NAPHTHALENE PLASMIDS IN PSEUDOMONADS

CENTRE FOR NEWFOUNDLAND STUDIES

TOTAL OF 10 PAGES ONLY
MAY BE XEROXED

(Without Author's Permission)

MARGARET ANNE MORRIS CONNORS

Naphthalene Plasmids in Pseudomonads

by

Margaret Anne Morris Connors B.Sc. (Hons)

A thesis submitted in partial fulfillment

of the requirements for the degree of

Master of Science

Department of Biochemistry

Memorial University of Newfoundland

August 1986

St. John's Newfoundland

Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.

ISBN 0-315-36980-9

ABSTRACT

The Pseudomonas strains NCIB 9816, P_G, ATCC 17483 and ATCC 17484 were examined for the involvement of plasmids in the degradation of naphthalene. The role of a plasmid was demonstrated in strains NCIB 9816 and P_G by conjugation. It was not possible to demonstrate the involvement of plasmids in the degradation of naphthalene in strains ATCC 17483 and ATCC 17484 either by curing or conjugation.

A published method was modified to permit the screening of wild type strains for the presence of plasmids. Strains NCIB 9816, P_G and ATCC 17484 contained multiple plasmids. It was not possible to demonstrate the presence of any plasmids in ATCC 17483. It was the largest of the pair of plasmids in NCIB 9816 and P_G (designated NAH2 and NAH3 respectively) which determined the degradation of naphthalene.

The patterns of restriction enzyme fragments from digestion with Hind III or BAM HI were identical for NAH2 and NAH3, but were very different from those of NAH, the plasmid described by Dunn and Gunsalus (1973). Nevertheless, NAH3 hydridized extensively with NAH.

Some aspects of the regulation of the naphthalene pathway were investigated. An examination of mutants generated by exposure to N-methyl-N'-nitro-N-nitrosoguanidine enabled the identification of salicylaldehyde as the first possible inducer of the pathway in strain ATCC 17483. The unusual regulatory characteristics of the meta enzymes for the degradation of catechol in strains NCIB 9816 and P_G were

transferred by conjugation, and are therefore specified by plasmids NAH2 and NAH3 respectively. However, differences in the regulation of plasmids NAH2 and NAH3 and plasmid NAH may not be as great as it would first appear.

dedicated to Harry, Michael and Mary Beth

ACKNOWLEDGEMENTS

To Dr. E.A. Barnsley, my supervisor, for his guidance and advice, Dr. P.G. Barnsley for her helpful advice on techniques, and Dr. S.S. Mookerjea, without whose persistent support and encouragement this thesis might not have been written, the writer wishes to express her appreciation, and also to thank those individuals, too numerous to mention, who gave a hand in the process.

TABLE OF CONTENTS

	Page
Introduction	1
The Microbial Degradation of Aromatic Compounds	1
The Degradation of Naphthalene by Pseudomonads	9
Purpose of the Study	22
Origin of the Naphthalene Metabolizing Strains used in this Study, and their Biochemical Characteristics	24
Materials and Methods	26
A. Bacterial Strains	26
B. Methods of Culture	26
C. Measurement of Enzymic Activities	30
a) Naphthalene Oxygenase	31
b) 1,2 Dihydroxynaphthalene oxygenase	31
c) 2'Hydroxybenzal pyruvate aldolase	32
d) Salicyaldehyde dehydrogenase	32
e) Salicylate hydroxylase	33
f) Catechol 2,3 dioxygenase	34
g) Catechol 1,2 dioxygenase	35
h) 2 Hydroxymuconic acid semialdehyde dehydrogenase	36
D. Curing	37
E. Isolation of Amino Acid Auxotrophs	38
F. Conjugation	41
G. Isolation of Plasmid DNA	43
H. Purification of Plasmid DNA by Density Gradient Centrifugation	44

TABLE OF CONTENTS CONTINUED

Page

I. Digestion with Restriction Endonucleases 46

J. Electrophoresis 47

K. Southern Blots 48

L. Nick Translation 49

M. Hybridization 50

Results 53

A. Curing 53

B. Mutagenesis and Selection for Amino Acid Auxotrophs 56

C. Conjugation Experiments 58

D. The Isolation and Purification of Plasmid DNA 64

E. Digestion of Plasmid DNA with Restriction Endonucleases 68

F. Hybridization Experiments 70

G. Examination of Naphthalene Pathway Mutants of Mutants of ATCC 17483 71

Discussion 77

A. Detection of Plasmids in Naphthalene-Metabolizing strains 77

B. Isolation of Plasmid DNA from Naphthalene-Metabolizing Strains 82

C. The Molecular Relationship among NAH, NAH2 and NAH3 84

D. The Regulation of Naphthalene Metabolism by Pseudomonads 87

Conclusions 90

TABLE OF CONTENTS CONTINUED

Page

Summary 92

References 96

List of Tables

		Page
Table I	Naphthalene-Metabolizing Pseudomonads	25
Table II	Phenotypes of Bacterial Strains	27
Table III	Curing of Naphthalene-Metabolizing Pseudomonads	54
Table IV	Activities of Naphthalene Pathway Enzymes in Wildtype and Cured Strains of Naphthalene-Metabolizing Pseudomonads	57
Table V	Reversion Rates and Enzymic Activities in Amino Acid Auxotrophs	59
Table VI	Conjugation of Naphthalene-Metabolizing Pseudomonads with Cured Strains	61
Table VII	Attempted Conjugation of Naphthalene-Metabolizing Pseudomonads with Cured Strains	62
Table VIII	Activities of Catechol 1,2 Dioxygenase and Meta Pathway Enzymes	63
Table IX	Activities of Enzymes Induced by 2-Aminobenzoate in Mutants of ATCC 17483 ..	74
Table X	Activities of Enzymes Induced in Mutants of ATCC 17483 Grown on Succinate in the Presence of Naphthalene	76

List of Figures

		Page
Figure 1.	The pathway for the degradation of naphthalene	10
Figure 2.	Mutagenesis and enrichment for amino acid auxotrophs	40
Figure 3.	Naphthalene plasmids in their original hosts and after transfer to PaW 330	65
Figure 4.	Plasmids in ATCC 17484	66
Figure 5.	Restriction patterns of plasmids NAH, NAH2 and NAH3 digested with Bam HI	69
Figure 6.	Restriction patterns of naphthalene plasmids NAH, NAH2 and NAH3 digested with Hind III	70
Figure 7.	Hybridization of Bam HI restriction fragments of NAH, NAH2 and NAH3 with NAH labelled with tritiated thymidine	72

List of Abbreviations

Chemicals

EDTA	Ethylene diaminetetraacetic acid
FAD	Flavin adenine dinucleotide
HOMASA	2-Hydroxymuconic acid semialdehyde
NAD^+	β Nicotinamide adenine dinucleotide
NADH	β Nicotinamide adenine dinucleotide, reduced
NG	N-methyl-N'-nitro-N-Nitrosoguanidine
PPO	2,5-Diphenyl oxazole
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TES	Tris-EDTA-salt buffer
Tris	Tris (hydroxymethyl) amino methane

Plasmids

CAM	Camphor
NAH	Naphthalene
OCT	Octane
SAL	Salicylate
TOL	Toluate

Phenotypes

Nah^+	able to grow on naphthalene
Nah^-	unable to grow on naphthalene
Sal^+	able to grow on salicylate
Sal^-	unable to grow on salicylate
Leu^-	requires leucine for growth
Met^-	requires methionine for growth
Trp^-	requires tryptophan for growth
Str^R	is resistant to streptomycin

INTRODUCTION

The Microbial Degradation of Aromatic Compounds

The early investigations into the degradation of aromatic compounds by micro-organisms arose out of practical considerations in the agricultural and petroleum-related industries. Many aromatic compounds, such as naphthalene, phenol or cresol, were used as pesticides in agriculture, and it was a common observation that after a second application of a compound, it disappeared more rapidly than the first (Tattersfield, 1928). The potential use of micro-organisms to degrade oil wastes or to indicate oil deposits were among the useful applications for the petroleum industry, but the degradation of cooling oils and lubricants, and of oiled and asphalted highways were among the less desirable effects of the microbial ability to degrade aromatic hydrocarbons (Zobell, 1946). The subsequent development of synthetic compounds with increased effectiveness as pesticides, lubricants etc. has resulted in the opposite problem, and current concerns are focussed on the vast quantities of synthetic compounds, particularly haloaromatic compounds, that are accumulating in the environment (Dagley, 1975). The degradative capabilities of micro-organisms are now being investigated as a means to reduce the concentrations of these chemicals in the environment. In addition, the agricultural, lumber, and papermaking industries create enormous quantities of lignocellulosic wastes, and current research is centred on the potential of micro-organisms to degrade the lignin in

these wastes, as it is the lignin which forms the barrier to the conversion of these wastes to industrially useful chemicals (Crawford, 1981).

Aromatic compounds are found ubiquitously in nature. Lignins and tannins, aromatic polymers, are synthesized continually by plants. Polycyclic aromatic hydrocarbons are present in soils and sediments around the world (Blumer, 1976) and are produced during the formation of petroleum, and as the result of the pyrolysis of organic matter during grass and forest fires. The recycling of the carbon from the otherwise inert benzenoid compounds is carried out almost exclusively by bacteria and fungi.

Aromatic compounds may be degraded oxidatively by aerobic micro-organisms, or in the absence of oxygen, by photometabolism, nitrate respiration or methanogenic fermentation by anaerobic micro-organisms. The anaerobic processes have only recently attracted attention, and their relative importance compared to the aerobic processes has not been determined (Evans, 1977). With anaerobic environments more available than aerobic environments, and the increase in the concentration of nitrates in the environment arising from the use of synthetic fertilizers, the contribution by anaerobic micro-organisms to the recycling of carbon may be significant. The aerobic processes required for the conversion of aromatic compounds to intermediates for the Krebs cycle or the fatty acid "spiral" are well known, and have been reviewed extensively

during the past 15 years (Cerniglia, 1981; Clarke, 1982; Crawford, 1981; Dagley, 1971, 1975, 1978; Gibson, 1971, 1984).

The ability of micro-organisms to degrade aromatic compounds is widespread. Samples from cultivated and uncultivated soils from several sites in Britain and Europe and soil samples from several uninhabitated islands in the South Atlantic were found to contain a wide variety of micro-organisms able to use aromatic compounds as substrates for growth (Gray and Thornton, 1928). Although pseudomonads are the micro-organisms most commonly associated with the degradation of aromatic compounds (Stanier et al., 1966) bacteria which form heat resistant spores and are able to grow on aromatic compounds are readily isolated from a variety of locations (Dagley, 1975). These findings are not very surprising in view of the widespread occurrence of benzenoid compounds in the environment and the fact that it is by the microbial degradation of these compounds that the carbon contained therein is returned to the biosphere.

Aromatic compounds are first converted to substrates which can be cleaved by dioxygenases to aliphatic compounds which are then degraded further to intermediates of the Krebs cycle. These substrates generally contain at least two hydroxyl groups which may be situated ortho or para to each other. A large number of dioxygenases have been described from many strains of bacteria, mainly pseudomonads (Dagley, 1971, 1975; Crawford, 1981). Catechol and protocatechuic

acid may be cleaved by ortho or meta fission dioxygenases. Methyl substituted catechols and homoprotocatechuic acid, are cleaved by meta fission dioxygenases. Gentisate and its homologues, dihydric phenols in which the hydroxyl groups are situated para to each other, and gallate, with three hydroxyl groups, and its homologues are also substrates for dioxygenases. The ortho fission dioxygenases and the pathways which follow cleavage of the aromatic ring have narrow substrate specificities. The meta fission dioxygenases and subsequent pathways have broader specificities and may tolerate methyl and halogen substituents in the aromatic ring. There is, however, very little cross reaction among the different pathways, for example protocatechuic acid is not cleaved by catechol 2,3 dioxygenase. There are some exceptions, for example protocatechuic 4,5 dioxygenase will cleave gallate and methoxy gallate, although their respective dioxygenases will not cleave protocatechuic acid. The substrates for the many dioxygenases are found in nature as products of the degradation of lignins and tannins, as metabolites of benzoic and mandelic acids, etc. Thus, during the evolution of bacteria, a large number of pathways have evolved in response to the presence of many different aromatic compounds present in the environment.

During the past 40 years, the environment has been flooded with large amounts of synthetic compounds with structural features not commonly found in nature, hence

these compounds are not readily degraded. Those synthetic compounds which have structures that are similar to naturally occurring compounds might be degraded microbially, or at least co-metabolized by microbes growing on other compounds. Although synthetic compounds, especially halogenated compounds have been present in the environment for a short period of time in evolutionary terms, the ability to degrade some of these compounds is appearing in microbial populations (Dagley, 1975; Ghosal et al., 1985). Nevertheless, concern has been expressed that the rate of microbial evolution may not be rapid enough to reduce the accumulation of synthetic compounds entering the environment.

The evolution of new pathways is dependent upon mutation and selection which results in alterations of the specificities of enzymes and regulatory elements. These processes may be accelerated in the laboratory with the use of continuous cultures in chemostats and by genetic manipulations. The discovery that the genes for the degradative pathways for many organic compounds were located on plasmids (reviewed in Chakrabarty, 1976) led to the understanding that the acquisition of a new degradative pathway by a microbial population could be a rapid process. The door was opened for the construction of microbial strains with increased degradative capabilities.

One of the earliest attempts was the construction, by Chakrabarty, of a pseudomonad strain into which were transferred a CAM-OCT, an NAH and a TOL plasmid (cited in Williams,

1981). Strains with new degradative capabilities may be constructed by the transfer of a plasmid which specifies the initial steps in a pathway to a strain which specifies the later steps in a pathway. This approach was first described by Reineke and Knackmus (1979) who transferred a TOL plasmid which specifies enzymes capable of degrading 2- and 4-chlorobenzoates but not the corresponding halocatechols, to strain B13 able to degrade halocatechols. The degradative capability of strain B13 was thus extended to include 2- and 4-chlorobenzoates. The phenotypic expression of the new degradative ability required a mutation in the TOL plasmid which rendered it unable to grow on p-toluate. The TOL plasmid was also used to extend the degradative capability of pseudomonad strains containing the plasmid pAC25 which codes for the degradation of 3-chlorobenzoate (Chatterjee et al., 1981). Both the TOL and pAC25 plasmids underwent structural rearrangements to generate new plasmids capable of degrading 4-chlorobenzoate and 3,5-dichlorobenzoate.

Plasmid pAC25 showed considerable homology with SAL and TOL, (Chatterjee et al., 1981; Kellogg et al., 1981) and it was concluded that pAC25 may have evolved in nature by recombination of fragments from other plasmids such as SAL and TOL. This finding suggested the possibility that the evolution of new plasmids might be accelerated in the laboratory under strong selective conditions (Kellogg et al., 1981) by a technique described as "plasmid assisted molecular breeding".

Soil samples from various waste dumping sites and Pseudomonas putida strains containing SAL, TOL, CAM, pAC21 (coding for the degradation of 4-chlorobiphenyl) and pAC25 were inoculated into a chemostat. At first, the substrates for growth were those of the defined degradative plasmids and a low concentration of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). The concentration of 2,4,5 T was gradually increased and the concentrations of the other substrates lowered until after six months, 2,4,5-T was the sole growth substrate available to the culture in the chemostat. Strain AC1100, Pseudomonas cepacia, able to grow on 2,4,5-T as the only carbon source was isolated from the mixed culture in the chemostat (Kilbane et al., 1982). The degradative ability for 2,4,5-T was unstable, which suggested the involvement of a plasmid or plasmids (Kilbane et al., 1982), however strain AC1100 is unable to transfer the 2,4,5-T degrading ability to other bacteria (Kilbane et al., 1983). Strain AC1100 contains at least two plasmids which appear to be involved in the degradation of 2,4,5-T, and there is also evidence for chromosomal involvement in the degradation of this compound (Ghosal et al., 1985). The precise roles of plasmid and chromosomal genes in the degradation of 2,4,5-T have not been determined. The initial tests of the ability of strain AC1100 to remove high concentrations of 2,4,5-T from soil were very successful (Chatterjee et al., 1982; Kilbane et al., 1983).

It is also possible to construct new strains by using

gene cloning techniques. The genes for toluate 1,2 dioxygenase and dihydrodihydroxy benzoic acid dehydrogenase from the TOL plasmid and the gene for salicylate hydroxylase from the NAH plasmid have been cloned and transferred to strain B13 to construct strains capable of utilizing 4-chlorobenzoate, 3,5-dichlorobenzoate, salicylate and chlorosalicylates as growth substrates (Lehrbach et al., 1984).

It remains to be seen if the development of strains with new degradative capabilities by the "plasmid assisted molecular breeding" technique of Chakrabarty and co-workers, or by gene cloning techniques will contribute substantially to the removal of synthetic compounds accumulating in the environment. Nevertheless an understanding of how micro-organisms are able to degrade aromatic compounds, and how genetic information is exchanged within and between microbial populations can lead to a wiser development of policy regarding the introduction of new compounds into the environment.

The Degradation of Naphthalene by Pseudomonads

The initial interest in the microbial degradation of naphthalene arose from its use in agriculture as a pesticide against protozoa and wireworms (the larvae of members of the Elateridae and Tipidulae families). It had been observed that the toxicity of naphthalene declined more rapidly than could be explained by vaporization of the compound. That the disappearance of naphthalene was due to microbial action was demonstrated by Tattersfield (1928). About the same time, Tausson (1928, cited in Walker and Wiltshire, 1953) isolated three strains of bacteria from soil from the Black Sea Oil fields that were able to degrade naphthalene. An identification of the micro-organisms involved in the degradation of naphthalene and other aromatic compounds was carried out by Gray and Thornton (1928). About 25% of the microbial strains isolated from various soil samples were pseudomonads. The pathway for the degradation of naphthalene by pseudomonads is shown in figure 1.

