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Characterization of genes from *Rhodococcus* sp. involved in the degradation of environmental pollutants

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ABSTRACT

The work described in this research report is an investigation into the microbial degradation of environmental pollutants, specifically : (a) the decolorization of azo dyes, (b) the desulfurization of dibenzothiophene and (c) the degradation of nylon monomers.

A Gram negative organism, SQ4, provisionally identified as a *Pseudomonas* sp. capable of using the nylon monomers caprolactam, diaminohexane, adipic acid and aminocaproic acid as a sole carbon and energy source was isolated from the soil. A BglII genomic library of this organisms was constructed in the positive selection vector, pEcoR251, and screened for genes involved in the degradation of the nylon monomers and aromatic compounds. The gene complementing the *lysA*23 mutation of *Escherichia coli* strain RK4904 was extracted from the BglII library and was expressed from its own promoter. A restriction map of the complementing DNA which codes for the enzyme diaminopimelate decarboxylase [E.C. 4.1.1.20], was constructed. A second HindIII library of SQ4 was also constructed.

An orange/red pigmented Gram positive organism was also isolated from the soil on its ability to use dibenzothiophene as a sulfur source. Preliminary work indicated that DBT was used as a sulfur source.

A mutant of *Rhodococcus erythroplis* ATCC 4277 unable to decolorize Amido Black was isolated. This mutant, SQ3, was complemented by plasmid pGSH1 and not by plasmid pGSH2. The BglII fragment from pGSH1 itself does not complement azo dye decolorization in GH1 and removal of this fragment results in a loss of decolorizing activity. Therefore, the gene/s involved are situated about the BglII sites of pGSH1.

DECLARATION

I declare that this research report is my own unaided work unless otherwise specified. It is being submitted to the University of the Witwatersrand, Johannesburg for the degree of Masters of Science in Biotechnology. It has not previously been submitted for any other degree or examination in this or any other university.

Selwyn Quan

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17th March 1993

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ABBREVIATIONS

AB:	Amido Black
amp:	ampicillin
asa:	arsenate
asi:	arsenite
ATCC:	American Type Culture Collection
BGSC:	*Bacillus* Genetic Stock Centre
BSM:	basal salts medium
bp:	base pairs
Cap:	caprolactam
Cm:	chloramphenicol
CsCl:	caesium chloride
DBT:	dibenzothiophene
Dig:	digoxigenin
DNA:	deoxyribonucleic acid
dNTP:	deoxynucleotide triphosphates
DSM:	Deutsche Sammlung von Mikroorganismem und Zellkulturen
EDTA:	ethylenediaminetetraacetic acid
EMBL:	European Molecular Biology Laboratory
EtBr:	ethidium bromide
GC:	gas chromotography
HPLC:	high pressure liquid chromotography
IPTG:	isopropyl-*β-D*-thiogalactoside
kb:	kilobase
kg:	kilogram
LA:	Luria Agar
M:	molar
ml:	millilitre
mm:	millimetre
MM:	minimal medium
mol:	molar
MS:	mass spectroscopy
MW:	molecular weight
NBT:	nitroblue tetrazolium salt
NTG:	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
O.D.:	optical density
OII:	Orange II
PEG:	polyethylene glycol
rif:	rifampicin
RNA:	ribonucleic acid
rpm:	revolutions per minutes
SDS:	sodium dodecyl sulfate
sm:	streptomycin
sp:	spectinomycin
SSC:	trisodium citrate

TES:	*N*-tris(hydroxymethyl)-methyl-2-amino-ethanesulfonic acid
tet:	tetracycline
Tris:	Tris(hydroxymethyl)-aminomethane
Tween-80:	polyoxyethylenesorbitan monooleate
X-phosphate:	*5*-bromo-*4*-chloro-*3*-indolyl phosphate
X-gal:	*5*-bromo-*4*-chloro-*3*-indolyl-*β*-*D*-galactoside

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1. INTRODUCTION

1.1 Environmental pollution

Increased industrialization in the last century has resulted in the escalating production of synthetic chemicals and consequential increase in the number and amount of environmental pollutants in the form of industrial chemicals, insecticides, noxious gases, heavy metals and detergents. Many of these environmental pollutants have had a detrimental effect not only on man but also the environment.

