzyme. A figure of 3.0μM was reported

as the Michaelis constant of catechol 2:3-dioxygenase for catechol in Pseudomonas putida (Nozaki et al 1968).

The apparent Michaelis constant of the dioxygenase for oxygen was considerably higher in the thermophile, 35μM compared to 7μM in P. putida (Nakai et al 1983).

7.5.4.The Effects of Inhibitors upon Catechol 2:3-Dioxygenase Activity

The activity of the thermophilic catechol 2:3-dioxygenase was not significantly decreased by any of the inhibitors used. Inhibition was observed with the enzyme from P. putida in the presence of p-chloromercuribenzoate, o-phenanthroline and 2,2'bipyridine, as well as the poor metal chelating nitrogen bases m-phenanthroline, 2-naphthoquinoline and pyridine (Nozaki et al 1963a, 1968). The nitrogen bases, in addition to inhibiting the enzyme competitively, also protected the ferrous ion from oxidation by hydrogen peroxide. The fact that three of these bases were poor chelators of ferrous ions, suggests that this protection/inhibition was due to hydrophobic interactions in the vicinity of the iron molecule (Nozaki et al 1979).

The thermophilic dioxygenase would appear to display different characteristics, when compared to its mesophilic counterpart. The main differences being the lower Michaelis constant for catechol and the higher constant for oxygen. The thermophilic dioxygenase was also resistant to inhibitors that decreased the activity of the enzyme from P. putida.

CHAPTER 8

THE METABOLISM OF RING FISSION PRODUCTS

This chapter concerns the degradation of ring fission products. Cell free extracts (CFE) were prepared in the manner described in Chapter 2, except that no bovine serum albumin was added.

8.1.Metabolism of 2-Hydroxymuconic Semialdehyde

The results in Table 13 show the rates of disappearance of the ring fission products of catechol and methyl catechols, and the effects of NAD on these rates.

8.1.1.Products of 2-Hydroxymuconic Semialdehyde Metabolism

In the absence of NAD, formate, when assayed by both chemical and enzymatic means, was found to be a metabolic product of 2-hydroxymuconic semialdehyde (2HMS).

A 4ml solution of 50mM phosphate buffer (pH7.0), containing 0.5mg/ml of CFE, was incubated with 2HMS for 1 hour at 55°C. During this time, 224nmols of 2HMS (calculated by the decrease in absorbance at 375nm) was metabolised. A total of 180nmols of formate was produced, when assayed by the chemical method described, a yield of 80%.

Formate was detected enzymatically using formate dehydrogenase. A 3ml sample, containing 0.4mg/ml CFE, metabolised a total of 48nmols of 2HMS in the absence of NAD. Forty two nmols of formate were produced, a yield of 88%.

The product of 2HMS degradation, in the presence of NAD, was found to be 4-oxalocrotonate (4OC). Cuvettes were

Table 13.Rates of Disappearance of Ring Fission Products

Ring Fission Product	Cell Free Extract		NADase Treated Cell Free Extract			
			In Absence Of NAD		In Presence Of NAD	
	Rate	%	Rate	%	Rate	%
2-Hydroxymuconic semialdehyde	4.25	91	3.34	83	67.6	100
2-Hydroxy-6-oxo-hepta-2,4-dienoate	4.69	100	4.04	100	4.3	6.3
2-Hydroxy-5-methyl-muconic semialdehyde	1.22	26	1.02	25	57.7	85

Notes:

(1) Rates are expressed as nmols/mg of protein/min.

(2) Extracts were treated with 0.056 units of NADase for 30 minutes at 37°C.

(3) NAD was added to a final concentration of 1mM.

(4) When NADP (1mM final concentration) was added to NADase treated CFE's, the rate of 2HMS disappearance increased to 11.4 nmoles/mg of protein/min, ie. 17% of the rate in the presence of NAD.

incubated at 55°C containing the following reaction mixture in 50mM, pH7.0, phosphate buffer (final concentrations): CFE 0.4mg/ml, NAD 1mM, EDTA 5mM. Fifty nmoles of 2HMS were completely metabolised. Upon addition of 100µl of 10M NaOH, a new peak at a wavelength of 350nm was observed, corresponding to 52nmoles of 4OC, a yield of 104%. EDTA was added as it was found to inhibit the 4-oxalocrotonate decarboxylase activity found in B. stearothermophilus IC3 (Table 14).

The identification of 4OC, as a product of 2HMS metabolism, was further verified by isolating a sample of the product and comparing it with authentic 4-oxalocrotonate. The following mixture was incubated at 55°C until the yellow colouration had disappeared (final concentrations are shown): CFE 0.2 mg/ml, EDTA 5mM, and catechol 100µM made up to 50mls with phosphate buffer (50mM, pH7.0). At the end of the incubation, the solution was acidified to pH1.5 with HCl, and the precipitate removed by centrifugation. The product was then extracted three times into 50mls of ethyl acetate. This was dried with anhydrous magnesium sulphate, and the ethyl acetate removed. The precipitate was dissolved in 1ml of ethanol and 50µl of this solution, added to 950µl of phosphate buffer, gave the UV spectrum shown in Figure 19. Also shown is the spectrum of authentic 4-oxalocrotonate.

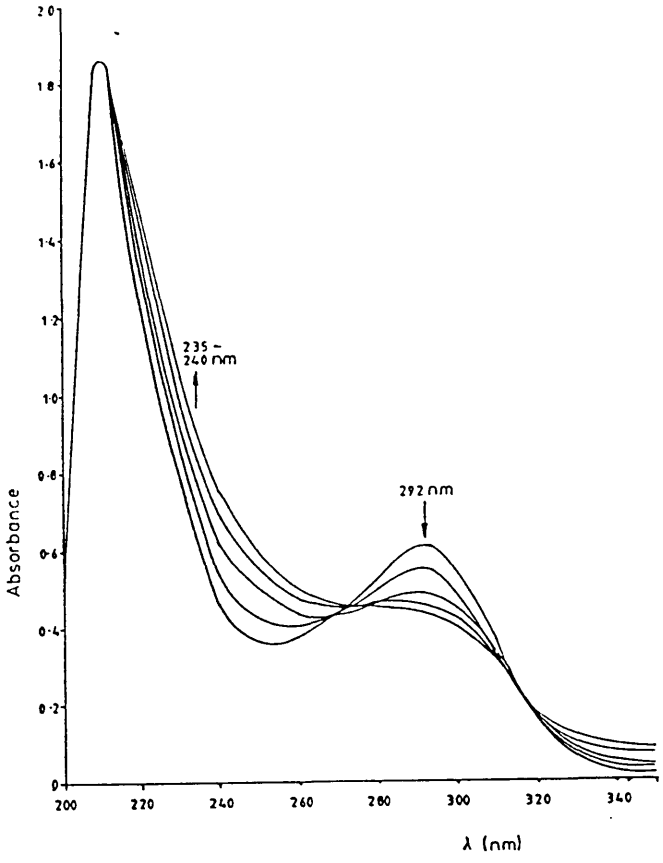
8.2.Metabolism of 4-Oxalocrotonate

8.2.1. 4-Oxalocrotonate Tautomerase

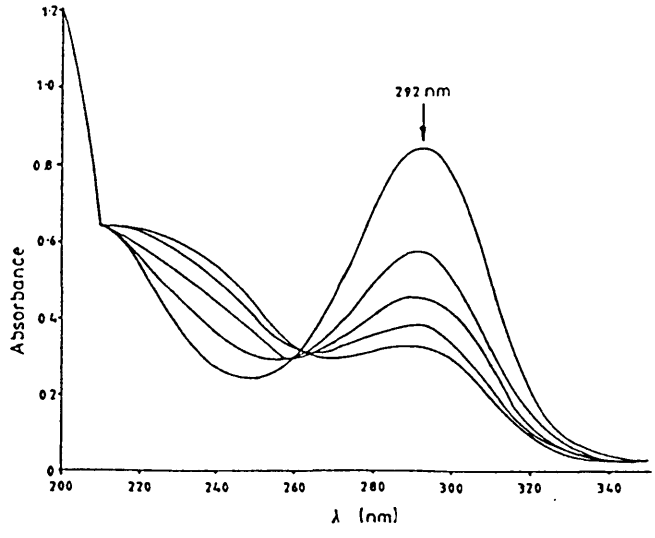
The first step in 4OC metabolism is a tautomerization

Figure 19.U.V. Spectra of Isolated and Authentic 4-Oxalocrotonate

a) Isolated



b) Authentic



between the enol and keto forms of this compound. This reaction occurs spontaneously, but is also catalyzed by cell free extracts.