The identification of the intermediates in the degradation of naphthalene was begun by Strawinski and Stone (1943, 1954) who isolated salicylic acid from cultures of Pseudomonas aeruginosa grown on naphthalene. Investigations by Walker and Wiltshire (1953) and Trecanni, Walker and Wiltshire (1954) identified naphthalene diol, salicyclic acid and catechol as intermediates in the degradation of naphthalene in several strains of bacteria including those which were

Figure 1. The pathway for the degradation of naphthalene

Pathway intermediates enzymes

Naphthalene
↓ naphthalene oxygenase
cis-1,2-Dihydrodihydroxynaphthalene
↓ cis-naphthalene dihydrodiol dehydrogenase
1,2-Dihydroxynaphthalene
↓ 1,2-dihydroxynaphthalene oxygenase
2-Hydroxychromene-2-carboxylic acid
↓ 2-hydroxychromene-2-carboxylic acid isomerase
2'Hydroxybenzal pyruvate
↓ 2'hydroxybenzal pyruvate aldolase
Salicylaldehyde + pyruvate
↓ salicylaldehyde dehydrogenase
Salicylate
↓ salicylate hydroxylase
Catechol

Ortho pathway

catechol 1,2 dioxygenase

Cis, cis-muconate
↓ lactonizing enzyme
Muconolactone
↓ isomerase
β-Ketoadipate enol lactone
↓ hydrolase
β-Ketoadipate
↓ transferase
Succinate + Acetyl CoA

Meta pathway

catechol 2,3 dioxygenase

2-Hydroxymuconic acid semialdehyde
↓ dehydrogenase
4-Oxalocrotonate (enol)
↓ tautomerase
4-Oxalocrotonate (keto)
↓ decarboxylase
Vinyl pyruvate ← hydrolase (from 2-Hydroxymuconic acid semialdehyde) **+ formate**
↓ hydratase
4-Hydroxy 2-ketovalerate
↓ aldolase
Acetaldehyde + pyruvate

later designated as ATCC 17483, ATCC 17484 and ATCC 17485.

The complete pathway was elucidated by Evans and co-workers (Fernley and Evans, 1958, Davies and Evans, 1964). It was demonstrated that 1,2-dihydroxynaphthalene was the product formed from the naphthalene diol, as naphthalene-grown cells readily metabolized 1,2-dihydroxynaphthalene. Fernley and Evans proposed that dihydroxynaphthalene underwent oxidative decarboxylation to o-carboxy-cis-cinnamic acid which itself was oxidatively decarboxylated to o-hydroxy-cis-cinnamic acid which was then converted to salicylate and then to catechol. However, Davies and Evans (1964) were unable to demonstrate that naphthalene grown cells were able to metabolize synthetic samples of o-carboxy-cis-cinnamate, and the oxygen uptake observed with o-hydroxy-cis-cinnamate was not sufficiently high enough to warrant its inclusion as an intermediate in the pathway. Davies and Evans (1964) undertook a more detailed investigation of the ring-fission of 1,2-dihydroxynaphthalene in order to identify the intermediates and the enzymes responsible for the reactions. 2-Hydroxybenzal-pyruvate was isolated from a reaction mixture in which 1,2-dihydroxynaphthalene was incubated with a dilute cell free extract from naphthalene grown cells. It was also demonstrated that the cell free extracts contained an aldolase which converted 2-hydroxybenzal pyruvate to salicyaldehyde and pyruvate. Salicylaldehyde was then converted to salicylate by an NAD^+-dependent dehydrogenase. Walker and Evans (1952)

had previously reported that salicylate was converted to catechol. The strains used by Davies and Evans were found to convert catechol to 2-hydroxymuconic acid semialdehyde by catechol 2,3-dioxygenase.

The naphthalene diol produced from naphthalene was originally identified as a D-trans-1,2 dihydroxydiol by Walker and Wiltshire (1953). The identification procedures used at the time (absorption spectrum and specific rotation) would not have distinguished between the cis and the trans isomer. Subsequent investigations by Jerina et al. (1971) and Jeffrey et al. (1975) demonstrated that the hydroxyl groups in the diol were in the cis conformation, and that both atoms of oxygen inserted into naphthalene came from a single molecule of oxygen. The actual conformation, based on results from TLC chromatography, proton-nuclear magnetic resonance spectroscopy, rates of dehydration and reduction with hydrogen, was established as (+) cis-1(R),2(S)-dihydroxy-1,2-dihydroxynaphthalene (Jerina et al. 1971). The cis isomer of the diol was metabolized at a much faster rate than the trans isomer of the diol by strain NCIB 9816 (P_G) (Catterall, Murray, and Williams, 1971), and radioactive trapping experiments, followed by identification by TLC identified the cis isomer as an intermediate in this strain. The cis isomer was found to be the intermediate in several strains, including ATCC 17483, ATCC 17484 and NCIB 9816.

The nature of the oxidation product of the action of

dihydroxynaphthalene oxygenase on 1,2 dihydroxynaphthalene was investigated by Barnsley (1976). It was observed that the absorption spectrum of the product was not consistent with that of a phenolic compound, but was consistent with that of 2-hydroxychromene-2-carboxylic acid. This compound was considered to be an artifact by Davies and Evans (1965) who reported that it was not metabolized by cell free extracts, and only slightly by whole cells. Although 2-hydroxychrome-2-carboxylic acid is converted to cis-2'-hydroxybenzal pyruvate at high pH, this conversion was carried out by an inducible enzyme in several strains of naphthalene-metabolizing pseudomonads, NCIB 9816, P_G, ATCC 17483, ATCC 17484 and PpG7. This enzyme was described as an isomerase. A later study by Patel and Barnsley (1980) concerned with the purification of 1,2 dihydroxynaphthalene oxygenase demonstrated that 3-methyl catechol was oxidized by 1,2 dihydroxynaphthalene oxygenase to 2 hydroxy-6-oxo heptadienoate, the structure of which is not analogous to 2-hydroxychromene-2-carboxylic acid. It was proposed that the product was formed as a result of ring fission of 1,2-dihydroxynaphthalene and underwent cyclization before the release of the product from the enzyme. The results of a series of polar mutants generated by transposon insertion into a strain derived from pPG7 also indicate that 1,2-dihydroxynaphthalene oxygenase and the isomerase are separate enzymes (Yen and Gunsalus, 1982). The role of 2 hydroxychrome-2 carboxylic acid isomerase is

unclear, and has been questioned by Cerniglia (1981) and Gibson (1984).

Salicylate was identified as an inducer of the naphthalene pathway by Shamsuzzaman and Barnsley (1974a) in several naphthalene metabolizing pseudomonads. Two structural analogues of salicylate, 2-aminobenzoate and 2-hydroxybenzyl-alcohol were found to induce naphthalene oxygenase gratuitously (Shamsuzzaman and Barnsley, 1974a). The early enzymes of the naphthalene pathway in NCIB 9816 were found to be co-ordinately induced en bloc by salicylate (Shamsuzzaman and Barnsley, 1974b). This study also demonstrated that 1,2-dihydroxynaphthalene oxygenase and catechol 2,3-dioxygenase were indeed distinct enzymes. Williams et al. (1974) reported that dihydroxynaphthalene oxygenase was not induced by salicylate in strain P_G, however the assay used by this group was probably not the best (see Shamsuzzaman and Barnsley, 1974b). The early enzymes of the naphthalene pathway were found to be induced by salicylate and 2-aminobenzoate in strains PpG7, ATCC 17483, NCIB 9816 and P_G (Barnsley 1975, 1976b).

Catechol is an intermediate in the degradation of several aromatic compounds, such as phenol, benzoate, salicylate, phenanthrene, anthracene, as well as of naphthalene (Stanier and Ornston, 1978). Two pathways have been described for the degradation of catechol, the ortho or β-ketoadipate pathway, and the meta pathway.

The steps and the regulation of the ortho pathway for

the degradation of catechol were elucidated over a period of 20 years, predominantly in the laboratories of Evans and co-workers and Stanier and co-workers, and have been reviewed by Evans et al. (1951) and by Stanier and Ornston (1973). The catechol ring is cleaved between the two hydroxyl groups to form <u>cis</u>, <u>cis</u>-muconate. <u>cis</u>, <u>cis</u>-Muconate is converted to (+)-muconolactone, which is converted to β-ketoadipate enol-lactone by an isomerase. The lactone is split by a hydrolase to form β-ketoadipate. β-ketoadipate: succinyl CoA transferase transfers CoA to β-ketoadipate, which is then cleaved by a thiolase to succinate and acetyl CoA.

The regulation of the β-ketoadipate pathway was examined in detail by Ornston (1966). Catechol 1,2-dioxygenase is induced by the product of the reaction it catalyses, <u>cis</u>, <u>cis</u>-muconate. <u>cis</u>, <u>cis</u>-Muconate also induces the muconate-lactonizing enzyme and muconolactone isomerase, which are co-ordinately synthesized. β-Ketoadipate is produced from the enol-lactone by high basal levels of the hydrolase, and is the inducer of the hydrolase and the transferase, as well as of two enzymes of the protocatechuate pathway, which converges at the enol-lactone.

The meta-cleavage of catechol was first reported by Dagley and Stopher (1959). Hydroxymuconic acid semialdehyde was formed from the cleavage of catechol between carbons 2 and 3 by catechol 2,3-dioxygenase (Dagley et al., 1960). Hydroxymuconic acid semialdehyde is degraded further either

by hydrolysis (Dagley and Gibson, 1965; Bayley and Dagley, 1969) or oxidative decarboxylation (Nishizuka et al., 1962; Catterall et al., 1972). Vinyl pyruvate and formate are formed from the hydrolysis of hydroxymuconic acid semialdehyde. Hydroxymuconic acid semialdehyde is also oxidized to 4-oxalocrotonate by an NAD^{+}-dependent aldehyde dehydrogenase. An enol-keto change catalysed by a tautomerase (Sala-Trepat and Evans, 1971) or an isomerase (Sparnins et al., 1976; the issue is apparently unresolved, Bayly and Barbour, 1984) preceeds the decarboxylation to form vinyl pyruvate. Vinyl pyruvate is hydrated to 2-keto-4-hydroxy valerate from which is formed acetaldehyde and pyruvate by the action of an aldolase (Dagley and Gibson, 1965). Catterall et al. (1971) demonstrated that the physiologically more important pathway for the degradation of hydroxymuconic acid semialdehyde was the oxidation to 4-oxalocrotonate by the NAD^{+}-dependent dehydrogenase. Pathways which produce catechol and 4-methylcatechol proceed by the dehydrogenase. Those which produce 3-methylcatechol proceed by the hydrolase.

The genes for the degradation of naphthalene have been mapped (Yen and Gunsalus, 1982) and cloned (Grund and Gunsalus, 1983) from strain PpG7. The genes are organized into two operons, one containing the genes for the degradation of naphthalene to salicylate, and one containing the genes for the degradation of salicylate via the meta pathway. The two operons are separated by a regulatory region. The gene

product of the regulatory region is required for the expression of the two operons. Double transformants were used to demonstrate that both operons are under positive control. The regulatory protein and salicylate are co-inducers of both operons.

In some bacterial strains, catechol is metabolized by the ortho pathway, and in others, by the meta pathway. Several strains, predominantly those of <u>Pseudomonas putida</u> can degrade catechol by both pathways, and it appears to be the precursor of catechol which determines which pathway is used. Several naphthalene-metabolizing pseudomonads were reported to metabolize the catechol produced during the degradation of naphthalene completely via the meta pathway. However, measurements for the activity of catechol 1,2-dioxygenase or the presence of intermediates of the ortho pathway were not reported (Davies and Evans 1964, Evans, et al., 1965). It was infered by Feist and Hegeman (1969) that only the meta pathway was induced by growth with naphthalene or salicylate, and that the ortho pathway enzymes were induced during growth on benzoate. Some <u>Pseudomonas aeruginosa</u> strains able to metabolize arylsulphonates, were reported to use the meta pathway. However, the enzymes or metabolites of the ortho pathway were not assayed. Other strains metabolized arylsulphonates by the ortho pathway only, and hydroxymuconic acid semialdehyde was not detected as a metabolite in these strains (Cain and Farr, 1968, Farr and Cain, 1968). The

ortho pathway was induced during growth on benzoate in all strains. The meta pathway, but not the ortho pathway is induced in Pseudomonas putida strain U during growth on phenol, while only the ortho pathway is induced during growth on benzoate. These observations, together with those described above led Feist and Hegeman to conclude that the meta pathway is induced by the primary substrate, which is not an inducer for the ortho pathway, while cis, cis-muconate, the product inducer of catechol 1,2-dioxygenase, does not induce the meta pathway. It was proposed that the catechol formed from phenol by phenol hydroxylase was rapidly metabolized by the high level of catechol 2,3-dioxygenase, and therefore was not metabolized by the low levels of catechol 1,2 dioxygenase. Catechol was observed to be a weak inducer of the meta pathway (Murray and Williams, 1974) and it was proposed that the lack of induction of the meta pathway during growth on benzoate was not due to the absence of an inducer, but due to the combination of catechol being a weak inducer, and that the intracellular concentrations of catechol never rose to a sufficient level to cause induction. In support of this proposal, it was reported by Williams et. al. (1974) that Pseudomonas putida strain P_G metabolized naphthalene via the meta pathway, with no induction of the ortho pathway, while salicylate, benzoate and catechol were metabolized solely by the ortho pathway. A rather different situation was observed in a copy of strain P_G sent to E.A. Barnsley by

P.A. Williams and in NCIB 9816, the accession number of strain P_G in the National Collection of Industrial Bacteria in Aberdeen. Catechol 2,3-dioxygenase was constitutive in the strains described by Barnsley (1976b), at a low level for NCIB 9816 and at a high level for P_G. Catechol 2,3 dioxygenase was inducible in the strain described by Williams et al. (1974). Austen and Dunn (1980) proposed that all three strains were regulatory mutants of a common strain. Catechol 1,2-dioxygenase and the enzymes of the ortho pathway were induced during growth on naphthalene or salicylate in NCIB 9816 and P_G as well as in strains PpG7 and ATCC 17484 to varying extents. The ortho pathway enzymes catechol 1,2-dioxygenase and muconate cycloisomerase were found to be absent in strain ATCC 17483. The meta enzymes were induced during growth on naphthalene or salicylate in those strains that had inducible catechol 2,3-dioxygenases, the enzymes were not induced further in those strains in which the enzymes were constitutive. It would seem that during growth on naphthalene or salicylate sufficient catechol is converted to <u>cis</u>, <u>cis</u>-muconate to cause the induction of the pathway. In view of the absence of assays for the presence of ortho pathway enzymes and metabolites in the reports of Davies and Evans (1964), Evans et al. (1965) and Cain and Farr (1968) the mutually exclusive occurrence of the ortho and meta pathways as described for <u>Pseudomonas putida</u> U may not be as universal as commonly reported in review articles (e.g. Clark,

1982, Bayly and Barbour, 1984). At the same time, it should be noted that where the ortho pathway may be induced in a strain which contains a full suite of meta enzymes, the activities of all of the ortho enzymes have not been measured, and other measures of the flux through the pathway have not been made. The point is worth noting, as there is an accumulation of coloured compounds in the culture medium during growth of the culture on naphthalene.

The involvement of plasmids in the degradation of naphthalene was first reported for strain PpG7 by Dunn and Gunsalus (1973). On the basis of curing and conjugation experiments, it was concluded that the genes for the degradation of naphthalene via the meta pathway were located on a plasmid, designated as NAH. That a plasmid was indeed involved, was confirmed by isolation and transformation experiments (Johnston and Gunsalus, 1977). Several other naphthalene-metabolizing strains have been investigated for the involvement of plasmids in the degradation of naphthalene. Boronin and co-workers have described several plasmids carrying genes for the degradation of naphthalene. Plasmid NPL1 (Boronin et al., 1976), isolated from _Pseudomonas putida_ strain 12A carries the genes for the early steps of the naphthalene pathway, but not for the degradation of salicylate. Catechol 2,3-dioxygenase is not present in strain 12A (Starovoitov et al., 1976), and the catechol resulting from the action of salicylate hydroxylase was found to be degraded by the ortho

pathway. The gene for salicylate hydroxylase is apparently located on the chromosome in this strain. The genes for the enzymes that catalyze the oxidation of naphthalene to salicylate and the gene for catechol 1,2 dioxygenase are carried on plasmid pBS2, which was isolated from Pseudomonas putida strain BS238 (Boronin et al. 1977). Plasmid pBS3, isolated from Pseudomonas fluorescens strain BS243 was found to be similar to NAH, in that the meta pathway is plasmid borne, but pBS3 was able to repress the expression of catechol 1,2 dioxygenase in a cured PpG7 strain (Boronin et al., 1977). Plasmids pNB 140 and pND 160 isolated from strains UB1-1 and UB2-1 respectively are also similar to NAH (Dunn et al. 1980), but have different inducer specificities. The genes for the degradation of naphthalene to salicylate appear to be chromosomally located in Pseudomonas putida strain PMD-1, while the genes for the degradation of catechol, and the genes for the meta-cleavage of catechol are located on plasmid pMWD-1 (Zuniga et al., 1981). It is evident that the pathway for the degradation of naphthalene may be coded entirely by a plasmid, or only partially with the rest of the pathway being coded by the chromosome. The location of the genes responsible for enzymes involved in naphthalene degradation varies in different strains of Pseudomonas.