For 4OC (75μM) in phosphate buffer, the spontaneous rate of tautomerization was 3.3 units/min. No detectable increase in this rate was observed on addition of CFE's derived from glucose grown cells, however, CFE's of phenol grown cells increased the rate of tautomerization by 400 units/mg of protein/min. (Table 14).

8.2.2. 4-Oxalocrotonate Decarboxylase

The activities of the 4OC degrading enzyme, under different conditions, are also shown in Table 14.

A product of the reaction was shown to be 2-oxopent-4-enoate by HPLC analysis (Table 3, conditions 4). In a 10ml phosphate buffer solution, containing 0.25mg/ml CFE and incubated at 55°C, the transient appearance of an intermediate with the same retention time as 2-oxopent-4-enoate was seen. This intermediate was also unstable in alkaline solution.

8.3.Metabolism of 2-Oxopent-4-enoate

The rates of metabolism of 2-oxopent-4-enoate by cell free extracts are shown in Table 15.

A product of the reaction was shown to be 4-hydroxy-2-oxovalerate. A solution (1ml) in 50mM, pH7.0, phosphate buffer, containing 0.5mg/ml CFE, 1mM $MgSO_4$, and 100μl of the 2-oxopent-4-enoate preparation, was incubated at 55°C and the

Table 14.Activities of 4-Oxalocrotonate Metabolizing Enzymes

Enzyme	Additions To Incubations	Cells Grown On: Phenol		Glucose	
		Rate	%	Rate	%
4-Oxalocrotonate Tautomerase	None	400	100	0	0
	EDTA	400	100	ND	-
4-Oxalocrotonate Decarboxylase	None	88.5	100	2.8	3
	EDTA	0	0	ND	-
	$MgSO_4$	963	1090	ND	-

Notes:

(1) ND = Not Determined.

(2) No spontaneous 4OC disappearance was observed in the absence of CFE's.

(3) EDTA was added to a final concentration of 5mM, and $MgSO_4$, 1mM.

(4) Controls performed of solutions containing EDTA and $MgSO_4$, without cell free extracts, showed no significant rate of metabolism above the background rate (Where present).

(5) Activities are expressed in the following units: 4-oxalocrotonate tautomerase, absorbance units (at 292nm) per mg of protein per min; 4-oxalocrotonate decarboxylase, nmols per mg of protein per min.

Table 15.Metabolism of 2-oxopent-4-enoate and 4-Hydroxy-2-oxovalerate

Enzyme	Addition To Incubations	Cells Grown On: Phenol Rate	Phenol %	Glucose Rate	Glucose %
2-Oxopent-4-enoate Hydratase (Units/mg/min)	None	0.170	100	0	0
	$CuSO_4$	0.244	144	ND	-
	$MgSO_4$	6.15	3600	ND	-
4-Hydroxy-2-oxovalerate Aldolase (nmoles/mg/min)	None	3.83	100	3.39	89
	NAD	3.58	93	ND	-

Notes:

(1) ND = Not Determined.

(2) $CuSO_4$ was added to a final concentration of 0.1mM. $MgSO_4$ and NAD were added to final concentrations of 1mM.

(3) Controls performed with $CuSO_4$, $MgSO_4$ and NAD, without cell free extracts, showed no significant rate of dissapearance above the background rate.

production of 4-hydroxy-2-oxovalerate monitered by HPLC (Table 3, conditions 5). After 1 minute, the valerate reached its maximum concentration (22.1μM), and was thereafter slowly metabolised.

8.4.Metabolism of 4-Hydroxy-2-oxovalerate

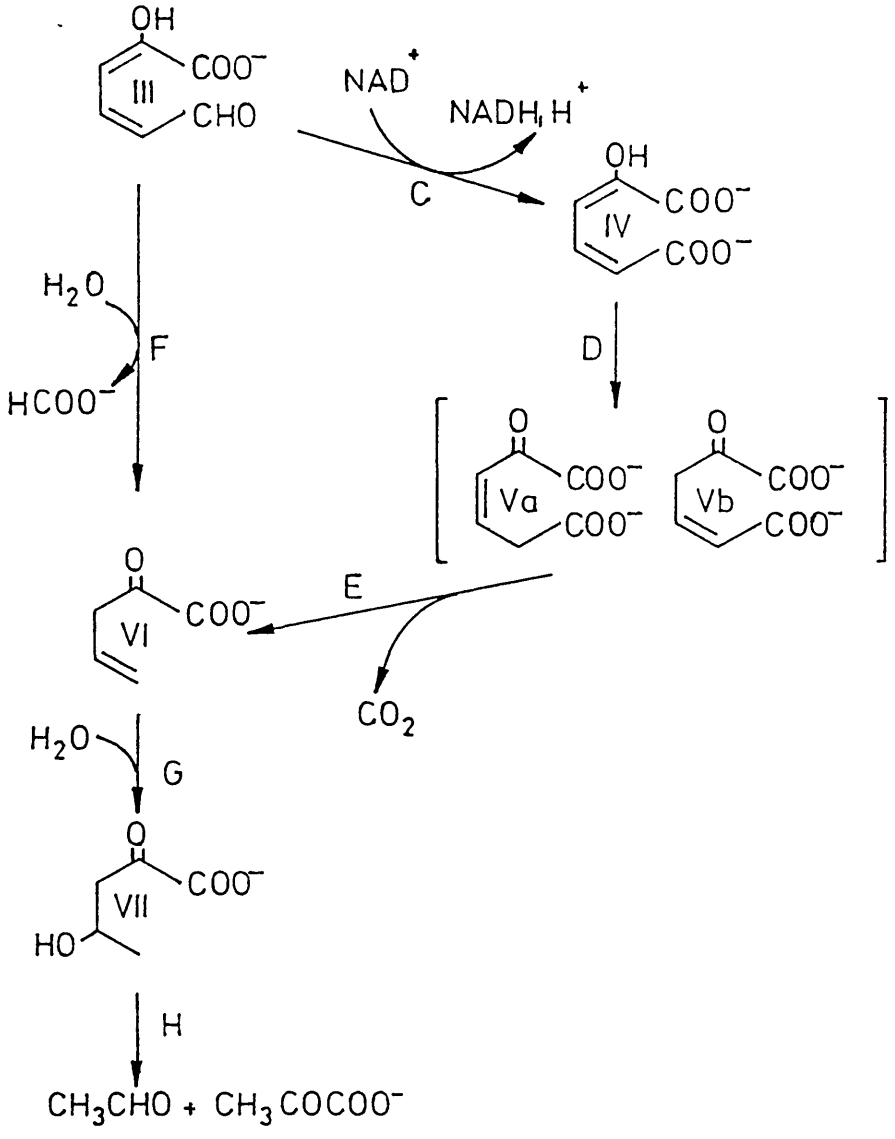
The rates of metabolism of 4-hydroxy-2-oxovalerate by cell free extracts are also shown in Table 15.

Pyruvate and acetaldehyde were both shown to be metabolic products of the valerate. Cell free extract solutions, in 50mM pH7.0 phosphate buffer, containing 500μM 4-hydroxy-2-oxovalerate, were incubated at 55°C for 30 minutes. The incubations were performed in sealed microfuge tube to prevent the escape of acetaldehyde (boiling point, 21°C). The solutions were then reassayed for valerate, and the pyruvate and acetaldehyde produced were determined. Three separate 30 minute incubations showed the following average results: valerate used, 78.3 nmols /ml; pyruvate formed, 37.7nmols /ml; acetaldehyde formed, 22.3nmols/ml. The results have been corrected for any acetaldehyde and pyruvate detected in controls. The control mixtures were identical to those above, but the 30 minute incubation at 55°C was omitted.

8.5.Discussion

The above results are consistent with the pathway shown in Figure 20. Similar pathways have been delineated for the

Figure 20. Degradation of Ring Fission Products



Letters represent enzymes as follows: C, 2-hydroxymuconic semialdehyde dehydrogenase; D, 4-oxalocrotonate tautomerase; E, 4-oxalocrotonate decarboxylase; F, 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase; G, 2-oxopent-4-enoate hydratase; H, 4-hydroxy-2-oxovalerate aldolase. Numerals represent chemicals as follows: III, 2-hydroxymuconic semialdehyde; IV, 4-oxalocrotonate (enol form); V, 4-oxalocrotonate (Keto form); VI, 2-oxopent-4-enoate; VII, 4-hydroxy-2-oxovalerate.

degradation of 2-hydroxymuconic semialdehyde, and its methylated derivatives, in pseudomonads (Murray et al 1972, Hopper and Taylor 1975) and Azotobacter species (Sala-Trepat and Evans 1971).