Purpose of the Study

This study was carried out from May 1979 to April 1981 and the results were published (Connors and Barnsley, 1980, 1982). Prior to this time, the existence of NAH was reported and the characterization of this plasmid was started in the laboratory of I.C. Gunsalus. In addition several plasmids involved in the degradation of naphthalene were reported by Boronin and co-workers. A plasmid was reported to exist in strain ATCC 17483 (Heinaru et al., 1978) but no data were provided to support this report. Plasmid involvement in the degradation of naphthalene by strain NCIB 9816 was proposed on the basis of the coordinate induction of the early enzymes in the pathway (Shamsuzzaman and Barnsley, 1974a). The loss of the ability to degrade naphthalene was associated with the loss of plasmid in strains ATCC 17484, P_G and NCIB 9816 (J.B. Johnston, personal communication to E.A. Barnsley).

Several studies have been carried out to examine the molecular relationships among a variety of catabolic plasmids (Heinaru et al., 1978, Farrell et al., 1978, Bayley et al., 1979) but with the exception of the statement that the plasmid in ATCC 17483 was identical to NAH (Heinaru et al., 1978) they were not concerned with the molecular relationships among naphthalene plasmids.

It was decided to examine strains NCIB 9816, P_G, ATCC 17483 and ATCC 17484 which previously had been characterized biochemically (Barnsley, 1975; 1976b) and several uncharacterized

naphthalene-metabolizing strains for the involvement of plasmids in the degradation of naphthalene, and to examine the molecular relationships among the napthalene plasmids.

Difficulties in obtaining reproducible isolations of plasmid DNA which were not resolved until the end of the time period available for this study, and the presence of multiple plasmids in several strains, reduced the scope of the study to those strains from which plasmids could be transferred to PaW 330, a cured *Pseudomonas putida arvilla* strain.

Origin of the Naphthalene-Metabolizing Strains Used in this Study, and their Biochemical Characteristics

Strains ATCC 17483 and ATCC 17484 were isolated at Rothampsted, England by N. Walker (Walker and Wiltshire, 1953). The strain designated PpG7 by Gunsalus (see Table II) is the strain 111 of Stanier, Palleroni and Doudoroff (1966). This organism was deposited in the American Type Collection as ATCC 17485 and was isolated by W.R. Sistrom, presumably at Berkley, California (Treccani et. al., 1954). Neither ATCC 17485 supplied by the American Type Culture Collection, nor strain 111 supplied to E.A. Barnsley by M. Doudoroff were able to grow on naphthalene. Strains NCIB 9816 and P_G are putatively identical and are apparently regulatory mutants of the same strain. The strain originally described as P_G and deposited in the National Collection of Industrial Bacteria as NCIB 9816 was isolated by E. Griffiths at Bangor, Wales (Evans et. al. 1965).

Strains PpG7, NCIB 9816, P_G, ATCC 17483 and ATCC 17484 are most easily distinguished by their catechol dioxygenases. There is, however, some confusion in the literature as to these characteristics (Feist and Hegeman, 1969; Williams et. al., 1975). The biochemical characteristics of the strains used in this study are those reported by E.A. Barnsley (1975 and 1976b) and are summarized in Table I.

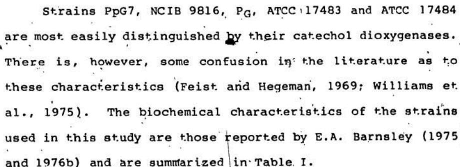

Table I. Naphthalene-Metabolizing Pseudomonads

Strain	Characteristics of Catechol Dioxygenases
PpG7	Both 1,2-dioxygenase and 2,3-dioxygenase are induced during growth on naphthalene
NCIB 9816	Low constitutive level of 2,3-dioxygenase; 1,2-dioxygenase induced during growth on naphthalene
P_G	High constitutive level of 2,3-dioxygenase; 1,2-dioxygenase induced during growth on naphthalene
ATCC 17483	2,3-Dioxygenase induced during growth on naphthalene; 1,2 dioxygenase not produced
ATCC 17484	Low constitutive level of 2,3-dioxygenase; 1,2-dioxygenase induced during growth on naphthalene

MATERIALS AND METHODS

A. Bacterial Strains

The strains used in this study belong to the genus Pseudomonas, and are listed in Table II. Pseudomonas putida PpG7 was obtained from Dr. I.C. Gunsalus. Pseudomonas sp strain P_G was obtained from Dr. P.A. Williams, PaW 330 and PaW 340 were obtained from Dr. C.J. Duggleby, and AC 545 was obtained from Dr. A.M. Chakrabarty. NCIB 9816 was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, ATCC 17483 and ATCC 17484 were obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A.

B. Methods of Culture

The strains were stored on slants of nutrient broth (0.3% peptone, 0.15% yeast extract) solidified with 2% agar.

For daily use, the strains were maintained on petri dishes containing mineral salts (9.3 mM $(NH_4)_2SO_4$, 25 mM K_2HPO_4, 25 mM NaH_2PO_4, 0.4 mM $CaCl_2.H_2O$, 2 mM $MgCl_2.6H_2O$, $2x10^{-4}$ mM $(NH_4)_3Mo_7O_{24}.4H_2O$, $7.2x10^{-3}$ mM $FeSO_4.7H_2O$, $5.1x10^{-3}$ mM $MnCl_2.4H_2O$, $4.2x10^{-3}$ mM $CoCl_2.6H_2O$) plus a carbon source (succinate at a final concentration of 7 mM, salicylate at a final concentration of 3 mM, or naphthalene as a solid added directly to the lid of the petri dish). Where required, amino acids were added at a final concentration of 100 µg/ml. Mineral salts solution A, containing $(NH_4)_2SO_4$,

Table II Phenotypes of Bacterial Strains

Strain	Derivation*	Phenotype	Plasmid
PpG7		Nah^+	NAH
P_G		Nah^+	NAH3
PaW 330		Nah^-Trp^-	plasmid deleted
PaW 340		$Nah^-Trp^-Str^R$	plasmid deleted
AC 545		Nah^+Met^-**	SAL
NCIB 9816		Nah^+	NAH2
ATCC 17483		Nah^+	plasmids not detected
ATCC 17484		Nah^+	plasmids not identified
MC 3	ATCC 17483	Nah^-Sal^+	
MC 4	ATCC 17483	Nah^-Sal^+	
MC 5	ATCC 17483	Nah^-Sal^+	
MC 7	ATCC 17483	Nah^+Sal^-	
MC 9	ATCC 17483	Nah^+Sal^-	
MC 10	ATCC 17483	Nah^+Sal^-	
MC 12	ATCC 17483	Nah^+Sal^-	
MC 17	ATCC 17483	Nah^-Sal^-	
MC 18	ATCC 17483	Nah^-Sal^-	
MC 125	cured PpG7	Nah^-	plasmid deleted
MC 119	cured NCIB 9816	Nah^-	plasmid deleted
MC 96	cured P_G	Nah^-	plasmid deleted
MC 86	PpG7	Nah^+Leu^-	NAH
MC 190	NCIB 9816	Nah^+Leu^-	NAH2
MC 192	P_G	Nah^+Leu^-	NAH3
MC 189	ATCC 17483	Nah^+Leu^-	plasmids not detected
MC 129	ATCC 17484	Nah^+Leu^-	plasmids not identified
MC 213	cured AC545	Nah^-Met^-	plasmid deleted
MC 234	MC 190 x MC 125	Nah^+	NAH2

Table II Phenotypes of Bacterial Strains – continued

Strain	Derivation*	Phenotype	Plasmid
MC 239	MC 192 x MC 125	Nah^+	NAH3
MC 250	MC 86 x MC 213	Nah^+Met^-	NAH
MC 256	MC 190 x MC 213	Nah^+Met^-	NAH2
MC 262	MC 192 x MC 213	Nah^+Met^-	NAH3
MC 281	MC 86 x PaW 330	Nah^+Trp^-	NAH
MC 286	MC 250 x PaW 330	Nah^+Trp^-	NAH
MC 274	MC 256 x PaW 330	Nah^+Trp^-	NAH2
MC 268	MC 262 x PaW 330	Nah^+Trp^-	NAH3

*Strains derived by conjugation are described as donor x recipient.

**Reported as Sal^+, was Nah^+.

KH_2PO_4 and NaH_2PO_4 were prepared at twice the required final concentration. Mineral salt solution B was prepared at 100x the required concentration. Solutions A and B were sterilized in the autoclave at 15 lbs/in^2 for 15 minutes. The agar was prepared in water at a concentration of 4% and was sterilized in the autoclave at 10 lbs/in^2 for 15 minutes. Solution A of the mineral salts was added to molten agar in a 1:1 proportion. Succinate and amino acids were prepared at 100x the required concentration, and were sterilized in the autoclave at 10 lbs/in^2 for 15 minutes, except for tryptophan, which was sterile filtered. Salicylate and benzoate were prepared at 100x the required concentration and were sterilized by filtration through a 0.22 m millipore filter.

For liquid cultures the following media were used: mineral salts plus a carbon source plus amino acids if required; L-broth (1.0% NaCl, 0.5% yeast extract, 1.0% tryptone); and nutrient broth (0.3% peptone, 0.15% yeast extract). L-broth and nutrient broth were sterilized in the autoclave at 10 lbs/in^2 for 15 minutes. The mineral salts solutions were sterilized in the autoclave at 15 lbs/in^2 for 15 minutes. Succinate and salicylate were sterilized as previously described. Aliquots of 1g of naphthalene were sterilized in a closed tube in the autoclave at 10 lbs/in^2 for 15 minutes. The sterile naphthalene was melted and added directly to flasks containing 250 ml of mineral salts plus a limiting amount of succinate (0.7 mM final concentration).

The flasks were swirled during the addition to disperse the naphthalene. Additional succinate to a final concentration of 7 mM was added to cultures growing on succinate in the presence of naphthalene.

C. Measurement of Enzymic Activities

Cells growing in mid-exponential phase were harvested by centrifugation (5000 rpm, 15 min, 4°C in a Sorvall RC-2B centrifuge) and were washed twice in phosphate buffer (0.05M KH_2PO_4 plus NaOH to pH 7.0). The final pellet was resuspended in 2-3 mls of phosphate buffer. A portion of this suspension was set aside to be used in assays requiring whole cell suspensions. The remainder of the suspension was sonicated (Sonifer, Model W185, Heat Systems - Ultrasonics Inc. Plain View, N.Y.). The micro tip probe and the sample to be sonicated were cooled in ice water. The cells were disrupted by three 30 second periods of sonication at 50W. Each period was followed by 60 seconds of cooling in ice water of both the probe and the sample. The sonicated cells were used directly without centrifugation.

All enzyme assays were carried out under conditions which were demonstrated to be linear with respect to time and protein concentration. Initial rates were measured and were used to calculate specific activities.

The concentration of protein was determined by the method of Lowry et. al. (1951). Samples from the suspensions

of whole cells and sonicated extracts were heated in centrifuge tubes with 5% TCA. The precipitated material was collected by centrifugation and used for the assay. Bovine serum albumin was used as the standard.

a) Naphthalene oxygenase

Naphthalene oxygenase was measured by the increase in the rate of oxygen uptake determined polarographically, when 15µl of 10 mM naphthalene in ethanol was added to 0.1 ml of a suspension of whole cells in 1.8 mls of phosphate buffer at 25°C (method reported in Connors and Barnsley, 1980). The endogenous rate was subtracted from the final rate. Although the increase in oxygen uptake indicates the presence of naphthalene oxygenase, this measurement is not quantitative as subsequent metabolic steps also require oxygen.

b) 1,2-Dihydroxynaphthalene oxygenase

1,2-Dihydroxynaphthalene oxygenase was measured polarographically at 25°C. The non enzymic rate of oxidation of 1,2-dihydroxynaphthalene was measured by the addition of 15µl of 40 mM 1,2-dihydroxynaphthalene in tetrahydrofuran to 1.8 mls of 0.05M acetate (adjusted to pH 5.5 with NaOH), and after 1 minute, sonicated cells were added. The rate in the presence of sonicated cells was corrected for the non enzymic rate (Patel and Barnsley, 1980).

1,2 Dihydroxynaphthalene was prepared from 1,2 naphthoquinone (obtained from Eastman Kodak Co., Rochester

N.Y.) by Dr. T.R. Patel according to a published method (Corner and Young, 1954) and was purified by vacuum sublimation.

c) <u>2'Hydroxybenzal pyruvate aldolase</u>

2'Hydroxybenzal pyruvate aldolase was measured by the disappearance of cis-2'hydroxybenzal pyruvate (Barnsley, 1976a). The reaction mixture contained 0.1M phosphate buffer pH 7.0 sufficient for a final volume of 3.0 ml and 60 µl of 2.085 mM cis 2'hydroxybenzal pyruvate. Sonicated cells were used to start the reaction. The rate of decrease in absorbance at 296 nm was measured. An extinction coefficient of 12.8 $mM^{-1}cm^{-1}$, the difference between that of cis 2'hydroxybenzal pyruvate and the product salicylaldehyde at 296 nm and pH 7.0, was used to calculate the rate of the reaction.

2-Hydroxychromene-2-carboxylic acid and 2'hydroxybenzal pyruvate were prepared by Dr. E.A. Barnsley by a published method (Barnsley, 1976a).

d) <u>Salicylaldehyde dehydrogenase</u>

Salicylaldehyde dehydrogenase was measured by the disappearance of salicylaldehyde (Barnsley, modification of the method of Shamsuzzaman and Barnsley, 1974). The reaction mixture contained 0.05M sodium pyrophosphate buffer (adjusted with HCl to pH 8.9) sufficient for a final volume of 3.0 mls, 50 µl of 9.0 mM salicylaldehyde in ethanol, 100 µl of 42 mM sodium pyruvate, 100 µl of 150 mM NAD^+ and 35 units of

lactic dehydrogenase (M_4, ammonium sulphate suspension obtained from Sigma Chemical Co., St. Louis Mo). Using a trial reaction mixture, the volume of 0.5M NaOH required to bring the pH to 8.9 was determined, and this volume was added to all reaction mixtures. This was needed because the ammonium sulphate suspensions of lactic dehydrogenase are quite acidic. The reaction was started with the addition of sonicated cells, and the rate of decrease in absorbance at 377 nm was measured. An extinction coefficient of 5.52 $mM^{-1}cm^{-1}$, the difference between that of salicylaldehyde and the product salicylate at 377 nm and pH 8.9, was used to calculate the rate.

The function of the excess of pyruvate and lactic dehydrogenase is to maintain the cofactor entirely in the form of NAD^+. A problem arises with the use of crude extracts, as subsequent reactions interfere with the measurements. Therefore by maintaining the cofactor in the form of NAD^+, the reaction is restricted to the conversion of salicylaldehyde to salicylate.

e) Salicylate hydroxylase (Salicylate, NADH:oxygen oxidoreductase E.C. 1.14.13.1)

Salicylate hydroxylase was assayed by measuring the amount of CO_2 released from salicylate (Barnsley, unpublished method). The reaction mixture contained 0.05M phosphate buffer pH 7.5 sufficient for a final volume of 3.0 ml,

200μl of 3.0 mM NADH, 100μl of 0.3 mM FAD and 200μl of a stock solution of salicylate containing 4.95 ml of 1.51 mM sodium salicylate and 50μl of [$^{14}COOH$] salicylate (the final specific activity was 0.095μCi/μ mol). The components of the reaction mixture were added to a 25 ml flask and incubated at 25°C in a shaking water bath for 1-2 minutes. A trap consisting of a strip of filter paper and 0.2 ml of NCS tissue solubilizer (obtained from Amersham, Oakville, Ont.) in a centre well, inserted into a rubber stopper (obtained from Mandel, Rockwood Ont.) was prepared. The reaction was started with the addition of sonicated cells, usually 20μl of a 5 fold dilution for extracts for induced cells. The stopper was immediately closed and the reaction mixture was incubated with gentle shaking for 5 min. The reaction was stopped by the injection of 1 ml of 2.5 M H_2SO_4 through the stopper. The shaking was continued for 1 hour. The stem of the centre well was cut, and the centre well was dropped into a scintillation vial containing 10 ml of Toluene-PPO (6 mg/ml), and counted in a scintillation counter (Beckman LS-233). A sample of the stock solution of salicylate was also counted to obtain the number of counts per μmol of salicylate. The rate was calculated as the number of μmols of CO_2 produced per minute.

f) Catechol 2,3-dioxygenase (catechol: oxygen 2,3 oxido reductase [decyclizing] E.C. 1.13.11.2)

Catechol 2,3-dioxygenase was measured by the accumulation

of 2-hydroxymuconic acid semialdehyde in the reaction mixture (Feist and Hegeman, 1969). The assay was the same as the published method except that a phosphate buffer was used. The reaction mixture contained 0.05M phosphate buffer pH 7.6 sufficient for a final volume of 3.0 ml and 67 µl of 3.0 mM catechol at 25°C. Sonicated cells were used to start the reaction. The increase in absorbance at 375 nm was measured. An extinction coefficient for 2-hydroxymuconic acid semialdehyde of 33 $mM^{-1}cm^{-1}$ at 375 nm was used to calculate the rate of the reaction.