8.5.1.Metabolism of 2-Hydroxymuconic Semialdehyde

Two different enzymes appear to be responsible for the degradation of 2HMS and its methyl substituted intermediates. One of the enzymes, which is not dependant on NAD for its activity, gives formate as a reaction product. This is consistent with the reaction of a hydroxylase enzyme, named 2-hydroxy-6-oxohepta-2,4-dienoate hydroxylase (HODH) because of the greater activity observed towards the ring fission product of 3-methyl catechol.

The other product of the reaction of HODH upon 2HMS has been shown to be 2-oxopent-4-enoate (Dagley and Gibson 1965). This is assumed to be the other product in B. stearothermophilus IC3, but has not been directly demonstrated as such.

The activity of the hydrolase of strain IC3 towards ring fission products, compared to previous reports, is shown in Table 16.

All the enzymes shown in Table 16 display maximum rates with 2-hydroxy-6-oxohepta-2,4-dienoate as a substrate. However, with the enzyme from strain IC3, the relative activities towards 2HMS and 2-hydroxy-5-methylmuconic semialdehyde are much higher than with the hydrolases from B. stearothermophilus PH24 and the mesophiles.

Table 16.Relative Activities of 2-Hydroxy-6-oxohepta-2,4-dienoate Hydrolases.

Strain	Source	Substrate 2HMS	HOD	2HMMS
B. stearothermophilus				
Strain IC3	1	83	100	25
Strain PH24	2	26	100	6
P. putida				
NCIB 10015	3	7	100	3
NCIB 9865	3	3	100	1
mt-2	4	6.9	100	3.2
A. eutrophus 345				
HODH I	5	31	100	3
HODH II	5	12	100	5

Notes:

(1) The sources are as follows: 1, this thesis; 2, Buswell 1974; 3, Bayly and Di Barardino 1978; 4, Duggleby and Williams 1986; 5, Bayly *et al* 1987.

(2) Abbreviations are as follows: 2HMS, 2-hydroxymuconic semialdehyde; HOD, 2-hydroxy-6-oxohepta-2,4-dienoate; 2HMMS, 2-hydroxy-5-methylmuconic semialdehyde.

(3) The data for the bacilli are from crude extracts. All other data are from purified enzymes.

The alternative route for the metabolism of ring fission products is an NAD mediated dehydrogenation. This enzyme, 2-hydroxymuconic semialdehyde dehydrogenase (HMSD), quantitatively converts 2HMS into 4-oxalocrotonate (IV). This enzyme also attacks 2-hydroxy-5-methylmuconic semialdehyde, although the reaction product has not been determined. Very little activity was seen towards HOD. These results are similar to those observed with HMSD from several Azotobacter species (Sala-Trepat and Evans 1971, 1971a) and P. putida mt-2 (Murray et al 1972).

Previous workers have suggested that the hydrolytic route, when the oxidative route is present, is utilized in the metabolism of compounds via HOD, the oxidative route being used for the degradation of 2HMS and 2-hydroxy-5-methylmuconic semialdehyde. Where no HMSD activity is present, 2HMS and its methyl substituted analogues can be metabolised by the hydrolytic route (Bayly and Dagley 1969).

The HODH of the TOL plasmid has a high affinity for HOD, and a relatively low affinity for the other two substrates. The latter substrates were only metabolised hydrolytically if they were allowed to accumulate to sufficiently high concentrations, and it is unlikely that they were hydrolysed in normal metabolism (Harayama et al 1987).

In contrast HMSD will only metabolise 2HMS and 2-hydroxy-5-methylmuconic semialdehyde, and organisms such as Azotobacter, that only display low, non inducible levels of

the enzyme, cannot degrade compounds via HOD (Sala-Trepat and Evans 1971).

The role of these enzymes in the metabolism of cresols in strain IC3 was not investigated beyond the degradation of ring fission products.

8.5.2. 4-Oxalocrotonate Metabolism

4-Oxalocrotonate was identified as a reaction product in the degradation of catechol by Nishizuka et al (1962). Subsequently, it was shown to be degraded by a tautomerization from the enol to the keto form (Va, Vb), followed by a decarboxylation, yielding 2-oxopent-4-enoate (VI) (Sala-Trepat and Evans 1971). This appears to be the pathway utilized by B. stearothermophilus IC3.

Fig 20

The results in Table 14 show that strain IC3 also possesses an inducible 4-oxalocrotonate tautomerase (OCTase) activity. The fact that this enzyme is required for growth, despite the rapid rate of spontaneous tautomerization, has been verified by a number of workers. Mutants defective in the tautomerase activity are unable to grow, or grow very poorly, on compounds usually metabolised via the oxalocrotonate pathway (Wigmore et al 1974, Barbour and Bayly 1980, Harayama et al 1987).

The exact structure of the OCTase product (Va, Vb) has not been conclusively demonstrated, although the favoured product from chemical considerations, which is also the most susceptible to decarboxylation, is Va (Sparnins et al 1974).

The 4-oxalocrotonate decarboxylase (OCD) of strain IC3,

as in previous reports (Sala-Trepat and Evans 1971), is stimulated by Mg^{2+} ions, and inhibited by EDTA. The product of the reaction is 2-oxopent-4-enoate.

8.5.3. 2-Oxopent-4-enoate Metabolism

The results in Table 15 show that strain IC3 possesses a 2-oxopent-4-enoate hydratase (OEH) activity, that is slightly stimulated by cupric ions and highly stimulated (36 fold) by magnesium ions. Collinsworth *et al* (1973) found that a similar enzyme in *P. putida* was not stimulated by magnesium ions, but was stimulated five fold by manganese ions. Another enzyme from *P. putida*, which catalysed the same reaction but showed important differences to the enzyme described by Collinsworth *et al*, was stimulated two fold by magnesium ions, and seven fold by manganese ions (Lampel 1979). The effect of manganese ions upon the OEH activity of strain IC3 was not investigated.

The slight stimulation of activity by cupric ions is in contrast to the complete inhibition observed with the OEH activity in *P. putida* (Lampel 1979). The presence of cupric ions in the CFE's of strain IC3, at concentrations of greater than 0.1mM, appeared to cause precipitation of the substrate, and also altered its absorption spectrum. The cupric ions (0.1mM) did not effect the assay of OEH activity by HPLC analysis.

The product of the OEH activity was found to be 4-hydroxy-2-oxovalerate. The OEH, and the following aldolase, were found to be stereospecific in other organisms.

Collinsworth *et al* (1973) concluded that L-(S)-4-hydroxy-2-oxovalerate was the biologically active enantiomer in *P. putida* NCIB 10015. The stereospecificity of these enzymes in *B. stearothermophilus* IC3 has not been investigated.

8.5.4. 4-Hydroxy-2-oxovalerate Metabolism

Strain IC3 metabolized 4-hydroxy-2-oxovalerate by an aldolase enzyme, yielding pyruvate and acetaldehyde. The yields of the products were significantly less than 100%. This may have been due to the background disappearance of the valerate at 55°C, further metabolism of the products, and loss of the aldehyde due to its low boiling point. However, pyruvate and acetaldehyde accumulated in sufficiently high amounts to suggest that they were the reaction products. A similar aldolase reaction has been reported previously (Dagley and Gibson 1965).

The metabolism of the valerate was not stimulated by NAD. An NAD dependent enzyme that degraded this compound, with acetate and pyruvate as products, has been described in a *Pseudomonas* species (Nishizuka *et al* 1962).

8.5.5.Production of Degradative Enzymes

Table 17 shows the activity of enzymes required for the metabolism of ring fission products, in cell free extracts of strain IC3 grown on phenol and glucose.

Most of the degradative enzymes were only present when the organism was grown on phenol. However, the HOD hydrolase and 4-hydroxy-2-oxovalerate aldolase, were present in significant amounts in CFE's from glucose grown cells. The

Table 17.Activities of the Degradative Enzymes when Grown on Different Carbon Sources

Enzyme	Growth Substrate			
	Phenol		Glucose	
	Rate	%	Rate	%
HOD Hydrolase (2)	3.34	100	2.1	63
HMS Dehydrogenase (2)	64.3	100	0	0
4OC Tautomerase (1)	400	100	0	0
4OC Decarboxylase	88.5	100	2.8	3
2OP Hydratase (1)	0.17	100	0	0
4HOV Aldolase	3.83	100	3.39	89

Notes:

(1) Activities are expressed as nmoles/mg of protein/min, except for the tautomerase and hydratase, which are expressed as absorbance units, at 292 and 262nm respectively, per mg of protein per min.

(2) The figures for the hydrolase and dehydrogenase were obtained with NADase treated CFE's, using 2-hydroxymuconic semialdehyde as a substrate.

non inducible production of HODH was noted in Azotobacter species (Sala-Trepat and Evans 1971a). These strains would not grow on compounds which would have produced 2-hydroxy-6-oxohepta-2,4-dienoate. Growth on compounds via HOD was observed in pseudomonads that possessed an inducible HODH activity (Murray et al 1972). B. stearothermophilus PH24 would grow on all of the cresol isomers, but the levels of HODH activity, compared to the HMSD activity in this organism, were much higher than those observed in strain IC3.

The non inducible nature of the aldolase enzyme has been observed in Azotobacter species (Sala-Trepat and Evans 1971), but not P. putida mt-2 (Murray et al 1972).

These results explain why, under certain conditions (Chapter 10), p-cresol is used as a carbon source. However, under similar conditions, o-cresol is also used and m-cresol used less efficiently. If o- and m-cresol were proceding via 3- and 4-methylcatechol respectively, as is the case in B. stearothermophilus PH24 (Buswell 1975), the reverse case to that observed would be expected in strain IC3. The reason for the results obtained remains unclear.

CHAPTER 9

EFFECTS OF OTHER CARBON SOURCES UPON AROMATIC METABOLISM

9.1.Glucose Repression Effects

When cells of B. stearothermophilus IC3 were grown in the presence of glucose and either phenol or benzoate, diauxic growth was observed, the aromatic compound not being utilized as a carbon source until the glucose was exhausted.

When strain IC3 was grown with ethanol and phenol, the initial rate of phenol disappearance was very low (0.4µmols/ml in the first 10 hours). However, phenol and catechol stimulated oxygen uptakes in washed cells, were present in much higher levels than was observed with cells grown on glucose and phenol (Table 20). No aromatic stimulated oxygen uptakes were observed with cultures grown on ethanol or glucose alone.

In cultures using two carbon sources, no non-aromatic compound was found that would allow normal rates of aromatic metabolism in the first growth phase. Raising the concentration of Brain Heart Infusion and Yeast Extract also resulted in growth with repression of the initial aromatic stimulated oxygen uptakes.

Based on the results in Chapters 5-7, it is not an unreasonable assumption, that the phenol and catechol stimulated oxygen uptakes, are the results of phenol hydroxylase and catechol 2:3-dioxygenase activities.

9.2.The Production of Derepressed Mutants

It had been observed that on completion of growth,

cells of strain IC3 rapidly lost phenol hydroxylase and catechol 2:3-dioxygenase activity. A rapid drop in the activities of these enzymes was also seen in whole cells after 1-2 hours. This produced a potential problem with further work, as it had been intended to produce mutants def-icient in the degradative pathway, and use washed whole cells to accumulate metabolites. The loss of enzyme activities in non-growing cells made this impractical.

An alternative was to use growing cells of mutants to accumulate products. However, the only carbon source that would allow reasonable expression of the initial oxygenases, ethanol, did not allow the metabolism of phenol. For this reason, mutants were sought that were "derepressed", i.e. did not exhibit glucose repression.

9.2.1. Selection for Derepressed Mutants by Growth on a Non Inducing Intermediate in the Presence of Glucose

The first strategy that was attempted was a two step procedure with the following rationale.

The first stage was to produce the enzymes responsible for aromatic degradation constitutively. This was done by selecting for growth on DL-allylglycine (Kunz *et al* 1981). In *Pseudomonas putida*, it was suggested that this compound was oxidized to 2-oxopent-4-enoate, a *meta* pathway intermediate, that did not induce the enzymes necessary for its metabolism.

The second stage of the strategy was to produce mutants of the above, that were also derepressed, by selecting for growth on DL-allyl glycine, as a main carbon and nitrogen

source, in the presence of glucose. Glucose represses the production of the enzymes necessary for aromatic (and therefore DL-allyl glycine) metabolism, but will not permit growth on nitrogen free media.

Mutagenesis, using NG, was performed on strain IC3, and the cells enriched in liquid media containing 25mM DL-allyl glycine. After several days, samples were plated out on plates also containing 25mM DL-allyl glycine. After several experiments, only one colony was isolated that would grow on allyl glycine, but not on no carbon controls. However, this mutant, labelled strain IC3.M5, would only utilize allyl glycine on solid, and not liquid, media. The growth characteristics of strain IC3.M5 are summarized in Table 18.

Experiments using the mutant in the second stage of the strategy were unsuccessful.

9.2.2.Selection for Derepressed Mutants with Glucose Analogues

It is now apparent that glucose repression, in a range of organisms, is exerted by lowering the intracellular levels of cyclic 3',5'-adenosine monophosphate (cAMP). In lowering the intracellular cAMP concentrations, glucose only needs to be phosphorylated, not extensively metabolised. Therefore, glucose analogues that can be phosphorylated, but not completely metabolised, can exert glucose repression. (Pastan and Adhya 1976).

A high proportion of mutants that grow on a carbon source that is normally repressed by glucose, in the presence of certain glucose analogues, are derepressed. Examples of

Table 18.Growth Substrates for Strain IC3.M5

Carbon Source	Media (Note 3)	Strain IC3		Strain IC3.M5	
		Liquid Media	Solid Media	Liquid Media	Solid Media
None	1	-	-	-	-
Glucose	1	+	+	+	+
Phenol	1	+	+	+	+
DL-Allyl Glycine	1	-	-	-	+
None	2	-	-	-	-
Glucose	2	-	-	-	-
Phenol	2	-	-	-	-
DL-Allyl Glycine	2	-	-	-	+

NOTE:

(1) + indicates growth, - indicates no growth

(2) Glucose and phenol were used at a concentration of 5mM, and DL-allyl glycine at 25mM.

(3) Medium 1 is that described in Chapter 2. Medium 2 (nitrogen free) was the same, with ammonium chloride being replaced by an equal weight of potassium chloride.

analogues that have been used in this way are α-methyl glucoside (Pastan and Adhya 1976), 2-deoxyglucose (Zimmermann and Scheel 1977) and, more recently, 5-thio-D-glucose (Egilsson et al 1986).

In experiments with strain IC3, α-methyl glucoside did repress phenol utilization, but in controls the organism also showed slow growth on this compound. 2-deoxyglucose (2DG), at a concentration of 2.5mM, inhibited growth on phenol and benzoate, allowed normal growth on glucose and did not itself act as a carbon source.

9.2.2.1.Production of Derepressed Mutants

Cells of strain IC3 were mutagenized with UV light, and enriched in a medium containing 2.5mM 2DG and 5mM phenol. After several days, 4 colonies were isolated from separate flasks that showed evidence of growth, by plating onto a similar solid medium.

One strain, labelled B. stearothermophilus IC3.M49, grew on the following compounds: phenol, benzoate, and both in the presence of 2DG. Slow but positive growth was observed on glucose and glycerol, and the mutant would not grow on 2DG alone.

9.2.2.2.Metabolism of Aromatic Compounds by Strain IC3.M49

A summary of its growth characteristics of strain IC3.M49, compared to strain IC3, is shown in Table 19. Aromatic stimulated oxygen uptakes of the mutant and parental strains are shown in Table 20.

Table 19.Growth Characteristics of Strain IC3.M49

		Strain IC3	Strain IC3.M49
Growth Substrates:			
Glucose		+	+
Glycerol		+	+
Phenol		+	+
Benzoate		+	+
2DG/Phenol		-	+
2DG/Benzoate		-	+
2DG		-	-
Growth Yields:			
Glucose	g/Mol	43.1	13.3
	g/g	0.239	0.074
Phenol	g/Mol	28.6	30.4
	g/g	0.304	0.323
Benzoate	g/Mol	31.2	35.2
	g/g	0.256	0.288
Doubling Times:			
Glucose		1.1	4.1
Phenol		2.4	1.8
Benzoate		6.8	4.8

NOTE:

(1) + indicates growth, - indicates no growth. Doubling times are expressed in hours.

Table 20. Oxygen Uptakes by Strains IC3 and IC3.M49

Growth Substrates	Aromatic Added	IC3		IC3.M49	
		nmols/mg /min	%	nmols/mg /min	%
Phenol	Phenol	593	100	703	100
	Catechol	984	100	849	100
Benzoate	Benzoate	136	100	178	100
	Catechol	310	100	219	100
Phenol and Glucose	Phenol	43	7	382	54
	Catechol	19	2	639	75
Benzoate and Glucose	Benzoate	45	33	168	94
	Catechol	65	21	230	105
Phenol and Ethanol	Phenol	125	21	ND	-
	Catechol	788	80	ND	-

Notes:

(1) ND=not determined

(2) Aromatics added to a final concentration of 1mM

(3) Percentages represent the percentage activity of the same strain when grown on the aromatic carbon source alone.