The following method was used to measure the activity of catechol 2,3 dioxygenase in suspensions of whole cells. A volume of 0.5 ml of culture was diluted into 2.0 ml of phosphate buffer pH 7.6 in a cuvette. The absorbance at 600 nm and 375 nm was measured. This suspension was incubated for 13 minutes at room temperature with hexadecyltrimethylammonium bromide. The final concentration of hexadecyltrimethylammonium bromide required to maximize the observed reaction was dependent on the concentration of cells. For a suspension with an absorbance of 0.2 at 600 nm, the final concentration of hexadecyltrimethylammonium bromide was 40 µg/ml. The reaction was started by the addition of 67 µl of 3.0 mM catechol, and the increase in absorbance at 375 nm was measured.

g) <u>Catechol 1,2-dioxygenase</u> (Catechol: oxygen oxido reductase [decyclizing] E.C. 1.13.11.1)

Catechol 1,2-dioxygenase was measured by the accumulation of cis, cis-muconate in the reaction mixture (Hegeman, 1966) after catechol 2,3-dioxygenase had been deactivated with a minimum of H_2O_2 (0.0005% final concentration) (Barnsley, 1976). The reaction mixture contained 0.05 phosphate buffer pH 7.0 sufficient for a final volume of 3.0 ml, 100 µl of 40 mM EDTA (to inactivate the muconate lactonizing enzyme) and 100µl of 3.0 mM catechol at 25°C. Sonicated cells were used to start the reaction. The increase in absorbance at 260 nm was measured. An extinction coefficient for cis,cis-muconate at 260 nm of 16.8 $mM^{-1}cm^{-1}$ was used to calculate the rate of the reaction.

h) 2-Hydroxymuconic acid semialdehyde dehydrogenase

2-Hydroxymuconic acid semialdehyde dehydrogenase was measured by the disappearance of 2-hydroxymuconic acid semialdehyde from the reaction mixture (Sala-Trepat and Evans, 1971).

2-Hydroxymuconic acid semi-aldehyde was prepared in the following way immediately before it was required.

Sonicated cells (about 0.1 ml) from a culture of Pseudomonas P_G, a strain constitutive for catechol 2,3 dioxygenase were added to 50 ml of 0.04 mM catechol in 0.05 M phosphate buffer pH 7.6. The absorbance at 375 nm was monitored at intervals. When the reaction was completed, indicated by no further increase in absorbance, the pH of the solution was

adjusted to 2.5 with HCl. The 2-hydroxymuconic semialdehyde was extracted into 50 ml of ether. The ether layer was then extracted into 20 ml of 0.05M phosphate buffer pH 7.6. Residual ether was evaporated by swirling a flask containing the product in a water bath at 37°C for a few minutes. A volume of this solution (usually 50-100 µl) was diluted with 0.05 M phosphate buffer pH 7.6 such that a final volume of 2.9 ml had an absorbance of 1.0 at 375 nm.

Sonicated cells were added to the solution of 2-hydroxymuconic semialdehyde in 0.05M phosphate buffer, pH 7.6. Sometimes a small, but significant, hydrolase activity was present, which was measured first. Then 100µl of 10 mM NAD^+ were added to start the dehydrogenase reaction. The rate due to the hydrolase was subtracted from the rate resulting from the addition of NAD to give the rate due to the dehydrogenase. An extinction coefficient for 2-hydroxymuconic acid semialdehyde at 375 nm of 33 $mM^{-1}cm^{-1}$ was used to calculate the rate of the reaction.

D. Curing

The method for curing was modified from a published method for curing with mitomycin C (Dunn and Gunsalus, 1973; Rheinwald et al., 1973), as suggested by J.B. Johnson (personal communication to E.A. Barnsley).

A culture was grown to stationary phase overnight in L-broth. The culture was diluted into three sets of tubes to

give each set respectively concentrations of 10^3, 10^4 and 10^5 cells per ml. To each set of tubes, a range of concentrations of curing agent was added, and the cultures were incubated with shaking at 25°C for 48 hours. The best curing agent and its concentration have to be established separately for each strain used. From tubes which showed no growth after 24 hours, but growth after 48 hours, the cultures which gave growth with the highest concentration of curing agent were selected for subculture on plates of L-broth. After growth, the colonies were replica plated on to selective media and scored.

E. Isolation of Amino Acid Auxotrophs

The production of and selection for amino acid auxotrophs was based on the method of Ornston et al. (1969). The procedure followed in this study is summarized in figure 2.

As the enrichment process does not result in the isolation of independent mutants, four separate cultures were used for each strain. At the beginning of each enrichment cycle the activity of catechol 2,3 dioxygenase was measured by the method for whole cell suspensions for all cultures of Pseudomonas P_G and Pseudomonas NCIB 9816 to confirm that the individual cultures as a whole still had the characteristic constitutive activity of catechol 2,3 dioxygenase. Those cultures which did not, were discarded.

Following selection, the amino acid auxotrophic strains

were tested for reversion. Cultures of the auxotrophs were incubated overnight on mineral salts containing a limiting amount of succinate (0.04%) and a limiting amount of the required amino acid (20 μg/ml). The following morning, additional succinate and the required amino acid were added to final concentrations of 0.2% and 100μg/ml respectively.

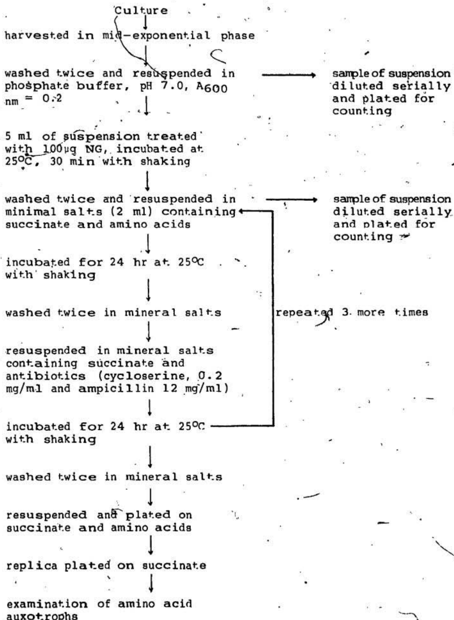

Figure 2. Mutagenesis and Enrichment for Amino Acid Auxotrophs

When the absorbances at 600 nm of the cultures had doubled, the cultures were centrifuged. The cells were resuspended in mineral salts medium to give an absorbance of 4 at 600 nm, which provided a concentration of cells of the order of 10^9 bacteria per ml. Volumes of 0.2 ml were spread on agar plates containing mineral salts plus succinate. The plates were incubated at 25°C for six days and then were scored.

The amino acid auxotrophic derivatives of P_G and NCIB 9816 were tested for the characteristic properties of catechol 2,3-dioxygenase.

F. Conjugation

Two methods for conjugating plasmid-bearing strains with plasmid-free strains were used. In one method, conjugation was carried out in liquid culture according to published procedures (Williams and Murray, 1974). In the other method, conjugation was carried out on agar plates containing L-broth (suggested by D. Bradley, personal communication to E.A. Barnsley).

a) Method I

Donor and recipient cultures were grown overnight on L-broth with shaking, at 25°C. Thirty minutes prior to the conjugation experiment, a sample of donor culture was diluted ten fold into fresh L-broth and was incubated with shaking at 25°C. After 30 minutes, 0.5 ml of the donor culture was

mixed with 0.5 ml of the recipient culture in the stationary phase, and the mixture was incubated without shaking at 25°C for 30 minutes. Sterile 1% saline (10 ml) was then added, and the mixture was centrifuged. The pellet was resuspended in 1.0 ml of sterile 1% saline, and the suspension was serially diluted. Samples of 0.1 ml from the undiluted suspension, 10, 10^2 and 10^3 fold dilutions were spread on plates selective for the exconjugates. Samples of 0.1 ml were spread from the 10^5, 10^6 and 10^7 fold dilutions on plates selective for recipients and on plates selective for donors. As controls, samples from the overnight cultures were centrifuged, washed with sterile 1% saline, and were spread on plates selective for exconjugates.

b) Method II

Donor and recipient cultures were grown overnight in L-broth with shaking at 25°C. Thirty minutes prior to the conjugation experiment, a sample of the donor culture was diluted ten fold into fresh L-broth, and was incubated with shaking at 25°C. After 30 minutes, 0.5 ml of the donor culture and 0.5 ml of the recipient culture were mixed on undried plates of L-broth agar. The plates were incubated at 25°C for two hours. Each plate was washed with 10 ml of sterile 1% saline, the suspension was centrifuged, and the pellet was resuspended in 1.0 ml of sterile 1% saline. Dilutions and plating were carried out as described for

Method I.

G. Isolation of Plasmid DNA

All solutions, plasticware and glassware required for the isolation of plasmid DNA were sterile.

a) Method I

The following method is a modification of the method of Birnboim and Doly (1979).

One litre of cells growing on L-broth was harvested when the turbidity reached an absorbance of 1.0-1.2. The cells were washed in sucrose/Tris buffer (25% sucrose w/v, 10 mM Tris, pH 8.0) to remove residual growth medium, and were resuspended in 65 ml of sucrose/Tris buffer. The cells were lysed by the addition of 200 ml of freshly prepared alkaline SDS-EDTA (0.2 M NaOH, 1% SDS, 4.4 mM EDTA). After the solution had stood at room temperature for 15 minutes, 113 ml of acidic sodium acetate (sodium acetate/acetic acid, 4 M with respect to sodium acetate, pH 4.8 when diluted 10 fold) were added, and the mixture was left on ice for 75 minutes. The suspension was centrifuged at room temperature at 10,000xg for 20 minutes. An equal volume of cold (-20°C) isopropanol was mixed with the supernatant, and the mixture was stored at -20°C overnight. The precipitated DNA was collected by centrifugation at 10,000xg rpm in a Sorvall RC2-B centrifuge at -10°C for 15 minutes. The pellet was dissolved in TES buffer (30 mM Tris, 5 mM EDTA,

50 mM NaCl pH 8.0). The sample was stored at -20°C until required.

This method can be scaled down to isolate plasmid DNA from samples of 1.5 ml of culture.

b) Method II

The method of Falkow (Guerry et al., 1973 and Meyers et al., 1976) was used as published, except that the reagents were scaled up proportionally for volumes of 1 litre of culture. This method differed from the above in that lysozyme, EDTA, and SDS were used to lyse the cells, and a high salt concentration (1M NaCl final concentration) was used to precipitate the chromosomal DNA-membrane complex. RNA was removed by treatment with RNase and protein was removed by extraction with Tris-saturated phenol.

H. Purification of Plasmid DNA by Density Gradient Centrifugation with Caesium Chloride-Ethidium Bromide

Crude preparations of plasmid DNA were purified by density gradient centrifugation with caesium chloride-ethidium bromide (Radloff et al., 1967).

The nucleic acid content of the preparation was measured by diluting 10µl of the preparation into 3.0 ml of phosphate buffer (pH 8.0) in a 1 cm cuvette and measuring the increase in absorbance at 260 nm. The absorbance of the undiluted preparation was calculated, and the volume used for purification was such that the product of the volume and the calculated

absorbance was 144.

The purification steps were carried out under a dull red light, and the DNA was not exposed to white light until after the final dialysis.

For centrifugation, 6.0 ml of a saturated aqueous solution of caesium chloride, 1.0 ml of an ethidium bromide solution (3 mg/ml TES) and the crude sample diluted to 2.0 ml with TES were mixed together. The refractive index of this solution was adjusted to 1.3900 ± 0.0005 with the solution of CsCl in TES, as required. The solution was centrifuged at 40,000 rpm for 40-43 hours at 15°C in a Beckman L3-50 centrifuge with a 50 Ti rotor. Fractions of 0.2 ml each were collected and a 20 μl sample from every second fraction was electrophoresed in a 0.5% agarose gel (type V agarose, Sigma Chemical Co.) in Tris-borate-EDTA buffer (89 mM Tris, 2.5 mM disodium EDTA, 89 mM boric acid, pH not adjusted, approximately 8.0) at a field strength of 5 volts/cm until a marker of bromophenol blue had run 10 cm.

The gel was stained with ethidium bromide in water (0.4 μg/ml) and viewed over a C-61 transilluminator (Ultra Violet Products Inc., San Gabriel, California).

Those fractions from the gradient containing plasmid DNA were pooled. The ethidium bromide was extracted with isopropanol equilibrated with saturated NaCl. The preparation was dialysed against three separate 1 litre lots of Tris-EDTA buffer (1 mM Tris - 0.1 mM EDTA pH 8.0) at 4°C in

bottles wrapped in aluminium foil to exclude light.

The contents of the dialysis bag were transferred to a test tube, and the dialysis bag was rinsed with a small amount of sterile water. The volume was reduced to approximately one tenth of the volume after dialysis by extraction of water with isobutanol. The preparation was divided into smaller portions before storing at -20ºC.

The quantities of plasmid DNA obtained following purification on a caesium chloride-ethidium bromide gradient were too small to determine the concentration. The volumes of the purified plasmid DNA required for digestion with restriction endonucleases and for nick translation were determined by electrophoresing small quantities from the sample and selecting a suitable volume that gave a clearly visible band in the gel after staining.

I. Digestion with Restriction Endonucleases

Bam HI and Hind III restriction endonucleases were obtained from Bethesda Research Laboratories (Rockville, Maryland, U.S.A.).

Digestion with the restriction endonucleases was carried out at 37ºC for seven hours. The medium for Bam HI contained 20 mM Tris-HCl, 100 mM NaCl, 7 mM $MgCl_2$, at pH 7.0, that for Hind III contained 20 mM Tris-HCl, 60 mM NaCl, 7 mM $MgCl_2$ at pH 7.4. The total volume of the reaction mixture was 50 µl. The buffers were prepared from stock solutions at concentrations

10x the final concentration to allow for dilution by the plasmid DNA and the restriction endonucleases. The stock solutions of buffers and the restriction endonucleases were stored at -20°C until required. The reaction was carried out in a 1.5 ml capped plastic centrifuge tube. Just prior to incubation, the tubes were centrifuged in an Eppendorf centrifuge for 1 second to ensure that all the reactants were mixed and were located at the bottom of the tube. After the period of incubation, the reaction was stopped by heating at 68°C for 10 minutes, 25 µl of agarose beads were added, and the sample was stored at -20°C.

J. Electrophoresis

Volumes of plasmid DNA (20 µl) isolated from crude preparations or from CsCl density centrifugation were electrophoresed in gels of 0.5% agarose (type V, Sigma) in Tris-EDTA-borate buffer (89 mM Tris, 2.5 mM disodium EDTA, 89 mM boric acid, pH not adjusted, approximately 8) at a field strength of 5 volts/cm. The gels were stained in ethidium bromide solution (0.4 µg/ml of H_2O) for 15 minutes, and viewed over an ultraviolet light source.

Volumes of 37µl from digestions by restriction endonucleases were electrophoresed in gels of 0.8% agarose containing 0.5 g/ml ethidium bromide, in Tris-EDTA-borate buffer at a field strength of 5 volts/cm.

K. Southern Blots

The BRL-DNA Blot System (Bethesda Research Laboratories, Rockville, Maryland, U.S.A.) was used to transfer the restriction fragments of plasmid DNA from the gel to a nitrocellulose filter according to the method of Southern (1975).

The gel was placed in a denaturation bath of NaOH (0.5 M)-NaCl (1.5 M). It was fully submerged and gently agitated in the bath for one hour. After denaturation the gel was immersed in a neutralizing bath of NaCl (3.0 M) - Tris-HCl (0.5 M pH 7.0) and agitated gently for one hour.

Excess buffer was blotted from the gel, the loading wells were trimmed off, and a wicking sheet was placed over the gel so that 3-4 cm extended on each side. The support was placed over the wicking paper. Air pockets between the gel and the wicking paper and between the wicking paper and the support were avoided. The apparatus was then inverted.

A nitrocellulose sheet was soaked in distilled H_2O for a few minutes, transferred to the blotting buffer for one minute, and then placed over the gel. Air pockets between the gel and the nitrocellulose were avoided. A stack of blotting pads was placed on top of the nitrocellulose and the assembly was placed in the buffer tray. The free ends of the wicking paper were placed in the blotting buffer. The blotting buffer was composed of NaCl (3.0 M) - Na citrate (0.3 M, pH 7.0). The time required for the transfer of DNA was about 20 hours. The gel was examined over a UV light

source to confirm that the DNA had been transferred.

The nitrocellulose filter was cut with a flamed blade to the required size for the hybridization chambers, and then soaked in dilute saline citrate (0.3 M NaCl, 0.03 M Na citrate, pH 7.0) for 10 minutes. After transfer of DNA the filter was dried at 37°C for 30 minutes and then baked at 80°C *in vacuo* for two hours, to bind the DNA to the filter.

L. Nick Translation

Plasmid DNA was labelled with [^{3}H]TTP using a nick translation kit from New England Nuclear (NEK-005). The method was based on that of Rigby et al. (1977).

The [^{3}H] TTP was lyophilized in a vacuum desiccator for 30-45 minutes. When the solvent had been removed completely, the required reagents were added to the tube containing the [^{3}H]TTP in the following order: 5 μl of nick translation buffer, 4 μl of unlabelled deoxynucleotide triphosphate mixture, up to 7 μl of plasmid DNA, and nick translation grade H_2O such that the total volume of plasmid DNA and H_2O was 7μl. To start the reaction, 2μl of DNase I were added. The reaction mixture was allowed to stand at room temperature for 2 min (Heinaru et al., 1978), prior to incubation at 12-14°C for three hours.

The extent of the labelling was measured at one hour intervals by the removal of 1 μl volumes of reaction mixture. The samples were added to tubes containing 100 μl of nick

translation stop buffer at 0°C. The DNA was precipitated by the addition of 1 ml of cold 5% TCA. To ensure the precipitation of all the DNA, the tubes were kept at 0°C for 15 minutes. The DNA was collected on fibre glass filter discs (Whatman GF/C) with a vacuum filtration apparatus. The filters were washed twice with 2 ml of 5% TCA, twice with 2 ml of 95% ethanol, and were dried under a heat lamp for 30 minutes. The filter discs were placed in vials containing toluene-PPO, and were counted in a scintillation counter.