9.3.Discussion

9.3.1.Glucose Repression Effects

From the results mentioned in the text and shown in Table 20, a number of conclusions can be drawn:

1. The expression of the initial oxygenases is repressed in the presence of glucose by over 90%.

2. Ethanol represses the expression of the phenol stimulated oxygen uptake by 79%.

3. Although ethanol represses the levels of the phenol stimulated oxygen uptakes, the catechol uptakes are only slightly affected. This would imply that the genes encoding these two activities are not located on the same operon. The genes for phenol hydroxylase and catechol 2:3-dioxygenase are located on separate operons in *Pseudomonas aeruinosa* (Ribbons 1970), *P. putida* (Wigmore and Bayly 1977, Bayly *et al* 1977) and *Alcaligenes eutrophus* (Hughes *et al* 1984).

4. The lack of catechol 2:3-dioxygenase repression in ethanol/phenol cultures indicates that the gene encoding the dioxygenase is either not directly repressed, or if it is, not to the same extent as the gene encoding phenol hydroxylase. The lack of dioxygenase activity in glucose grown cells may have been caused by the absence of an inducer, due to the lack of phenol metabolism.

5. Phenol, or one of its metabolites, induces the enzymes necessary for its degradation.

The lack of phenol metabolism by cells grown on phenol and ethanol, despite the presence of the enzyme necessary for its degradation, has not been further investigated. It may be the case that the phenol hydroxylase is allosterically inhibited by ethanol, or one of its products. Alternatively, the hydroxylase, which has a relatively high Michaelis constant for oxygen (Chapter 7), may not be able to compete with the respiratory enzymes for this gas. The respiratory enzymes of B. stearothermophilus have previously been described as having a high affinity for oxygen (Coultate and Sundaram 1975).

Attempts at directly demonstrating a role for cyclic AMP were inconclusive. Cyclic AMP (50μM) did not derepress phenol metabolism in strain IC3, when cells were cultured in 2DG and phenol. A similar method derepressed amylase synthesis in a B. stearothermophilus strain (Srivastava and Mathur 1984).

9.3.2.The Production of Derepressed Mutants

The first procedure used in an attempt to produce derepressed mutants was not a success. This procedure relied on certain assumptions: firstly, that allyl glycine was metabolised to 2-oxopent-4-enoate by B. stearothermophilus; that this compound could not induce the enzymes necessary for its degradation; and finally, that all the enzymes responsible for aromatic degradation were encoded in the same operon, or at least induced by a common regulatory protein.

Little can be concluded from the results obtained,

except that the technique for growing mutants on allyl glycine as a sole carbon and nitrogen source appeared to work.

The reason for strain IC3.M5 only utilizing allyl glycine on solid media is unclear. However, mutants of *P. putida*, defective in phenol metabolism, constitutively produced the enzymes for *meta* cleavage only on solid media (Wigmore and Bayly 1977).

The second procedure used in the isolation of derepressed mutants was successful. From the results shown in Tables 19 and 20, the following points are apparent:

1. The rates of growth and cell yields on glucose are reduced in the mutant IC3.M49. The tendancy of regulatory mutants, isolated in this way, to show reduced growth on glucose, has been observed in *Saccharomyces cerevisiae* (Zimmermann and Scheel 1977).

It is likely that strain IC3.M49 is a regulatory mutant, and not a mutant defective in glucose phosphorylation. It should also be pointed out that the remaining 3 isolates that grew on 2DG and phenol, were completely unable to utilize glucose. These mutants presumably were able to metabolise phenol, in the presence of 2DG, because they were unable to partially metabolise the sugar analogue.

2. The growth rates and cell yields, on aromatic compounds, were higher in the mutant than in the parental

strain. This may have been the result of the increased enzyme activites observed.

3. The phenol and benzoate stimulated oxygen uptakes, when grown on the aromatic alone, are increased in the derepressed mutant. These comparisons are made despite the different growth rates of the parent and mutant strains. A faster growth rate may lower specific enzyme activities by a dilution effect. However, higher activities were seen in faster growing cultures. More meaningful comparisons of enzyme levels could be made if the strains were growing at the same rates, i.e. in a chemostat.

Derepressed mutants, showing increased activities of enzymes that are usually derepressed, have also been observed in Saccharomyces cerevisiae (Zimmermann and Scheel 1977).

4. Catechol stimulated oxygen uptakes are decreased in the mutant strains growing on aromatic compounds. This is interesting, as it contrasts with the effect on phenol hydroxylase, and could be a result of a faster growth rate. This supports the conclusion drawn in Section 9.3.3.1 that the genes for the two enzymes are encoded in separate operons, and the catechol dioxygenase is not directly repressed by glucose.

5. In cultures of strain IC3.M49 grown on an aromatic compound and glucose, the glucose repression effect is reduced.

6. At no time was any reversion of strain IC3.M49

observed, despite the strong selection pressures for normal growth on glucose. It may be that the lesion in this mutant is a deletion. Deletions are frequently produced if UV light is used as a mutagen (Schwartz and Beckwith 1969).

Overall, the following points can be made: Firstly, the genes encoding the two oxygenases are located in separate operons. The genes are induced by phenol, or one of its metabolic products, although the two genes do not necessarily share the same inducer. Finally, expression of the phenol hydroxylase gene is repressed in the presence of glucose. Catechol 2:3-dioxygenase is also repressed in the presence of non-aromatic carbon sources, but not to the same extent as the hydroxylase gene. This could be due to direct glucose repression, or the lack of an inducer due to the low hydroxylase activity.

The organisation and regulation of the enzymes responsible for phenol degradation via a *meta* cleavage pathway have been studied in some detail in other organisms. The inducers of *meta* cleavage, in *Pseudomonas putida* PpU, were phenols and a number of substituted analogues. Catechol was not an effector for *meta* cleavage, but would act as an inducer for the *ortho* cleavage pathway. (Feist and Hegeman 1969).

In *P. aeruginosa* T1, the genes encoding phenol hydroxylase and catechol 2:3-dioxygenase, were encoded on separate operons. The hydroxylase was induced by phenol, cresols and catechol, whereas the dioxygenase was only

induced by phenol and catechol (Ribbons 1970).

The genes encoding catechol 2:3-dioxygenase and the further *meta* cleavage enzymes of *P. putida* NCIB 10015, were all located on the same operon. The gene for phenol hydroxylase was encoded separately (Bayly *et al* 1977). All of the enzymes were induced by phenol and cresols (Sala-Trepat *et al* 1972, Bayly *et al* 1977). In *P. putida* however, the two operons appeared to be controlled by one protein, which, in the absence of an effector, negatively controlled the *meta* cleavage genes, and in the presence of an effector, positively controlled the phenol hydroxylase gene (Wigmore *et al* 1977). A similar system was also observed in *Alcaligenes eutrophus* 335 (Hughes and Bayly 1983).

It is probable that strain IC3.M49 is a regulatory mutant. However, there is a possibility that the derepression is a result of defective glucose metabolism. Whatever the cause, strain IC3.M49 has the desired phenotype, i.e. it metabolises aromatic comounds in the presence of another carbon source.

CHAPTER 10

BASIC GENETIC EXPERIMENTS

10.1.The Production of Mutants Defective in Aromatic Metabolism

Mutants of Pseudomonas species, defective in aromatic metabolism, have previously been enriched by exposure to halogenated aromatic intermediates (Wigmore and Ribbons 1981). Using a similar method for B. stearothermophilus IC3, a number of mutants, defective in phenol metabolism, were obtained after selection on halogenated phenols. Cells, that had been treated with NG, were incubated in flasks containing 2.5mM o-chlorophenol or p-fluorophenol. After 2 days, samples were plated out onto a glycerol containing medium, and phenol defective colonies selected by replica plating. Approximately 90% of the survivors after incubation with p-fluorophenol, and 50% of the survivors with o-chlorophenol, were unable to grow on phenol. These were all confirmed as mutants defective in phenol metabolism by culturing in liquid media.

Two flasks containing p-fluorophenol yielded 30 mutants (strains IC3.M9-IC3.M38), and one flask containing o-chlorophenol 7 mutants (strains IC3. M39-IC3.M45). A further 2 mutants were isolated after UV mutagenesis by enrichment on p-fluorophenol (strains IC3.M50 and IC3.M51).

None of the above mutants would metabolise phenol, even partially, when used as a sole carbon source. Neither, unlike the parent strain, would mutants utilize phenol when it was supplied in conjunction with benzoate. All of the defective strains would grow on benzoate.

Two mutants chosen at random, IC3.M39 and IC3.M40, were tested for reversion. A NG reversion test proved positive, and spontaneous reversion frequencies of 3.1×10^{-6} (IC3.M39) and 5.0×10^{-7} were later calculated.

Using a similar procedure to that above for benzoate grown cells, with enrichment in the prsence of o-, m-, or p-fluorobenzoate, or o, or m-chlorobenzoate, failed to give any mutations defective in benzoate metabolism.

No phenol or benzoate defective mutants were isolated when a D-cycloserine/penicillin G enrichment procedure was used, following UV mutagenesis with strain IC3, or NG mutagenesis with strain IC3.M49.

10.2.Experiments to Detect the Presence of Plasmids

The genes encoding the enzymes responsible for aromatic degradation, in some organisms, have been shown to be plasmid bo-rne (See Chapter 1 for examples). However, no evidence was found for either the existence of plasmid DNA, or the encoding thereupon of the genes involved in aromatic metabolism, in strain IC3. Plasmids were not visible on gels of DNA prepared by the method of Eckhardt (1978), and attempted heat and acridine orange curing revealed no strains defective in phenol metabolism. Thirteen colonies isolated after "heat curing", and 200 after "acridine curing", all grew on phenol.

10.3.Growth on Substituted Phenols

The growth of strain IC3 on cresols was investigated

following the observation that methyl substituted phenols and catechols were metabolised by the initial oxygenases present in cell free extracts (Chapters 6 and 7).

It was originally thought that the lack of growth on cresols may have been due to the production of non-metabolisable intermediates at some further stage in the pathway. To test this hypothesis, cells of *B. stearothermophilus* IC3 were grown in the presence of phenol and one of the cresol isomers. With the *o*- and *p*- isomers, the cresol was completely metabolised (Figure 21), yielding cell mass in approximately equal amounts to phenol. However, *m*-cresol did not appear to be used as quickly, or as efficiently, as the other isomers. Nevertheless, these results suggest that at least two of the cresols, and their intermediates, were fully metabolised.

From the results shown in Figure 21, assuming that phenol was assimilated with a yield of 29 g/mol in the presence of cresols, the following yields for *o*- and *p*-cresols can be calculated: *o*-cresol = 33.9g/Mol (0.314g/g) and *p*-cresol = 34.7g/Mol (0.321g/g). These yields are slightly better than those of 28.6g/Mol (0.304g/g) obtained with phenol.

Another hypothesis that would explain the lack of growth on cresols, was that they were unable to induce the enzymes necessary for their metabolism. If this was the case, then it should have been possible to select for mutants, growing on cresols, that were altered in their ability to