The main nick translation reaction was stopped by the addition of 10 μl of 0.25 M EDTA, pH 7.4 and heating at 68°C for 10 minutes to inactivate the enzyme (Rigby et al., 1977), and the preparation was then stored at -20°C.

The unreacted triphosphates were removed by passing the mixture through a column of Sephadex G 50 (fine, 20-80 pore size, Pharmacia) with a bed volume of 7 ml equilibrated with Tris-HCl (10 mM, pH 8.0) - NaCl (10 mM) - EDTA (2 mM). The fractions containing the plasmid DNA were detected by counting 1 μl volumes from each fraction. The fractions containing the labelled DNA were pooled, and stored at -20°C.

M. Hybridization

The conditions for hybridization were described in Heinaru et al. (1978). The experiments were carried out in duplicate.

The nitrocellulose filters were pre-incubated for six

hours at 65°C in a solution of SSC at a three fold concentration (SSC is 0.15M NaCl and 0.015 M sodium citrate, pH 7.0) containing 0.02% w/v each of ficoll, polyvinylpyrolidone and bovine albumin fraction V (Denhardt, 1966).

Samples of the radioactively labelled nucleic acid containing 10^6 cpm were denatured for five minutes in a boiling water bath and were cooled rapidly in iced water.

The hybridization solution contained the single stranded labelled DNA, 0.02% each of ficoll, polyvinyl, pyrolidone and bovine albumin fraction V, and 0.2% w/v SDS in SSCP at a four fold concentration (SSCP is 0.12 M NaCl, 15 mM sodium citrate, 15 mM KH_2PO_4, 1 mM EDTA, pH 7.2). The final volume of this solution was 10 ml. The hybridization was carried out in hybridization chambers which measured 4 cm x 9.5 cm x 2.5 cm. The nitrocellulose filter was placed in the hybridization chamber with the side to which the DNA was bound facing upwards. The incubation was carried out at 65°C for 40 hours.

The filter was washed in SSC buffer at a four fold concentration containing 0.05% w/v SDS at 65°C for two hours, and then in SSC buffer at a four fold concentration at 65°C for 30 minutes. The filter was briefly rinsed in SSC buffer at a two fold concentration at room temperature, and then was dried at 37°C for one hour.

The filter was dipped in a solution of 20% w/v PPO in toluene, and placed against X-ray film (Curix RPI X-Ray

film, Agfa - Gavaert, Toronto, Ontario) (Randerath, 1970; Bonner and Laskey, 1974). The film and filter were held together between two glass plates. The package was wrapped in aluminium and was stored at -70°C for a minimum of two weeks. At the end of two weeks, one of the pair was developed. If necessary, the second was left at -70°C for a longer period.

RESULTS

A. Curing

The method reported in Rheinwald et. al. (1973) and Dunn and Gunsalus (1973) was used to try to cure the naphthalene metabolizing strains NCIB 9816, P_G, ATCC 17483 and ATCC 17484. PpG7, the strain of Dunn and Gunsalus was used as a control for the curing method. Instead of curing, treatments with mitomycin C resulted in the generation of slowly growing mutants, with frequencies for PpG7 which ranged from 2-5%, dependent upon the concentration of mitomycin C. The mutants all showed a partial reversion to the wild type. The examination of a single clone grown on succinate and induced with salicylate showed that the level of naphthalene oxygenase was 54-57% of that in the wild type.

The method of curing was modified to that described in the section on methods. Cells in late stationary phase instead of early stationary phase were used to inoculate the medium containing the curing agent, and a range of bacterial concentrations was used instead of a single concentration. Mitomycin C and ethidium bromide were used as the curing agents. The results are shown in Table III.

The effective concentration of curing agent was particular to each strain. The percentage of curing was low for NCIB 9816 when mitomycin C was used as the curing agent, and P_G did not cure at all. Good percentages of curing were obtained for NCIB 9816 and P_G when ethidium bromide was used as the

Table III Curing of Naphthalene Metabolizing Pseudomonads

Strain	Curing agent	concentration	frequency of curing
PpG7	Mitomycin C	10 μg/ml	12-49%
	Ethidium bromide	10 μg/ml	<0.1%
NCIB 9816	Mitomycin C	3 μg/ml	2-3%
	Ethidium bromide	10 μg/ml	3-17%
P_G	Mitomycin C	1 μg/ml	<0.4%
	Ethidium bromide	10 μg/ml	5-40%
ATCC 17483	Mitomycin C	0.1 μg/ml	<0.4%
	Ethidium bromide	10 μg/ml	<0.2%
ATCC 17484	Mitomycin C	3 μg/ml	<0.4%
	Ethidium bromide	5 μg/ml	<0.05%
AC 545	Mitomycin C	10 μg/ml	<0.06%
	Ethidium bromide	300 μg/ml	4%

curing agent, but PpG7 did not cure. Neither ATCC 17483 nor ATCC 17484 were cured by mitomycin C or ethidium bromide. Both mitomycin C and acridine orange were very toxic to ATCC 17483, the concentrations which permitted growth were very low. A single attempt to cure these strains with acridine orange was not successful. Exposure to acridine orange merely resulted in the production of slowly growing mutants. Attempts to cure ATCC 17483 and ATCC 17484 with NG also resulted in the production of slowly growing mutants. Treatment of ATCC 17483 with NG did produce some clones that were Nah$^-$, but the enzymes of the naphthalene pathway were found to be inducible, although at lower levels than in the wild type. This situation was investigated further (see section G).

Potentially cured clones were selected at random and were streaked out to single colonies on agar plates plus succinate. A single clone from each strain was tested for growth on benzoate, salicylate and naphthalene. A small number of potentially cured strains either reverted to growth on naphthalene or showed a Sal$^+$ phenotype and therefore were mutants. In addition to colony colour and morphology, growth on benzoate indicated that the strain was probably a derivative of the parent strain, and not a contaminant.

Strains which grew on benzoate (with the exception of ATCC 17483 derivatives) and were Nah$^-$Sal$^-$ were selected to be assayed for the enzymes of the naphthalene and meta

pathways. The results for the derivatives of PpG7, NCIB 9816 and P_G that were used in later experiments are shown in Table IV, but in all strains tested the whole block of enzymes was deleted. Those strains which reverted, or which were inducible or grew slowly were deemed to be mutants. Those strains which had lost a block of enzyme activities were assumed to have been cured.

B. Mutagenesis and Selection for Amino Acid Auxotrophs

The viability of the cells after treatment with NG was about 5% for all strains. Exposure to NG can result in the production of amino acid auxotrophs, but at very low frequencies. This situation was improved upon by using an enrichment process following the mutagenesis. As the enrichment process does not produce independently generated mutants, four separate mutagenesis experiments were carried out for each strain. Due to the method used, it was not possible to calculate the frequency of mutation. However, despite the enrichment process, amino acid auxotrophic mutants were not produced in very great numbers.

The constitutive properties of catechol 2,3 dioxygenases in strains NCIB 9816 and P_G appeared to be very sensitive to the process of mutagenesis and enrichment, as the enzyme became inducible in some of the mutants of these strains. It became necessary to monitor the activity of catechol 2,3 dioxygenase in these strains during the enrichment process.

Table IV Activities of Naphthalene Pathway Enzymes in Wildtype and Cured Strains of Naphthalene Metabolizing Pseudomonads

	Specific Activity (µmol/min per mg protein) of:					
	Naphthalene Oxygenase	1,2 Dihydroxy Naphthalene Oxygenase	Salicylaldehyde Dehydrogenase	Salicylate Hydroxylase	Catechol 2,3 Dioxygenase	HOMASA* Dehydrogenase
PpG7	0.003	0.01	0.121	0.002	0.011	-
PpG7-induced	0.29	1.5	1.67	0.072	0.59	-
MC 125	N.D.	N.D.	N.D.	N.D.	N.D.	-
MC 125-induced	N.D.	N.D.	N.D.	N.D.	N.D.	-
NCIB 9816	0.014	0.242	0.214	0.005	0.05	0.010
NCIB 9816-induced	0.204	1.39	1.35	0.044	0.04	0.008
MC 119	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
MC 119-induced	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
P_G	0.009	0.299	0.039	0.006	0.512	0.07
P_G-induced	0.225	1.60	1.28	0.080	0.638	0.07
MC 96	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
MC 96-induced	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

The strains were grown in liquid culture with succinate as the carbon source, induced cultures were grown in the presence of a gratuitous inducer, 2-aminobenzoate, (final concentration, 0.3 mM) for 1.5 - 2 generations.

*Abbreviations: HOMASA - Hydroxymuconic acid semialdehyde; N.D. Not Detected; -, means not measured.

Catechol 2,3 dioxygenase was assayed by the method for whole cell suspensions. Of the four separate populations of cells for each strain, one lost the constitutive property of catechol 2,3 dioxygenase.

Following selection for the amino acid auxotrophs, those clones which had shown good growth on the selective plates were tested for reversion. Those strains which grew at rates comparable to the wild type strains, had low reversion rates and showed the characteristic enzymic activities of catechol 2,3 dioxygenase, were selected for use in further experiments (Table V).

C. Conjugation Experiments

Attempts were made to conjugate naphthalene-metabolizing strains with strains in which the enzymes of the naphthalene pathway were absent. The results of successful and unsuccessful conjugations are shown in Tables VI and VII respectively.

Amino acid auxotrophic derivatives of the naphthalene metabolizing strains with phenotypes $Nah^{+}Leu^{-}$ were crossed with cured derivatives of PpG7, NCIB 9816 and P_G with phenotypes Nah^{-} as described in method I. Selection was made for Nah^{+} phenotypes. Strains MC 86, MC 190 and MC 192 were found to conjugate with the cured strains with high frequency. As the plasmid in pPG7 had been designated as NAH (Dunn and Gunsalus, 1973) the presumptive plasmids in MC 190 and MC 192 were designated as NAH2 and NAH3 respectively. Strains

Table V Reversion Rates and Enzymic Activities in Amino Acid Auxotrophs Derived from Naphthalene-Metabolizing Pseudomonads

Strain and Phenotype		Reversion Rate	Specific Activity (in µmols/min per mg protein) of		
				Naphthalene Oxygenase	Catechol 2,3 Dioxygenase
MC 86	$Nah^{+}Leu^{-}$	$\leq$ 6 in 10^9	non-induced	0.017	0.008
			induced	0.210	0.542
MC 190	$Nah^{+}Leu^{-}$	$\leq$ 1 in 10^9	non-induced	0.018	0.050
			induced	0.371	0.046
MC 192	$Nah^{+}Leu^{-}$	$\leq$ 5 in 10^9	non-induced	0.012	0.836
			induced	0.406	-
MC 189	$Nah^{+}Leu^{-}$	$\leq$ 5 in 10^9	non-induced	0.01	0.002
			induced	0.19	0.432
MC 129	$Nah^{+}Leu^{-}$	$\leq$ 2 in 10^8		-	-

Strains were grown on succinate, induced with 2-aminobenzoate for one generation and were then harvested.

- means not measured

MC 189 and MC 129 failed to conjugate with the cured strains.

Strains PpG7, NCIB 9816, P_G, ATCC 17483, ATCC 17484 and AC 545 with phenotypes of Nah^+ were crossed with PaW 340 with a phenotype of Nah^-Str^R as described in method I and selection was made for Nah^+Str^R phenotypes. Only strains PpG7 and AC545 were able to conjugate with PaW 340, and the strains NCIB 9816, P_G, ATCC 17483 and ATCC 17484 failed to conjugate even when the period of incubation of the conjugation mixture was increased to two hours, or when the conjugation was carried out as described in method II. Strain MC 234, a derivative of PpG7 containing NAH2, was able to conjugate with PaW 340 only when method II was used. Strain MC 239, a derivative of PpG7 containing NAH3 failed to conjugate with PaW 330.

Strains MC 86, MC 190 and MC 192 conjugated with MC 213, a cured derivative of AC 545 with high frequencies. Exconjugates from these crosses were used as donor strains for conjugation with PaW 330. Strain MC 256 containing NAH2 and strain MC 262, containing NAH3, conjugated readily when method I was followed. Strains MC 86 and MC 250, containing NAH, conjugated with PaW 330 only when method II was followed.

The enzymic activities of catechol 1,2 dioxygenase and the first two enzymes of the meta cleavage pathway were examined in exconjugates containing NAH, NAH2 and NAH3 (Table VIII). Catechol 2,3 dioxygenase was inducible in strain MC 281 at a level comparable to MC 86. Thus the

Table VI Conjugation of Naphthalene-Metabolizing Pseudomonads with Cured Strains

Donor strain/plasmid	Recipient strain	Method	Frequency of conjugation*	Exconjugates selected for later work
MC 86/NAH	MC 125	I	5×10^{-3}	
MC 190/NAH2	MC 125	I	2×10^{-3}	MC 234
MC 192/NAH3	MC 125	I	3×10^{-3}	MC 239
MC 86/NAH	MC 119	I	5×10^{-3}	
MC 190/NAH2	MC 119	I	1×10^{-3}	
MC 192/NAH3	MC 119	I	9×10^{-4}	
MC 86/NAH	MC 96	I	3×10^{-3}	
MC 190/NAH2	MC 96	I	1×10^{-3}	
MC 192/NAH3	MC 96	I	8×10^{-4}	
PpG7/NAH	PaW 340	I	2×10^{-5}	
AC 545/SAL	PaW 340	I	5×10^{-4}	
PpG7/NAH	PaW 340	II	5×10^{-6}	
MC 234/NAH2	PaW 340	II	1×10^{-7}	
MC 86/NAH	MC 213	I	1×10^{-2}	MC 250
MC 190/NAH2	MC 213	I	3×10^{-4}	MC 256
MC 192/NAH3	MC 213	I	1×10^{-3}	MC 262
MC 86/NAH	PaW 330	II	5×10^{-6}	MC 281
MC 250/NAH	PaW 330	II	1×10^{-6}	
MC 256/NAH2	PaW 330	I	1×10^{-3}	MC 274
MC 262/NAH3	PaW 330	I	4×10^{-4}	MC 268

*defined as the number of exconjugates per number of donor cells.

Table VII. Attempted Conjugation of Naphthalene-Metabolizing Pseudomonads with Cured Strains

Donor strain	Recipient strain	Upper limit of frequency*
MC 189 (17483)	MC 125	<1 in $3.0x10^{7}$
MC 189	MC 119	<1 in $3.0x10^{7}$
MC 189	MC 96	<1 in $3.0x10^{7}$
MC 129 (17484)	MC 125	<1 in $3.8x10^{7}$
MC 129	MC 119	<1 in $3.8x10^{7}$
MC 129	MC 96	<1 in $3.8x10^{7}$
NCIB 9816	PaW 340	<1 in $4.7x10^{8}$
P_G	PaW 340	<1 in $3.9x10^{8}$
ATCC 17483	PaW 340	<1 in $4.1x10^{7}$
ATCC 17484	PaW 340	<1 in $7.6x10^{8}$
MC 239 (NAH3)	PaW 340	<1 in $2.4x10^{8}$

*defined as number of exconjugates per number of donor cells.

Table VIII Activities of Catechol 1,2 Dioxygenase and Meta Pathway Enzymes in Strains Containing NAH, NAH2 and NAH3.

Strain	Plasmid	Carbon Source	Activity (µmol/min per mg protein) of: Catechol 1,2 Dioxygenase	Catechol 2,3 Dioxygenase	HOMASA Dehydrogenase
MC 86	NAH	Naphthalene	0.015	1.07	–
		Succinate	<0.001	0.008	
MC 281	NAH	Naphthalene	0.147	0.99	–
		Succinate	<0.001	0.002	
MC 190	NAH2	Naphthalene	0.202	0.017	0.005
		Succinate	<0.002	0.049	0.007
MC 256	NAH2	Naphthalene	0.188	0.018	–
		Succinate	<0.002	0.008	–
MC 274	NAH2	Naphthalene	0.300	0.031	0.006
		Succinate	<0.002	0.021	0.005
MC 192	NAH3	Naphthalene	–	0.418	0.130
		Succinate	–	0.405	0.136
MC 262	NAH3	Succinate	–	0.356	–
MC 268	NAH3	Naphthalene	–	0.410	0.125
		Succinate	–	0.425	0.131
MC 213	none	Succinate	–	<0.001	0.001
PaW 330	none	Succinate	–	<0.001	0.001

– means not measured

characteristic regulatory properties associated with NAH2 and NAH3 were transferred with these plasmids. The low constitutive levels of catechol 2,3 dioxygenase and hydroxymuconic acid semialdehyde dehydrogenase are similar in MC 190 and MC 274 and are not induced further during growth on naphthalene. The high constitutive levels of catechol 2,3 dioxygenase and hydroxymuconic acid semialdehyde dehydrogenase are similar in MC 192 and MC 268, and are not induced further during growth on naphthalene. The chromosomally located catechol 1,2 dioxygenase was inducible in all strains tested.

D. The Isolation and Purification of Plasmid DNA

Plasmid DNA was isolated from PpG7, MC 281, NCIB 9816, MC 274, P_G, MC 268 and ATCC 17484 according to the method outlined in the section on materials and methods (Figures 3 and 4). Plasmid DNA was not present in PaW 330, the background strain for MC 281, MC 274 and MC 268. PpG7 and MC 281 each contained one plasmid. Strains NCIB 9816 and P_G each contained two plasmids. It was the larger of the two plasmids that was transferred to MC 274 and MC 268 respectively, and was associated with the ability to degrade naphthalene. The transfer of plasmids NAH, NAH2 and NAH3 from their original strains into PaW 330 did not appear to result in a reduction of molecular weight. These three plasmids were not separated by electrophoresis in agarose gels. Strain ATCC 17484

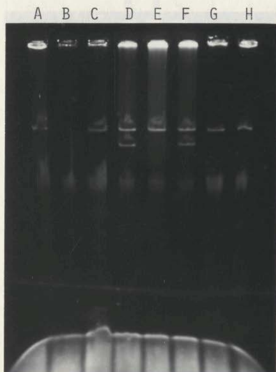

Figure 3. Naphthalene plasmids in their original hosts and after transfer to PaW 330. The lanes contain: A, PpG7, B, PaW 330; C, MC 268; D, P_G, E, MC 274; F, NCIB 9816; G, MC 281; H, PpG7.