Figure 21.Metabolism of Cresols in the Presence of Phenol

a) Phenol only

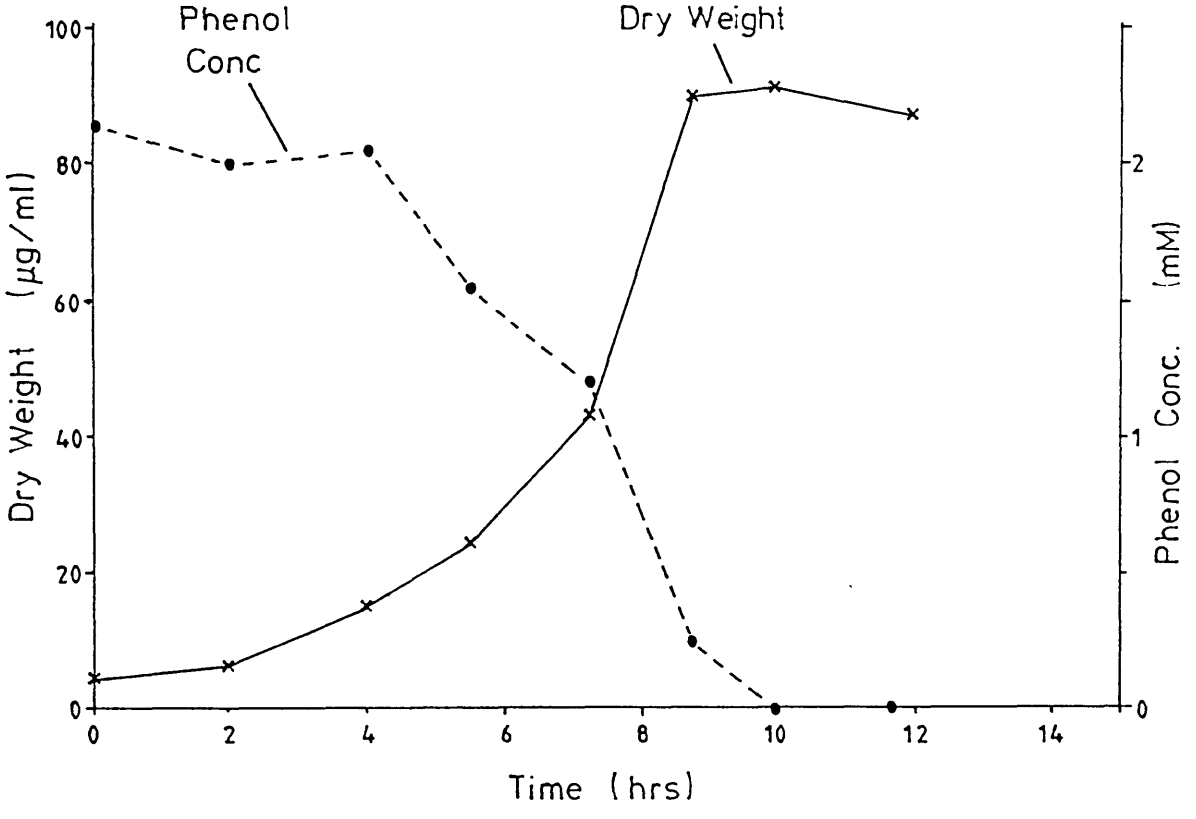


Figure 21.Continued

b) Phenol and o-cresol

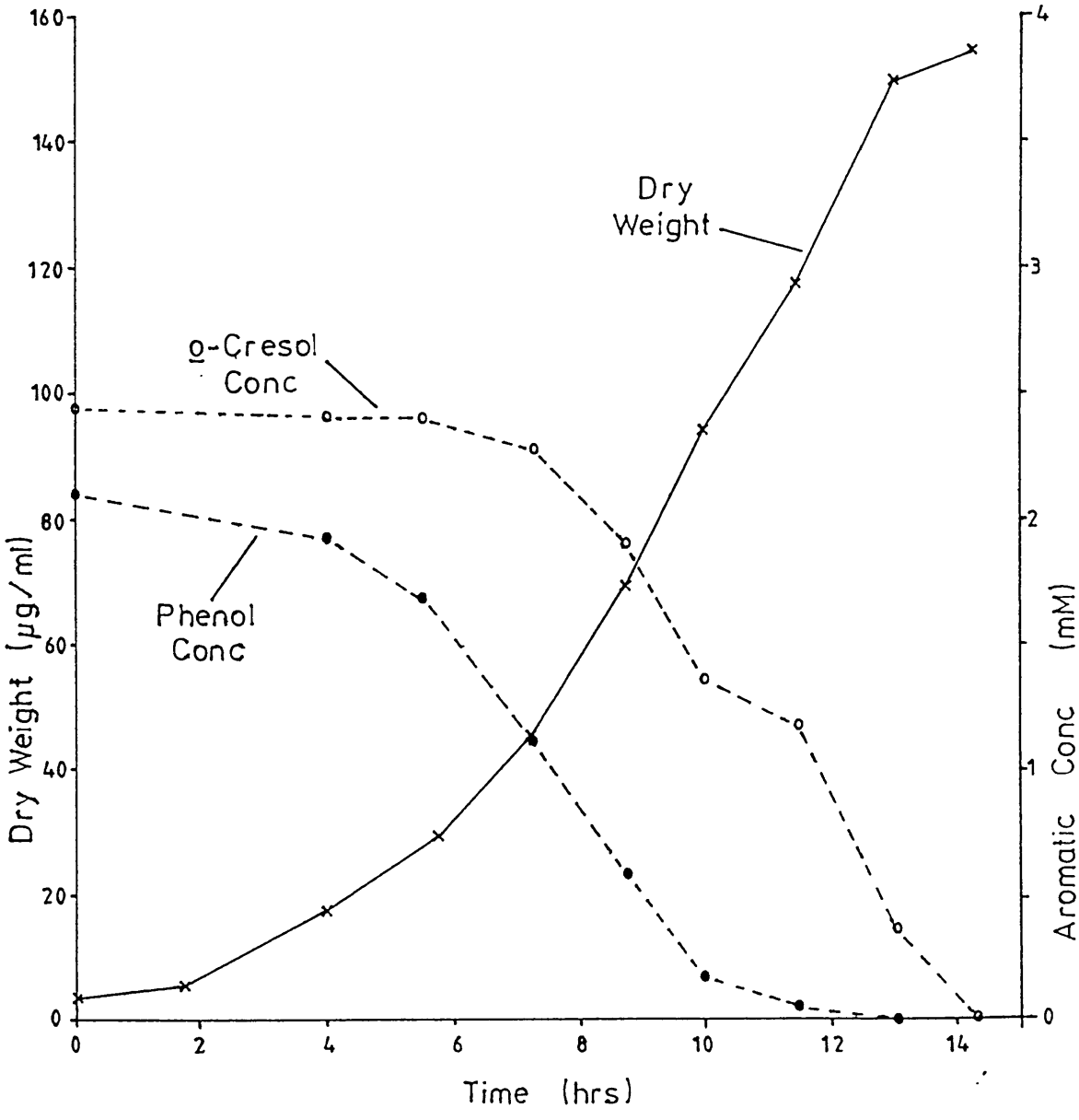


Figure 21.Continued

c) Phenol and m-cresol

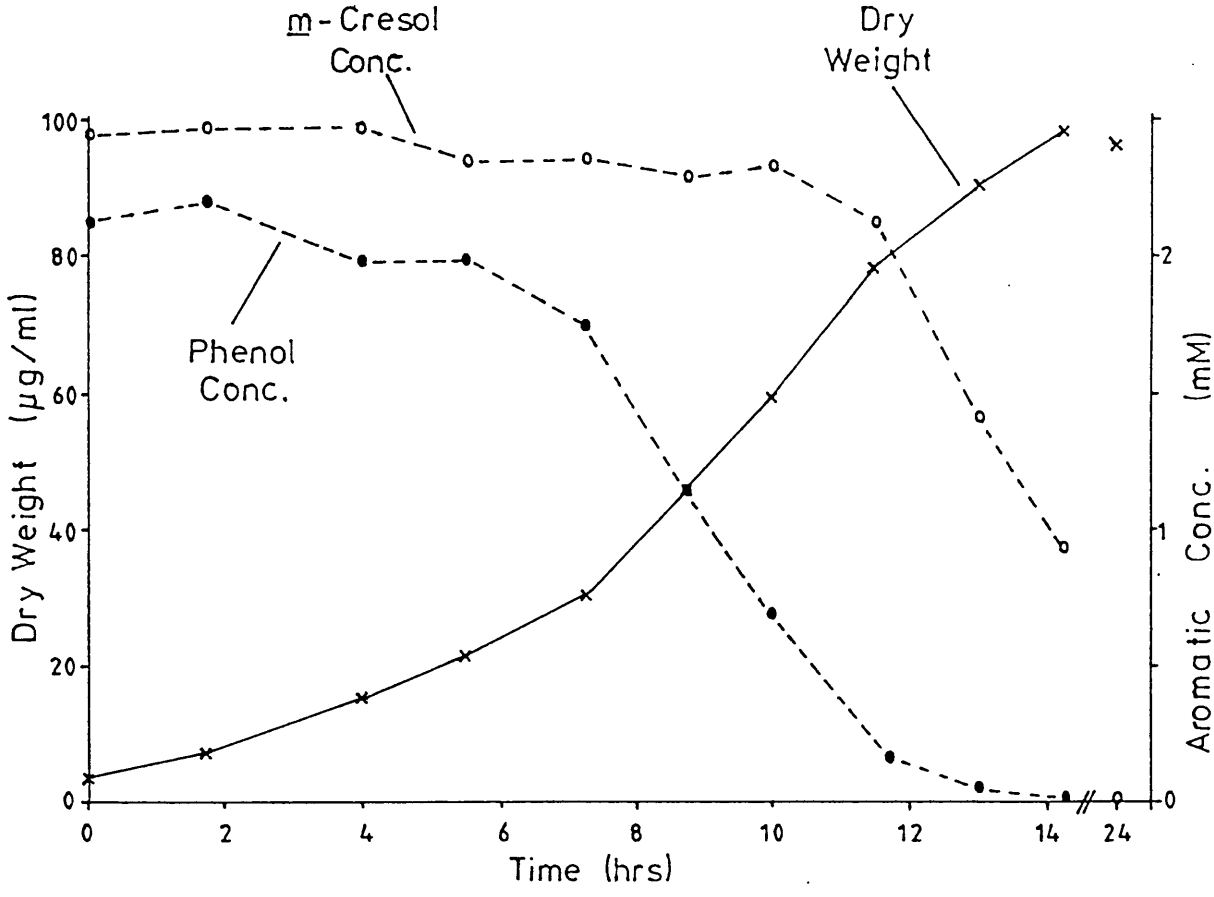
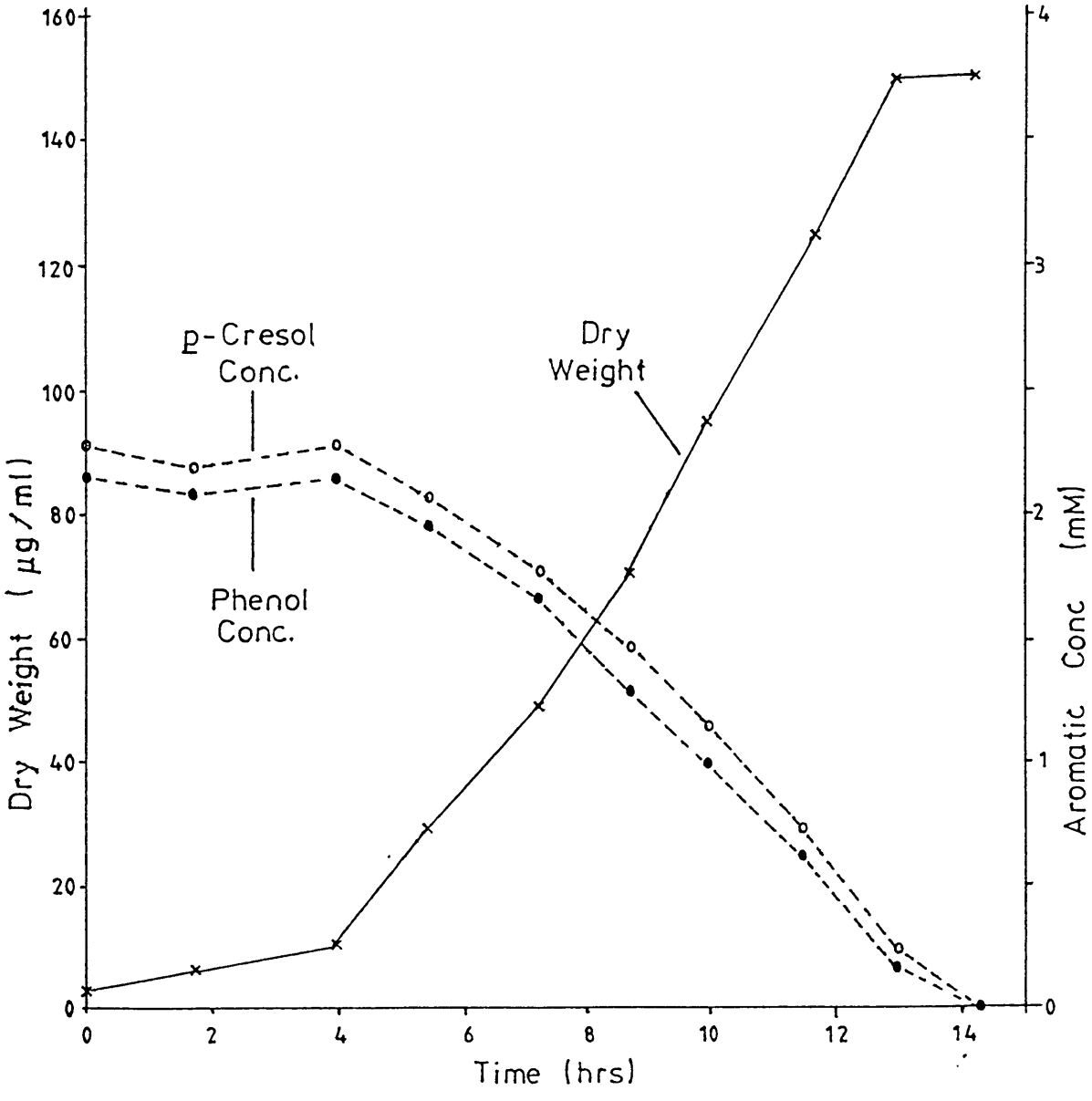


Figure 21.Continued

d) Phenol and p-cresol



induce the phenol metabolising enzymes, producing them constitutively, or with a different range of inducers. However, mutagenesis of the strain with NG, UV light or ICR191, followed by enrichment on o-cresol, failed to produce any mutants that would grow on the cresol as a sole carbon source. This result indicated that the inability to grow on cresol was not a result of a failure to induce the necessary enzymes. This was confirmed when it was discovered that when any of the cresols were used as a carbon source in conjunction with ethanol, a compound that on its own did not induce any aromatic stimulated oxygen uptakes, the aromatic was completely metabolised.