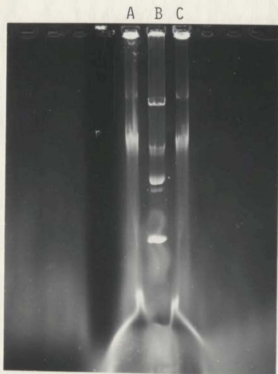

Figure 4. Plasmids in ATCC 17484. Lanes A and C contain PpG7, lane B contains plasmids from ATCC 17484.

contained multiple plasmids, the largest plasmid appeared to be slightly smaller than NAH on the basis of electrophoretic mobility. It was not possible to detect any plasmids in ATCC 17483 by this method, or any other.

The samples of plasmid DNA isolated from MC 281, MC 268 and MC 274 were contaminated by unidentified pieces of DNA, presumably sheared low molecular pieces of chromosomal DNA, and RNA. The plasmid DNA was purified by caesium chloride-ethidium bromide density gradient centrifugation.

The initial yields of purified plasmid DNA, based on the intensity of the bands after electrophoresis, were low. The yield was improved by carrying out the fractionation of the gradient and the removal of ethidium bromide and CsCl in the dark. This presumably reduced nicking of the plasmid DNA caused by light.

Difficulties in separating the band containing the plasmid DNA from the band containing linear and nicked circular DNA were resolved in two ways. A peristalic pump was used to fractionate the gradient at a slow rate (1 ml per 4 minutes 25 seconds). Some mixing of the two bands still occurred during fractionation, but it was possible to obtain fairly clean fractions of plasmid DNA. The amount of crude DNA that was applied to the CsCl-ethidium bromide was restricted, as described in the section on materials and methods. When larger amounts of a crude preparation was used, the number of fractions containing plasmid DNA only

was few, and many fractions containing plasmid DNA were still contaminated with linear and nicked circular DNA. When smaller amounts of a crude preparation were used, an insufficient amount of purified plasmid DNA was obtained.

It was not possible to remove completely the contaminating DNA with one density gradient centrifugation. A second centrifugation removed more of the contaminating material but not all of it, and the quantity of purified plasmid DNA obtained was reduced further. As a compromise, only one density gradient centrifugation was used to purify the plasmid DNA.

E. Digestion of Plasmid DNA with Restriction Endonucleases

Plasmids NAH, NAH2 and NAH3, isolated from strains MC 281, MC 274 and MC 268 respectively, were digested with the restriction endonuclease Bam HI and Hind III. The pattern of fragments obtained from NAH2 and NAH3 were identical with both restriction endonucleases. The patterns obtained from NAH differed substantially from those obtained from NAH2 and NAH3 (Figures 5 and 6). Digestion with Hind III produced 11 fragments from NAH and 20 from NAH2 and NAH3. Of these fragments, only two appear to be common to all three strains. Digestion with Bam HI produced 10 fragments from NAH and 4 fragments from NAH2 and NAH3. Of these fragments, one might be common to all three plasmids.

Assuming that the first intense band resulting from

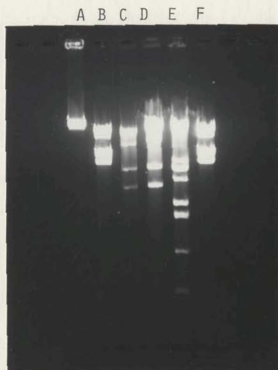

Figure 5. Restriction patterns of naphthalene plasmids NAH, NAH2 and NAH3 digested with Bam HI. The lanes contain: A, undigested λ; B, λ; C, NAH2; D, NAH3; E, NAH; F λ

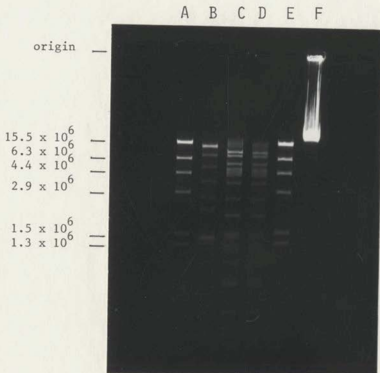

Figure 6. Restriction patterns of naphthalene plasmids NAH, NAH2 and NAH3 digested with Hind III. The lanes contain: A, λ , B NAH; C, NAH2; D, NAH3; E, λ ; F, undigested

digestion with Hind III is two unresolved fragments (Farrell et al, 1978), the sum of the fragments of NAH is $52x10^6$. The sum of the fragments resulting from digestion with Hind III of NAH2 and NAH3 is $77x10^6$.

F. Hybridization Experiments

Plasmids NAH and NAH3 were labelled with tritiated thymidine by nick translation, and were denatured to provide single stranded DNA probes. The restriction fragments from Bam HI digests of NAH, NAH2 and NAH3 were transferred from agarose gels to nitrocellulose filters by means of Southern blots. The hybridizations were carried out as described in the section on materials and methods.

All the fragments from NAH2 and NAH3 hybridized with the labelled NAH DNA (figure 7). Similarly, all the fragments from NAH hybridized with the labelled NAH3 DNA. As would be expected, all the fragments from NAH hybridized with the labelled NAH DNA and all the fragments from NAH2 and NAH3 hybridized with the labelled NAH3 DNA. These latter hybridizations were included to provide an internal control of the degree of hybridization, as permitted by the degree of stringency specified by the method of hybridization.

G. Examination of Naphthalene Pathway Mutants of ATCC 17483

Attempts to cure ATCC 17483 with NG resulted in the production of strains with a Nah^- phenotype. This suggested

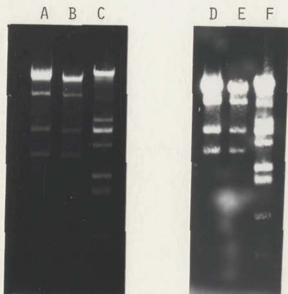

Figure 7. Hybridization of Bam HI restriction fragments of NAH, NAH2 and NAH3 with NAH labelled with tritiated thymidine. Lanes A, B and C contain NAH3, NAH2 and NAH respectively, in the original gel, lanes D, E, and F show the autofluorograph of NAH3, NAH2 and NAH respectively.

that curing had taken place. However, further examination of the Nah$^-$strains revealed that many of these mutants were of a Nah$^-$Sal$^+$ phenotype and that enzyme of the naphthalene pathway could be induced by 2-aminobenzoate in strains of the Nah$^-$Sal$^-$ phenotype.

A series of mutants, each mutant having a defect at a different step in the naphthalene pathway, was generated by the exposure of a culture of ATCC 17483 to NG, as previously described (the enrichment process was omitted). Mutants of Nah$^-$Sal$^+$, Nah$^+$Sal$^-$ and Nah$^-$Sal$^-$ phenotype were produced at frequencies of 0.4, 0.6 and 0.5% respectively.

Nah$^-$ mutants were grown on agar plates containing succinate as a carbon source, and then exposed to naphthalene, added as a solid to the lid. Clones which produced different colours on exposure to naphthalene vapour were selected for further examination.

The activities of four of the enzymes of the naphthalene pathway induced by 2-aminobenzoate in the wild type and selected mutants are shown in Table IX. Strains MC 3, MC 4 and MC 5 each have a Nah$^-$Sal$^+$ phenotype, but are blocked at different steps in the pathway. MC 3 is blocked at naphthalene oxygenase, MC 4 is blocked at 2'-hydroxybenzal pyruvate aldolase and MC 5 is blocked at 1,2-dihydroxynaphthalene oxygenase. Of the strains with a Nah$^+$Sal$^-$ phenotype, strains MC 7 and MC 9 have low levels of salicylaldehyde dehydrogenase, and strains MC 10 and MC 12 may have a block in salicylate

Table IX Activities of Enzymes Induced by 2-Aminobenzoate in Mutants of ATCC 17483.

Strain	Specific Activity* of: Naphthalene Oxygenase	1,2-Dihydroxy Naphthalene Oxygenase	2'-Hydroxybenzal-pyruvate Aldolase	Salicyaldehyde Dehydrogenase
ATCC 17483	100 (0.31)	100 (0.93)	100 (0.25)	100 (1.33)
MC 3	<1	75	69	78
MC 4	30	80	1	118
MC 5	64	3	1	< 1
MC 7	70	97	52	8
MC 9	53	88	64	1
MC 10	122	104	75	106
MC 12	127	66	68	112
MC 17	91	7	25	14
MC 18	129	81	66	91

*Activities are given as percentages of the activity in the wild type. The actual specific activities for the wild type, expressed as µmols/min per mg protein are given in parenthesis.

metabolism. Of the strains with a Nap⁻Sal⁻ phenotype, MC 17 has low levels of 1,2-dihydroxynaphthalene oxygenase and salicylaldehyde dehydrogenase, and MC 18 appears to have a block in salicylate metabolism.

ATCC 17483 and the mutants described above were grown on succinate in the presence of naphthalene. The activities of naphthalene oxygenase, 1,2 dihydroxynaphthalene oxygenase, 2'hydroxybenzalpyruvate aldolase and salicylaldehyde dehydrogenase were measured (Table X). Only in strains MC 7, MC 9, MC 10, MC 12 and MC 18 were some or all of these enzymes induced in the presence of naphthalene.

Table X Activities of Enzymes Induced in Mutants of ATCC 17483 Grown on Succinate in the Presence of Naphthalene.

Strain	Specific Activity (μmols/min per mg protein) of			
	Naphthalene Oxygenase	1,2-Dihydroxy Naphthalene	2'Hydroxybenzal-pyruvate Aldolase	Salicyaldehyde Dehydrogenase
ATCC 17483	0.29	0.96	0.22	0.89
ATCC 17483 (non-induced)	0.005	0.009	0.008	0.020
MC 3	0.009	<0.005	0.005	0.017
MC 4	0.006	<0.005	<0.005	0.108
MC 5	0.025	<0.005	0.007	0.005
MC 7	0.35	0.96	0.034	0.005
MC 9	0.15	0.89	0.15	0.012
MC 10	0.36	1.10	0.25	0.73
MC 12	0.42	1.66	0.21	0.90
MC 17	0.017	<0.005	0.013	0.040
MC 18	0.40	1.15	0.15	0.83

DISCUSSION

A. Detection of Plasmids in Naphthalene-Metabolizing Strains

A plasmid is usually defined as a genetic element which specifies processes which are not essential for growth under normal environmental conditions. Thus it may be acquired or lost without lethal effect to its host (Clowes, 1972). Under unusual environmental conditions, the presence of one or more plasmids may confer a selective advantage, such as the ability to grow in the presence of antibiotics, as in the case of resistance plasmids, or the ability to utilize unusual substrates for growth, as in the case of catabolic plasmids.

Curing

The loss of a plasmid is called curing. Occasionally, in the laboratory, a plasmid may be lost when a strain containing a plasmid has been maintained on a medium which is not selective for the plasmid (Williams and Murray, 1974). Spontaneous curing is usually rare. However, the frequency of curing can be enhanced when a strain containing a plasmid is grown in the presence of cytotoxic chemicals such as mitomycin C (Dunn and Gunsalus, 1973), ethidium bromide, acridine orange or NG (Novik, 1969). A high frequency of curing with no reversion to the original phenotype may provide the initial evidence for the presence of a plasmid in a bacterial strain.

The results of the curing experiments were consistent with reports of the presence of plasmids in strains PpG7, NCIB 9816 and P_G associated with the ability to degrade naphthalene. The absence of curing in strains ATCC 17483 and ATCC 17484 cannot be used to rule out the presence of a plasmid in these two strains. As is apparent from the results, different strains responded to different curing agents, and to different concentrations of curing agent. It has been reported that ATCC 17484 can be cured (J.B. Johnston, personal communication to E.A. Barnsley), and experiments discussed later provided direct evidence for the presence of plasmids in ATCC 17484, so it may be that an appropriate curing agent was not identified for ATCC 17483 and ATCC 17484.

Conjugation

Many plasmids contain the genes which specify a mechanism for conjugation, a process by which a plasmid is transferred from one strain to another. Such plasmids are called conjugative or self transmissible plasmids (Clowes, 1972). The conjugative transfer of a phenotype suspected of being carried on a plasmid is strong evidence for the involvement of a plasmid. Not all plasmids are self-transmissible, however, and the absence of conjugative transfer of a particular phenotype does not rule out the involvement of a plasmid.

A current model for the mechanism of conjugation involves the transfer of one strand of the plasmid DNA from the donor

cell to the recipient cell, through a pore that is formed in the cell envelope following cell to cell contact (Willetts, 1981). The complementary strand is synthesized by each cell. There are three requirements for conjugation (Willetts, 1981); the plasmid must contain an origin of transfer sequence, the host cell must synthesize a DNA transfer and replication system that recognizes the origin of transfer sequence, and the cell must synthesize a system for a stable mating pair formation. These requirements are coded for by self-transmissible plasmids, but the latter two can be supplied in trans to transfer non self-transmissible plasmids.

Pili appear to be involved in the formation of stable mating pairs. The role of the pili in this regard has not been fully explained, but they seem to be involved in bringing about cell to cell contact. Pili have been identified from all incompatibility groups of plasmids (Bradley, 1981) and may be classified on the basis of morphology and environmental conditions required for conjugation.

Conjugations were attempted for two reasons. The first was to determine if the ability to degrade naphthalene was transmissible. The second arose out of practical considerations, as at the outset of the work, plasmids could only be isolated reproducibility from hosts PaW 330 and PaW 340.

The results of the conjugation experiments demonstrated the role of a plasmid in the degradation of naphthalene in strains NCIB 9816 and P_G and that NAH2 and NAH3 were self-

transmissible. Although strains ATCC 17483 and ATCC 17484 or their amino acid auxotrophic derivatives did not conjugate with any of the cured strains, the involvement of plasmids can not be ruled out. If plasmids are involved in the degradation of naphthalene in these strains, the plasmids may not be self transmissible, or the strains used as recipients in the conjugation may not have been compatible with strains ATCC 17483 or ATCC 17484. Another possibility is that only part of the pathway is plasmid borne, and the selection technique would not have been able to select for such a plasmid.

In order to obtain reproducible isolations of plasmids NAH, NAH2 and NAH3 it was necessary to transfer these plasmids to strains PaW 330 or PaW 340 (C.J. Duggleby, personal communication to E.A. Barnsley). Strains PaW 330 and PaW 340 are cured Trp$^-$ derivatives of a TOL bearing Pseudomonas putida arvilla) strain. PaW 340 differs from PaW 330 in that the strain has a streptomycin resistance marker. Direct crosses from the wild type strains into PaW 340 were attempted, but only NAH from PpG7 was transferred. The absence of conjugation by strains NCIB 9816 and P_G was unexpected as NAH2 and NAH3 are self-transmissible plasmids.

It was reported that the conjugational pili specified by the plasmids of the same incompatibility group as NAH (P-9) are rigid, and because of this property, strains containing plasmids from this group conjugate with higher frequency on agar plates than in liquid culture (Bradley, 1981). Conjugation

experiments between the strains under investigation and PaW 330 were carried out on agar plates. PpG7 did conjugate, but not with increased frequency, and the other strains did not conjugate. It was possible to transfer NAH2 from strain MC 234 to PaW 340 by this method, but not NAH3 from strain MC 239.

As strain AC 545 (reported to contain a SAL plasmid, but which was of a transmissible Nah^+ phenotype) was able to conjugate with PaW 340, NAH, NAH2 and NAH3 were transferred to strain MC 213, a cured derivative of AC 545. Exconjugates from these crosses were used as donors in conjugations with PaW 330. Thus NAH2 and NAH3 could be transferred to PaW 330 only indirectly via an intermediate strain. NAH could be transferred either directly from an amino acid auxotrophic derivative of the wild type, or indirectly through an intermediate strain. Although the genes for the mechanism of conjugation are specified by the plasmid, the host strain appears to influence the process of conjugation.

The characteristic properties of catechol 2,3-dioxygenase and 2-hydroxymuconic acid semialdehyde dehydrogenase were transferred with plasmids NAH2 and NAH3. These properties therefore are determined by the plasmid, and not by the host strain.

B. Isolation of Plasmid DNA from Naphthalene-Metabolizing Strains.

Plasmids exist in the bacterial cell as covalently closed circles of double stranded DNA (Clowes, 1972). It is this configuration that makes the plasmid resistant to denaturation, and techniques for the isolation of plasmid DNA make use of this property. As a result of difficulties in the isolation of large plasmids, several methods have been reported. The methods have common features: the bacterial cells are lysed and chromosomal DNA is denatured and precipitated as a chromosomal DNA-membrane complex, leaving the plasmid DNA in the supernatant. The difficulties in the isolation of catabolic plasmids appear to be due to the difficulty in separating large plasmids from the chromosomal DNA-membrane complex (Palchaudhuri and Chakrabarty, 1976), and primarily due to nuclease activity released during cell breakage.

It was not possible to isolate plasmid DNA reproducibly from the wild type strains by the use of several methods (Guerry et al. 1973 and Meyers et al., 1976; Birnboim and Doly, 1979, Hansen and Olsen, 1978). This difficulty was circumvented by the transfer of NAH, NAH2 and NAH3 to PaW 330. The isolation of plasmid DNA from a PaW 330 background was easily accomplished by any of the above methods. However, this method is confined to those plasmids and strains that are able to conjugate with PaW 330.

The method of Birnboim and Doly (1979) was modified to

permit the detection of plasmids in the wild type strains. The modifications evolved during several large scale isolations of plasmid DNA and are described as follows. Cultures in late exponential or early stationary phase were diluted 10-fold into fresh medium and were grown until the absorbance at 600 nm reached 1-1.2. The cells were washed in an iso-osmolar buffer solution to remove residual growth medium, the iso-osmolar buffer was used to pervent premature lysis of the cells. The treatment with lysozyme was omitted as it was unnecessary for pseudomonads. Rapid lysis was effected with NaOH which would also denature nucleases. Steps to remove RNA by treatment with RNase (heated at 100°C for 10 minutes prior to use to inactivate DNases) and to remove protein by extraction with Tris-saturated phenol or phenol/chloroform solutions can be incorporated into the method, and initially were routinely carried out. The plasmid DNA isolated by this method was not pure enough for digestion with restriction endonucleases, and purification on a caesium chloride-ethidium bromide gradient was necessary. Plasmid DNA was separated from RNA and protein on the gradient, and so these steps were omitted in the initial isolation procedure.

The isolation of plasmid DNA from the wild type strains demonstrated that PpG7 contained a single plasmid, and that NCIB 9816, P_G and ATCC 17484 contained multiple plasmids. Although the method permitted the detection of plasmids in

several uncharacterized strains isolated from soil, in addition to the strains described above, it was not possible to detect any plasmids in ATCC 17483 by any method.

NAH2 and NAH3 are the larger of the two plasmids present in NCIB 9816 and P_G respectively, because their transfer to plasmid-free strains is accompanied by the ability to degrade naphthalene. It is possible that the largest plasmid in ATCC 17484 might be responsible for the degradation of naphthalene, but there is no evidence for this. On the basis of electrophoretic mobility, the largest plasmid in ATCC 17484 appeared to be smaller than NAH, it may be that this plasmid lacks genes required for the conjugational transfer of the plasmid. NAH was the only plasmid in PpG7.

As NAH2 and NAH3 were the only plasmids to transfer to PaW 330, NAH, NAH2 and NAH3 were isolated from the PaW 330 background. The conjugational transfer of these plasmids to a second or third strain did not appear to result in a decrease in molecular weight, as determined by electrophoretic mobility in agarose gels. (The isolation of NAH and SAL from different background strains has resulted in the isolation of plasmids having different molecular weights (Heinaru et al., 1978; Farrell et al., 1978).

C. The Molecular Relationship Among NAH, NAH2 and NAH3

Restriction endonucleases are part of the restriction and modification systems used by bacteria as defence mechanisms

against the uptake of foreign DNA (Nathans and Smith, 1975). Class II restriction endonucleases recognize specific nucleotide sequences in double stranded DNA and cleave both strands. The number and size of the fragments produced by a particular endonuclease is dependent upon the number and distribution of the specific nucleotide sequences in the plasmid DNA. Thompson et al (1974) showed that fragments produced by a digest with restriction endonucleases could be resolved into specific patterns in agarose gels, and that this method could be used to distinguish among different plasmids. Duggleby et al. (1977) used this technique to look at the molecular relationships among a variety of TOL plasmids. Closely related plasmids can be expected to retain a similar distribution of the specific sequences for cleavage by the restriction endonucleases and thus produce similar patterns of restriction fragments during electrophoresis.

The patterns of restriction fragments from digestions with Hind III and Bam HI were identical for NAH2 and NAH3. This result confirmed that NAH2 and NAH3 were essentially the same plasmid and were regulatory mutants. The patterns of restriction fragments from NAH were substantially different from those of NAH2 and NAH3, indicating that the molecular relationships between the two plasmids were not very close.

Although NAH, NAH2 and NAH3 were not separated by electrophoretic mobility in agarose gels the molecular weights as determined by the summation of the restriction

fragments generated by digestion with Hind III are different. NAH was found to have a molecular weight of 52×10^6 daltons, which was in close agreement with a previously published molecular weight of 50×10^6 daltons (Farrell et al, 1978). NAH2 and NAH3 were found to have a molecular weight of 77×10^6 daltons. This is somewhat puzzling as TOL, with a molecular weight of 75×10^6 daltons in strain AC 137 (Chakrabarty et al. 1978) and 77×10^6 daltons in strain *Pseudomonas putida arvilla* mt-2 (Duggleby et al., 1977) and SAL (no published molecular weight for SAL in AC 545, but SAL has been reported to be larger than NAH [Johnson and Gunsalus, 1977, Farrell et al., 1978]), are readily separated from NAH by electrophoretic mobility in agarose gels. A possible explanation of this behaviour may be that NAH2 and NAH3 are more tightly supercoiled than NAH.

A more direct way to examine molecular relationships among plasmids is by the method of DNA:DNA hybridization between the DNA from one plasmid with the restriction endonuclease fragments from another plasmid. Although the patterns of restriction fragments were very different for the two plasmids, all the fragments from NAH2 and NAH3 hybridized with NAH, and all the fragments from NAH hybridized with NAH3. NAH and NAH2/NAH3 are structurally more similar than a comparison of the patterns of the restriction fragments would suggest. It could not be ascertained from this experiment whether the homology lay in the structure of the genes that

specify the enzymes for the degradation of naphthalene or other parts of the plasmid or both. The homology among the plasmids appears to be extensive, under the conditions specified by the method of hybridization. The hybridization were carried out according to a published method to see if there was any homology between NAH and NAH3. The extent of the hybridization was not expected, and more stringent conditions of hybridization were not used. The filters could have been washed with lower concentrations of saline-citrate in order to promote the dissociation of duplexes formed between imperfectly matched fragments. A more rigorous test of homology would be to carry out hybridization between the catabolic genes cloned from each plasmid.

D. The Regulation of Naphthalene Metabolism by Pseudomonads

The enzymes of the naphthalene pathway are induced in cultures growing on succinate in the presence of 2-aminobenzoate in PpG7, NCIB 9816, P_G, ATCC 17483 and ATCC 17484 (Barnsley, 1975 and 1976b). Only in ATCC 17483 are these enzymes induced when a culture is grown on succinate in the presence of naphthalene. This property made it possible to examine a series of mutants with blocks at different steps in the early stage of the naphthalene pathway for the effect of induction by naphthalene. The mutants were characterized by the enzymic activities induced by 2-aminobenzoate, and then were examined for the induction of the enzymes of the naphthalene

pathway by naphthalene.

Although the low activities of some enzymes in MC 3, MC 4, MC 5 and MC 17 could account for the Nah$^-$ phenotype, the relative activities were not consistent with polar effects resulting from a single mutation. The Nah$^+$Sal$^-$ phenotypes of MC 7 and MC 9 may have been due to a reduced uptake of salicylate rather than a block in salicylate metabolism. Although the pyruvate produced from 2-hydroxybenzal pyruvate by the aldolase can support growth, the low levels of salicylaldehyde dehydrogenase in these strains would result in the accumulation of salicylaldehyde, which is toxic.

The enzymes of the naphthalene pathway were not induced in the presence of naphthalene in those mutants (MC 3, MC 4, MC 5 and MC 17) which were blocked in the steps preceeding salicylaldehyde dehydrogenase. Only in those mutants which did not have blocks in the early stage of the naphthalene pathway (MC 10 and MC 12) or may have had a block in salicylate metabolism (MC 18) or had low activities of salicylaldehyde dehydrogenase (MC 7 and MC 9) were the enzymes of the naphthalene pathway induced in the presence of naphthalene. These results can be explained if induction in the presence of naphthalene requires the conversion of naphthalene to salicylaldehyde. Thus only when the pathway is intact as far as salicylaldehyde is the pathway induced.

As salicylate is also an inducer of the naphthalene pathway (Barnsley 1975, 1976b) it can not be ruled out that

salicylate may be reduced to salicylaldehyde in vivo, or that salicylaldehyde may be non-enzymatically oxidized to salicylate. However, salicylaldehyde and salicylate are structurally similar to the gratuitous inducers 2-amino benzoate and 2-hydroxybenzyl alcohol and may both be inducers of the pathway. It would appear that salicyladehyde is the first possible inducer of the pathway and that naphthalene itself or the earlier metabolites are not inducers.

The mutants of NCIB 9816 and P_G which had lost the constitutive properties of catechol 2,3 dioxygenase were not examined closely, except to note that the levels of induced and non-induced catechol 2,3 dioxygenase were very similar to those of PpG7. This observation supports the proposal of Austen and Dunn (1980) that the regulation of the naphthalene pathway in these two strains, and the strains of Williams et al (1975) was essentially the same as that specified by NAH in PpG7, and that in each strain, a mutation had altered the control of the pathway. This situation is in contrast to that in ATCC 17483, in which repeated attempts to isolate mutants constitutive for catechol 2,3 dioxygenase have been unsuccessful (unpublished observations).

CONCLUSIONS

The involvement of plasmids in the degradation of naphthalene was demonstrated in strain NCIB 9816 and P_G by curing, conjugation and plasmid isolation experiments. The examination of the naphthalene plasmids in these two strains by restriction endonuclease digestion showed that the two plasmids are essentially the same plasmid, being regulatory mutants of each other. The presence of several plasmids was demonstrated in strain ATCC 17484, and it is assumed that the largest of these plasmids, similar in electrophoretic mobility to NAH, is the one involved in the degradation of naphthalene. It was not possible to demonstrate the presence of any plasmids in strain ATCC 17483. Firm conclusions may be premature, but it would seem that the genes for the degradation of naphthalene are located on the chromosome in this strain. In any case, ATCC 17483 is very different from the other strains used in this study, as it alone induces the enzymes for the degradation of naphthalene in the presence of naphthalene while growing on excess succinate, and also has the property of giving, with high frequency and without selection, different mutants in naphthalene metabolism.

The strains containing plasmid NAH and plasmid NAH2 and NAH3 were originally isolated on separate continents. It was not surprising then, to observe that the patterns of restriction endonuclease fragments were very different. Plasmids NAH2 and NAH3 are larger than NAH. Nevertheless,

NAH and NAH2/NAH3 hybridize extensively with each other, and therefore have considerable homology. A similar situation exists among the TOL plasmids (Duggleby et al., 1977) and homology has been demonstrated among NAH, SAL and TOL (Heinaru et al., 1978). It would appear that certain molecular structures are geographically widespread. The parent strain of NCIB 9816 and P_G degrades phenanthrene via dihydroxynaphthalene and the remainder of the naphthalene pathway (Evans et al., 1965), although these strains do not grow on phenanthrene (Barnsley, unpublished observations). It may be that genes for the degradation of phenanthrene to dihydroxynaphthalene are also located on the plasmid.

The differences in the regulation of the pathways specified by NAH, NAH2 and NAH3 may not be as great as would first appear. The constitutive properties of catechol 2,3-dioxygenase are easily destroyed in NAH2 and NAH3 by treatment with NG, and mutants of NAH with a constitutive catechol 2,3 dioxygenase appear to be readily isolated (Austen and Dunn, 1980).

From the examination of a series of mutants of strain ATCC 17483, it can be concluded that salicylaldehyde is the first possible inducer of the naphthalene pathway. The regulation of naphthalene pathway is unusual compared to that of other hydrocarbons (Clarke and Ornston, 1975) in that a relatively large number of enzyme catalyzed steps are required to produce the first inducer.

SUMMARY

Four strains of naphthalene metabolizing pseudomonads were examined for the involvement of plasmids in the degradation of naphthalene. The plasmid NAH in the strain PpG7 of I.C. Gunsalus which had been characterized previously in his laboratory was used both as a control and for the purpose of comparison. Of the strains under investigation, PpG7, NCIB 9816, P_G and ATCC 17484 were classified as Pseudomonas putida, ATCC 17483 was an unclassified fluorescent pseudomonad. These strains had been characterized biochemically in the laboratory of E.A. Barnsley. NCIB 9816 and P_G were putatively identical and were assumed to be regulatory mutants of the same strain (Barnsley, 1976b).

The initial evidence of the involvement of plasmids in NCIB 9816 and P_G came from curing and conjugation experiments. The ability to degrade naphthalene was lost upon curing, and subsequent investigation showed that the whole block of enzymes from naphthalene oxygenase to the first two enzymes of the meta pathway were completely deleted. Cured strains regained the ability to degrade naphthalene only after conjugation. The plasmids in NCIB 9816 and P_G, designated NAH2 and NAH3 respectively were shown to be self-transmissible plasmids. The regulation of the meta pathway enzymes was transferred with the plasmids, thus the genes for the regulation of these enzymes are plasmid borne. It was not possible to demonstrate the involvement of plasmids in the degradation

of naphthalene in ATCC 17483 or ATCC 17484 either by curing or by conjugation.

Difficulties in obtaining reproducible isolations of plasmid DNA from the strains under investigation were circumvented in two ways. Plasmids associated with the degradation of naphthalene were transferred from their host strain to a cured strain PaW 330, by conjugation either directly or indirectly via a second cured strain. It was then possible to isolate plasmid DNA easily by several methods. The major draw back to this technique is that it is limited to self-transmissible plasmids that are able to conjugate with strain PaW 330. The method of Birnboim and Doly (1979) was modified, and the resulting method enabled the isolation of plasmid DNA from the wild type strains. Strains NCIB 9816, P_G and ATCC 17484 contained multiple plasmids. It was not possible to demonstrate the presence of any plasmids in strain ATCC 17483. The largest plasmid in strains NCIB 9816 and P_G was the plasmid involved in the degradation of naphthalene, perhaps the largest plasmid in strain ATCC 17484 also determined the degradation of naphthalene. The roles of the smaller plasmids in these strains was not determined. NAH appeared to be the only plasmid in strain PpG7.

As strains MC 281, MC 274 and MC 268 each contained only one plasmid, NAH, NAH2 and NAH3 were isolated from these hosts, respectively, and were purified by caesium chloride-ethidium bromide density gradient centrifugation.

The molecular relationships among NAH, NAH2 and NAH3 were analysed by the comparison of the patterns of fragments generated by digestion with restriction endonucleases, and by an in situ hybridization of single stranded labelled plasmid with restriction fragments bound to nitrocellulose filters.

The patterns of restriction endonuclease fragments of NAH2 and NAH3 were identical, which was not unexpected as the two plasmids were from putatively identical organisms. The patterns of restriction endonucleases fragments from NAH differed considerably from the patterns from NAH2 and NAH3. However, the restriction endonuclease fragments from NAH2 and NAH3 hybridized extensively with NAH, and the restriction endonuclease fragments from NAH hybridized extensively with NAH3, demonstrating an extensive structural homology that was not indicated from the comparison of the patterns of restriction endonuclease fragments.

Some observations on the regulation of the naphthalene pathway were made. The enzymes of the naphthalene pathway are induced in ATCC 17483 while a culture is growing on succinate in the presence of naphthalene. This property, peculiar to ATCC 17483, made it possible to identify salicylaldehyde as the first possible inducer of the pathway, and to demonstrate that naphthalene itself or earlier metabolites are not inducers. The regulation of the naphthalene pathway in PpG7 and NCIB 9816 and P_G is probably similar, as it was possible

to isolate mutants of NCIB 9816 and P_G with an inducible catechol 2,3-dioxygenase.

REFERENCES

1. Austen, R.A. and N.W. Dunn (1980) "Regulation of the plasmid-specified naphthalene catabolic pathway of Pseudomonas putida". J. Gen. Microbiol. 117: 521-528.

2. Barnsley, E.A. (1975) "The induction of the enzymes of naphthalene metabolism in pseudomonads by salicylate and 2-aminobenzoate". J. Gen. Microbiol. 88: 193-196.

3. Barnsley, E.A. (1976a) "Naphthalene metabolism by pseudomonads: The oxidation of 1,2 dihydroxynaphthalene to 2-hydroxychrome-2-carboxylic acid and the formation of 2'-hydroxybenzalpyruvate". Biochem. Biophys. Res. Commun. 72: 1116-1121.

4. Barnsley, E.A. (1976b) "Role and regulation of the ortho and meta pathways of catechol metabolism in Pseudomonas metabolizing naphthalene and salicylate". J. Bacteriol. 125: 404-408.

5. Bayley, S.A., D.W. Morris and P. Broda (1979) "The relationship of degradation and resistance plasmids of Pseudomonas belonging to the same incompatibility group". Nature 180: 338-339.

6. Bayly, R.C. and M.G. Barbour (1984) "The degradation of aromatic compounds by the meta and gentisate pathways: biochemistry and regulation" in Microbial Degradation of Organic Compounds. D.T. Gibson ed. Marcel Dekker Inc. New York and Basal. p.253-294.

7. Bayly, R.C. and S. Dagley (1969) "Oxoenoic acids as metabolites in the bacterial degradation of catechols". Biochem. J. 111: 303-307.

8. Birnboim, H.C. and J. Doly (1979) "A rapid alkaline extraction procedure for screening recombinant plasmid DNA". Nucleic Acids Research 7: 1513-1523.

9. Blumer, M. (1976) "Polycyclic aromatic compounds in nature". Sci. Am. 234: 34-45.

10. Bonner, W.M. and R.A. Laskey (1974) "A film detection method for tritium - labelled proteins and nucleic acids in polyacrylamide gels". Eur. J. Biochem. 46: 83-88.