Following the discovery that cresol isomers were metabolised in the presence of phenol, other substituted phenols were supplied in a similar manner. The results of these experiments are shown in Table 21.

10.4.Discussion

10.4.1.Mutants Defective in Aromatic Metabolism

All of the mutants isolated, that were defective in aromatic metabolism, were lacking a functional phenol hydroxylase. No mutants were isolated that were defective at a further stage in the pathway. Similar results were also obtained by Ribbons (1970), when all the mutants of *Pseudomonas aeruginosa* T1 defective in phenol/cresol metabolism, were found to be lacking phenol hydroxylase activity. It was suggested at the time, that this was due to the pseudomonad having an isofunctional pathway for catechol

Table 21.The Metabolism of Substituted Phenols in the Presence of Phenol

Substituted Phenol	Growth	Phenol Metabolism	Substituted Phenol Metabolism
o-cresol	+	+	+
m-cresol	+	+	+
p-cresol	+	+	+
o-chlorophenol	+	+	+
m-chlorophenol	-	-	-
p-chlorophenol	-	-	-
o-fluorophenol	-	ND	ND
m-fluorophenol	-	ND	ND
p-fluorophenol	-	ND	ND
o-methoxyphenol	+	+	-
m-methoxyphenol	w	w	-
p-methoxyphenol	w	w	-
o-nitrophenol	-	-	w
m-nitrophenol	-	-	-
p-nitrophenol	-	-	-

Notes:

(1) Growth and metabolism are denoted by: + positive; - negative; w weak; ND not determined.

(2) The experiment was performed with 40ml liquid cultures, containing 5mM phenol and 1mM substituted aromatic.

(3) The aromatic concentrations were measured after 0, 8, 20, 44, and 100 hours by HPLC analysis (Table 2, conditions 2 and 3).

(4) No growth was observed with any of the substituted aromatics alone at concentrations of 1, 2 and 5mM.

metabolism. This may be the case for strain IC3, and has been observed in the phenol degrading pathway of Alcaligenes eutrophus 345 (Hughes et al 1984).

No mutants defective in benzoate metabolism were obtained, despite the fact that the o-, m- and p-fluoro substituted analogues of benzoate all inhibited growth. Preliminary experiments, with benzoate grown whole cells, suggest that the substituted benzoates were not metabolised, which may explain why the enrichment procedure failed.

10.4.2.The Presence of Plasmids

There was no evidence for the presence of plasmids, or the encoding upon plasmids, of genes involved in aromatic degradation in strain IC3. However, the work involved was not extensive, and the "heat curing" experiment also needs repeating, as only 13 colonies were originally screened.

Antibiotic resistant plasmids have previously been isolated from thermophilic Bacilli (Bingham et al 1979,1980, Imanaka et al 1981, Hoshino et al 1985)), but these have all been of a relatively small size.

More recently, techniques have been developed for plasmid manipulation in thermophilic Bacilli (Imanaka et al 1982, Imanaka 1983, Imanaka and Aiba 1986), as well as alternative methods of genetic analysis such as protoplast fusion (Chen et al 1986). This may provide a means of genetic analysis in strain IC3, where conventional techniques have yielded little information.

10.4.3.Growth on Substituted Phenols

The reason for the lack of growth of strain IC3 on cresols is unclear. It is not the result of an inability to metabolise methylated intermediates, or to induce the enzymes necessary for growth. It would be useful to confirm the latter point by growing the organism in a chemostat, with a cresol as a sole carbon source after initial growth.

With regard to the results shown in Table 21, it was interesting to see that o-chlorophenol was completely metabolised, and o-nitrophenol partially so. This suggests that the phenol is being dechlorinated at some stage in its metabolism, since the degradation of halogenated aromatic compounds usually produces toxic products. This has even been used as a method for selecting mutants def-icient in aromatic metabolism (Wigmore and Ribbons 1981). It should be noted however, that o-chlorophenol was successfully used for the selection of mutants in B. stearothermophilus.

The enzyme most likely to be responsible for this dechlorination is the initial hydroxylase, since chlorocatechols inhibit enzymes by chelating ferric ions. Also, the ring cleavage product of 3-chlorocatechol irreversibly inhibits catechol 2:3-dioxygenase (Knackmuss 1983).

Alternatively, the catechol 2:3-dioxygenase may have a high Michaelis constant for chlorocatechols, and therefore, the unsubstituted catechol may be preferentially metabolised.

To date, all organisms that successfully degrade

halogenated catechols do so by ortho cleavage, not by the meta cleavage route observed in strain IC3. (Knackmuss 1983).

Overall, the following conclusions about the metabolism of aromatic compounds by thermophiles were made. Firstly, a large proportion of thermophilic Bacilli were able to utilize aromatic compounds as sole carbon sources. One of these strains, B. stearothermophilus IC3, degraded phenol to pyruvate and acetaldehyde via the meta fission of catechol. Both branches of the meta pathway were present. The route involved has previously been observed in mesophiles, however, significant differences were seen in the stability of the enzymes, their activity at different temperatures, the effect of metal ions upon activity and the Michaelis constants of dioxygenases for aromatic compounds.

The genes encoding the initial oxygenases involved in phenol metabolism were located on separate operons. The production of these oxygenases was induced by phenol or one of its metabolic products and the production of phenol hydroxylase was inhibited in the presence of glucose.

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APPENDIX

This appendix concerns the identification of strain IC2 and IC3 by the National Collection of Industrial and Marine Bacteria, Torry Research Station, 135 Abbey Road, Aberdeen AB9 8DG.

Strain IC2

This strain was identified as a *Bacillus* species and had the following characteristics:

Cell Morphology: Gram variable; spores formed; non motile.

Colony Morphology (24 hours): irregular; semi-translucent; granular; raised; off white; 2mm diameter.

Growth Temperatures: growth was observed at 50 and 55°C, but not at 45, 60 or 65°C.

Biochemical Characteristics: catalase positive; oxidase (Kovacs) negative: no gas or acid produced from growth on glucose.

Strain IC3

This strain was identified as a *Bacillus stearothermophilus* strain, atypical in growing at pH5.7. It appeared to be a member of "Group 1" as described by Walker and Wolf (1971).

Cell Morphology: gram negative; spores present (eliptical or cylindrical, distended, terminal/subterminal); no intracellular globules.

Colony Morphology (24 hours): round; regular; entire; smooth; white; opaque; low convex; 1mm diameter.

Growth Temperatures: growth was observed at 50, 55, 60, 65 and 70°C, but not at 37°C.

Biochemical Characteristics: no anaerobic growth; no growth in the presence of 5, 7 or 10% NaCl; acid but no gas produced from glucose; growth observed in pH5.7 broth; no casein or tyrosine decomposition; gelatin was decomposed; starch hydrolysed (beneath colony only); catalase and oxidase (Kovacs) positive; VP (acetoin), nitrate reduction, and citrate test (Kosers) all negative.

The following compounds supported growth: glycerol; ribose; D-glucose; D-fructose; D-mannose; mannitol; aesculin (weak growth); maltose; sucrose; trehalose; starch; and D-turanose.

The following compounds did not support growth:D- or L-arabinose; cellobiose; lactose; thamnose; D- or L-xylose; erythritol; adonitol; β-methyl xyloside; galactose; L-sorbose; rhamnose; dulcitol; inositol; sorbitol; α-methyl-D-mannoside; α-methyl-D-glucoside; N-acetylglucosamine; amygdalin; arbutin; salicin; melibiose; inulin; melezitose; D-raffinose; glycogen; xylitol; β-gentiobiose; D-lyxose; D-tagatose; D- or L-fucose; D- or L-arabitol; gluconate; 2- or 5-keto-gluconate.