11. Boronin, A.M., V.V. Kochetkov, I.I. Starovoitov and G.K. Skryabin (1977) "Plasmids pBS2 and pBS3, controling the oxidation of naphthalene in bacteria of the genus Pseudomonas. Dokl. Akad. Nauk. SSR 237: 1205-1207. Eng.

transl. 487-485.

12. Boronin, A.M., I.I. Starovoitov, A.N. Borisoglebskaya, and G.K. Skryabin (1976) "Plasmid of Pseudomonas putida which controls the initial stages of naphthalene oxidation". Dokl. Akad. Nauk. SSSR, 228: 962-965. Eng. Transl. 226-228.

13. Bradley, D.E. (1981) "Conjugative pili of plasmids in Escherichia coli K-12 and Pseudomonas species" in Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids. S.B. Levy, R.C. Clowes, E.L. Koenig eds. Plenum Press, New York and London, p.217-226.

14. Cain, R.B. and D.R. Fair (1968) "Metabolism of arylsulphonates by micro-organisms". Biochem. J. 106: 859-877.

15. Catterall, F.A., K. Murray and P.A. Williams (1971) "The configuration of the 1,2-dihydroxy-1,2-dihydroxy-naphthalene formed in the bacterial metabolism of naphthalene". Biochim. Biophys. Acta 237: 361-364.

16. Catterall, F.A., J.M. Sala-Trepat, and P.A. Williams (1971) "The coexistence of two pathways for the metabolism of 2-hydroxymuconic semialdehyde in a naphthalene-grown pseudomonad". Biochem. Biophys. Res. Commun. 43: 463-469.

17. Cerniglia, C.E. (1981) "Aromatic hydrocarbon metabolism by bacteria, fungi and algae". Rev. Biochem. Toxicol. 3: 321-361.

18. Chakrabarty, A.M. (1976) "Plasmids in Pseudomonas". Ann. Rev. Genet. 10: 7-30.

19. Chakrabarty, A.M., D.A. Friello, and L.H. Bopp (1978) "Transposition of plasmid DNA segments specifying hydrocarbon degradation and their expression in various microorganisms". Proc. Natl. Acad. Sci. USA 75: 3109-3112.

20. Chatterjee, D.K., S.T. Kellogg, D.R. Watkins and A.M. Chakrabarty (1981) "Plasmids in the biodegradation of chlorinated aromatic compounds." in Molecular Biology, Pathogenicity and Ecology of Bacterial Plasmids. S.B. Levy, R.C. Clowes and E.L. Koenig eds. Plenum Press, New York p. 519-528.

21. Chatterjee, D.K., J.J. Kilbane and A.M. Chakrabarty (1982) "Biodegradation of 2,4,5-trichloropenoxyacetic acid in soil by a pure culture of Pseudomonas cepacia". Appl. Environ. Microbiol. 44: 514-516.

22. Clarke, P.H. (1982) "The metabolic versatility of pseudomonads". Antonie van Leeuwenhoek 48: 105-130.

23. Clarke, P.H. and L.N. Ornston (1975) "Metabolic pathways and regulation I" in Genetics and Biochemistry of Pseudomonas. P.H. Clarke and M.H. Richmond (eds) John Wiley Sons, Inc., New York p. 191-216.

24. Clowes, R.C. (1972) "Molecular structure of bacterial plasmids". Bacteriol. Rev. 36: 361-405.

25. Connors, M.A. and E.A. Barnsley (1980) "Metabolism of naphthalene by pseudomonads: salicylaldehyde as the first possible inducer in the metabolic pathway". J. Bacteriol. 141: 1052-1054.

26. Connors, M.A. and E.A. Barnsley (1982) "Naphthalene plasmids in pseudomonads". J. Bacteriol. 149: 1096-1101.

27. Corner, E.D.S., and L. Young (1954) "Biochemical studies of toxic agents. The metabolism of naphthalene in animals of different species". Biochem. J. 58: 647-655.

28. Crawford, R.L. (1981) "Lignin biodegradation and transformation". John Wiley and Sons, New York, Chichester, Brisbane, Toronto.

29. Dagley, S. (1971) "Catabolism of aromatic compounds by micro-organisms". Adv. Microbiol. Physiol. 6: 1-46.

30. Dagley, S. (1975) "A biochemical approach to some problems of environmental pollution". Essays in Biochemistry 11: 81-138.

31. Dagley, S. (1978) "Pathways for the utilization of organic growth substrates". The Bacteria VI: 305-388. L.N. Ornston, J.R. Sokatch eds.

32. Dagley, S., W.C. Evans and D.W. Ribbons (1960) "New pathways in the oxidative metabolism of aromatic compounds by micro-organisms". Nature 188: 560-566.

33. Dagley, S. and D.T. Gibson (1965) "The bacterial degradation of catechol". Biochem. J. 95: 466-474.

34. Dagley, S. and D.A. Stopher (1959) "A new mode of fission of the benzene nucleus by bacteria". Biochem. J. 73: 16P.

35. Davies, J.I., and W.C. Evans (1964) "Oxidative metabolism of naphthalene by soil pseudomonads the ring-fission mechanism". Biochem. J. 91: 251-261.

36. Denhardt, D.T. (1966) "A membrane-filter technique for the detection of complementary DNA". Biochem. Biophys. Res. Commun. 23: 641-646.

37. Duggleby, C.J., S.A. Bayley, M.J. Worsey, P.A. Williams and P. Broda (1977) "Molecular sizes and relationships of TOL plasmids in Pseudomonas". J. Bacteriol. 130: 1276-1280.

38. Dunn, N.W., H.M. Dunn, and R.A. Austen (1980) "Evidence for the existence of two catabolic plasmids coding for the degradation of naphthalene". J. Gen. Microbiol. 117: 529-533.

39. Dunn, N.W. and I.C. Gunsalus (1973) "Transmissible plasmid coding early enzymes of naphthalene oxidation in Pseudomonas putida". J. Bacteriol. 114: 974-979.

40. Evans, W.C. (1977) "Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments". Nature 270: 17-22.

41. Evans, W.C., H.N. Fernley and E. Griffiths (1965) "Oxidative metabolism of phenanthrene and anthracene by soil pseudomonads: The ring fission mechanism". Biochem. J. 95: 819-831.

42. Evans, W.C., B.S.W. Smith, R.P. Linstead, and J.A. Elvidge (1951) "Chemistry of the oxidative metabolism of certain aromatic compounds by micro-organisms". Nature 168: 772-775.

43. Farr, D.R. and R.B. Cain (1968) "Catechol oxygenase induction in Pseudomonas aeruginosa". Biochem. J. 106: 879-885.

44. Farrell, R., I.C. Gunsalus, I.P. Crawford, J.B. Johnston, and J. Ito (1978) "Restriction endonuclease sites and aromatic metabolic plasmid structure". Biochem. Biophys. Res. Commun. 82: 411-416.

45. Feist, C.F., and G.D. Hegeman (1969) "Phenol and benzoate metabolism by Pseudomonas putida: Regulation of tangential pathways". J. Bacteriol. 100: 869-877.

46. Fernley, H.N. and W.C. Evans (1958) "Oxidative metabolism of polycyclic hydrocarbons by soil pseudomonads". Nature 182: 373-375.

47. Ghosal, D., I.-S. You, K. Chatterjee, A.M. Chakrabarty (1985) "Microbial degradation of halogenated compounds". Science 228: 135-142.

48. Gibson, D.T. (1971) "The microbial oxidation of aromatic hydrocarbons". Crit. Rev. Microbiol. 1: 199-223.

49. Gibson, D.T. ed. (1984) "Microbial degradation of organic compounds". Marcel Dekker Inc. New York and Basel.

50. Gibson, D.T. and V. Subramanian (1984) "The microbial degradation of aromatic hydrocarbons" in Microbial Degradation of Organic Compounds. D.T. Gibson ed. Marcel Dekker Inc. New York and Basel. p. 181-252.

51. Gray, P.H.H., and H.G. Thornton (1928) "Soil bacteria that decompose aromatic compounds". Zbl. Bakt. 73: 74-96.

52. Grund, A.D. and I.C. Gunsalus (1983) "Cloning of genes for naphthalene metabolism in Pseudomonas putida". J. Bacteriol. 156: 89-94.

53. Guerry, P., D.J. LeBlanc, and S. Falkow (1973) "General method for the isolation of plasmid deoxyribonucleic acid". J. Bacteriol. 116: 1064-1066.

54. Hansen, J.B. and R.H. Olson (1978) "Isolation of large bacterial plasmids and characterization of the P_2 incompatibility group plasmids pMG1 and pMG5". J. Bacteriol. 135: 227-238.

55. Hegeman, G.D. (1966) "Synthesis of the enzymes of the mandelate pathway by Pseudomonas putida I. Synthesis of enzymes of the wild type". J. Bacteriol. 91: 1140-1154.

56. Heinaru, A.L., C.L. Duggleby and P. Broda (1978). "Molecular relationships of degradative plasmids determined by in situ hybridisation of their endonuclease - generated fragments". Molec. Gen. Genet. 160: 347-351.

57. Jeffrey, A.M., H.J.C. Yeh, D.M. Jerina, T.R. Patel, J.F. Davey, and D.T. Gibson (1975) "Initial reactions in the oxidation of naphthalene by Pseudomonas putida". Biochemistry 14: 575-584.

58. Jerina, D.M., J.W. Daly, A.M. Jeffrey, and D.T. Gibson (1971) "Cis-1,2 Dihydroxy-1,2-Dihydronaphthalene: A bacterial metabolite from naphthalene". Arch. Biochem. Biophys. 142: 394-396.

59. Johnston, J.B., and I.C. Gunsalus (1977) "Isolation of metabolic plasmid DNA from Pseudomonas putida". Biochem. Biophys. Res. Commun. 75: 13-19.

60. Kellogg, S.T., D.K. Chatterjee and A.M. Chakrabarty (1981) "Plasmid-assisted molecular breeding: New technique

for enhanced biodegradation of persistent toxic chemicals". Science 214: 1133-1135.

61. Kilbane, J.J., D.K. Chatterjee and A.M. Chakrabarty (1982) "Detoxification of 2,4,5-Trichlorophenoxyacetic Acid from contaminated soil by Pseudomonas cepacia". Appl. Environ. Microbiol. 44: 72-78.

62. Kilbane, J.J., D.K. Chatterjee and A.M. Chakrabarty (1983) "Detoxification of 2,4,5-Trichlorophenoxyacetic acid from contaminated soil by Pseudomonas cepacia". Appl. Environ. Microbiol. 45: 1697-1700.

63. Lehrbach, P.R., J. Zeyer, W. Reineke, H.J. Knackmuss, and K.N. Timmis (1984) "Enzyme recruitment in vitro: Use of cloned genes to extend the range of haloaromatics degraded by Pseudomonas sp. strain B 13". J. Bacteriol. 158: 1025-1032.

64. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) "Protein measurement with the folin-phenol reagent". J. Biol. Chem. 193: 265.

65. Meyers, J.A., D. Sanchez, L.P. Elwell, and S. Falkow (1976) "Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid". J. Bacteriol. 127: 1529-1537.

66. Murray, K. and P.A. Williams (1974) "Role of catechol and the methylcatechols as inducers of aromatic metabolism in Pseudomonas putida". J. Bacteriol. 117: 1153-1157.

67. Nathans, D. and H.O. Smith (1975) "Restriction endonucleases in the analysis and restructuring of DNA molecules". Ann. Rev. Biochem. 44: 273-293.

68. Nishizuka, Y., A. Ichiyama, S. Nakamura and O. Hayaishi (1962) "A new metabolic pathway of catechol". J. Biol. Chem. 237: PC 268-270.

69. Novick, R.P. (1969) "Extrachromosomal inheritance in bacteria". Bacteriol. Rev. 33: 210-235.

70. Ornston, L.N. (1966) "The conversion of catechol and protocatechuate to -ketoadipate by Psuedomonas putida IV Regulation". J. Biol. Chem. 241: 3800-3810.

71. Ornston, L.N., M.K. Ornston, and G. Chou (1969) "Isolation of spontaneous mutant strains of Pseudomonas putida". Biochem. Biophys. Res. Commun. 36: 179-184.

72. Palchaudhuri, S. and A.M. Chakrabarty (1976) "Isolation

of plasmid deoxyribonucleic acid from Pseudomonas putida". J. Bacteriol. 126: 410-416.

73. Patel, T.R. and E.A. Barnsley (1980) "Naphthalene metabolism by pseudomonads: purification and properties of 1,2 dihydroxynaphthalene oxygenase". J. Bacteriol. 143: 668-673.

74. Radloff, R., W. Bauer, and J. Vinograd (1967) "A dye-bouyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in He La cells". Proc. Natl. Acad. Sci. U.S.A. 57: 1514-1522.

75. Randerath, K. (1970) "An evaluation of film detection methods for weak -emitters, particularly tritium". Anal. Biochem. 34: 188-205.

76. Reineke, W. and H-J. Knackmus (1979) "Construction of haloaromatics utilizing bacteria". Nature 277: 385-386,

77. Rheinwald, J.G., A.M. Chakrabarty, and I.C. Gunsalus (1973) "A transmissible plasmid controlling camphor oxidation in Pseudomonas putida". Proc. Natl. Acad. Sci. U.S.A. 70: 885-889.

78. Rigby, P.W.J., M. Dickmann, C. Rhodes and P. Berg (1977) "Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I". J. Mol. Biol. 113: 237-251.

79. Sala-Trepat, J.M. and W.C. Evans (1971) "The meta cleavage of catechol by Azotobacter species". Eur. J. Biochem. 20: 400-413.

80. Shamsuzzaman, K.M. and E.A. Barnsley (1974a) "The regulation of naphthalene oxygenase in pseudomonads". J. Gen. Microbiol. 83: 165-170.

81. Shamsuzzaman, K.M. and E.A. Barnsley (1974b) "The regulation of naphthalene metabolism in pseudomonads". Biochem. Biophys. Res. Commun. 60: 582-589.

82. Southern, E.M. (1975) "Detection of specific sequences among DNA fragments separated by gel electrophoresis". J. Mol. Biol. 98: 503-517.

83. Sparnins, V.L., P.J. Chapman, and S. Dagley (1974) "Bacterial degradation of 4-hydroxyphenylacetic acid and homoprotocatechuic acid". J. Bacteriol. 120: 159-167.

84. Stanier, R.Y., N.J. Palleroni, and M. Doudoroff (1966)

"The aerobic pseudomonads: a taxonomic study". J. Gen. Microbiol. 43: 159-271.

85. Stanier, R.Y. and L.N. Ornston (1978) "The β-ketoadipate pathway". Adv. Microbiol. Physiol. 9: 89-151.

86. Starovoitov, I.I., A.M. Boronin, and G.K. Skryabin (1976) "A comparative study of routes for naphthalene catabolism in two strains of Pseudomonas putida". Dokl. Acad, Nauk SSSR 228: 228-231. Engl. Transl. 213-215.

87. Strawinski, R.J. and R.W. Stone (1943) "Conditions governing the oxidation of naphthalene and the chemical analysis of its products". J. Bacteriol. 45: 16.

88. Strawinski, R.J. and Stone, R.W. (1954) "Biological oxidation of naphthalene". Can. J. Microbiol. 1: 206-210.

89. Tattersfield, F. (1928) "The decomposition of naphthalene in the soil and the effect upon its insectidal action". Ann. Appl. Biol. 15: 57-80.

90. Tausson, W.O. (1928) "Naphthalin als Kohlenstoffquelle fur bakterine". Planta 4: 214.

91. Thompson, R., S.G. Hughes and P. Broda (1974) "Plasmid identification using specific endonucleases". Molec. Gen. Genet. 133: 141-149.

92. Treccani, V., N. Walker and G.H. Wiltshire (1954) "The metabolism of naphthalene by soil bacteria". J. Gen. Microbiol. 11: 341-348.

93. Walker, N. and W.C. Evans (1952) "Pathways in the metabolism of the monohydroxy benzoic acids by soil bacteria". Biochem. J. 52: XXIII.

94. Walker, N., and G.H. Wiltshire (1953) "The breakdown of naphthalene by a soil bacteria". J. Gen. Microbiol. 8: 273-276.

95. Wigmore, G.J., R.C. Bayly, and Di Berardino (1974) "Pseudomonas putida mutants defective in the metabolism of the products of meta fission of catechol and its methyl analogues". J. Bacteriol. 120: 31-37.

96. Willetts, N. (1981) "Sites and systems for conjugal DNA transfer in bacteria" in Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids. S.B. Levy, R.C. Clowes, E.L. Koenig eds. Plenum Press, New York and London p. 207-215.

97. Williams, P.A. (1981) "Catabolic plasmids". TIBS 6: 23-26.

98. Williams, P.A., F.A. Catterall and K.Murray (1975) "Metabolism of naphthalene 2 methylnaphthalene, salicylate and benzoate by Pseudomonas P_G: Regulation of tangential pathways". J. Bacteriol. 124: 679-685.

99. Williams, P.A. and K. Murray (1974) "Metabolism of benzoate and the methylbenzoates by Pseudomonas putida arvilla) mt-2". Evidence for the existence of a TOL plasmid. J. Bacteriol. 120: 416-423.

100. Yen, K.-M. and I.C. Gunsalus (1982) "Plasmid gene organization: naphthalene/salicylate oxidation". Proc. Natl. Acad. Sci. USA 79: 874-878.

101. Zobell, C.E. (1946) "Action of microorganisms on hydrocarbons". Bacteriol. Rev. 10: 1-49.

102. Zuniga, M.C., D.R. Durham and R.A. Welch (1981) "Plasmid and chromosome-mediated dissimiliation of naphthalene and salicylate in Pseudomonas putida PMD-1". J. Bacteriol. 147: 836-843.