An often cited example is that of the organochloride insecticide DDT (1,1-*bis(p*-chlorophenyl)2,2,2-trichloroethane) which was indiscriminately used in vector control, industry medicine and in the household shortly after the second World War. Subsequently it has been found that this compound accumulates in the fatty tissue due to its lipophilic structure and therefore undergoes bioconcentration in the food chain. Since then the industrial countries of North America, Europe and Asia have passed legislation prohibiting the use of this and other organochlorine insecticides like aldrin and dieldrin (Munnecke, 1981). Polychlorinated biphenyls (PCBs) are another example of a synthetic chemical with an enormous pollution potential. PCBs were first manufactured in 1929 for use in transformer oils, capacitor dielectrics and heat transformer fluids under trade names such as Aroclor, Clophen and Phenoclor (Leisinger and Brunner, 1986). These compounds have since been identified in the wildlife samples from many industrialized countries and reports regarding its toxicity to man and animal have prompted a ban on the production of PCBs in most of these countries.

Another prime example is atmospheric pollution principally as a result of the burning of fossil fuels such as coal and oil. Carbon monoxide, sulfur dioxide, hydrogen sulfide, hydrogen fluoride and nitrogen dioxide are a few examples of some of the gases that are released from the combustion of fossil fuels that can cause serious pollution problems. Not only is there a health risk associated with these gases but effects such as acid rain, the greenhouse effect and the depletion of the ozone layer are also a source of concern.

1.2 Microbial biodegradation of environmental pollutants

Xenobiotics compounds are defined as man-made compounds with chemical structures to which microorganisms have not been exposed to in the course of evolution (Hutzinger and Veerkamp, 1981). With regards to environmental pollution the concern is not so much whether a particular chemical has a xenobiotic structure but whether it is readily biodegradable. Pollutants which are not biodegradable or slowly biodegradable tend to persist in the environment and these so-called recalcitrant compounds are a major source of concern. A generalization of a number of structural features which determine a compounds recalcitrance in the environment have been suggested (Leisinger and Brunner, 1986): (1) oligomerization as is the case with synthetic polymers increases the recalcitrance of the compound. (2) Introduction of chlorine, nitro and sulfo groups or of branched carbon chains into a molecule and (3) an increasing number of substituents also enhance recalcitrance. The microbial biodegradation of these compounds is an aspect of biotechnology that is currently receiving wide interest (Slater and Bull, 1982 Leisinger *et al.* 1981; Ghisalba, 1983; Bouwer, 1992).

Microorganisms play an important role in the degradative processes of many of the naturally occurring organic compounds and they are an integral element of the carbon, nitrogen and sulfur cycles. Microorganisms have in the course of evolution adapted to use a wide variety of naturally occurring chemical compounds as carbon and energy sources but the increasing number of xenobiotic compounds produced every year prompts the question of how microorganisms have acquired novel metabolic functions to deal with them (Mortlock, 1982; Boyle, 1992). By looking specifically at several well characterized biodegradation pathways for aromatic compounds, van der Meer and coworkers (1992) suggested that a number of molecular mechanisms are responsible for accelerating the evolution of new catabolic pathways including recombination, transposition and gene transfer.

A number of organic xenobiotic compounds which are slowly biodegradable or persist in the environment have been identified and these include the halogenated aromatics, halogenated aliphatics and several pesticides (Alexander, 1981; Leahy and Colwell, 1990). The persistence of these compounds may be due to unfavourable physicochemical

conditions such as temperature, pH and redox potential or may be a result of nutrient availability or predation (Goldstein *et al.*, 1985). On the other hand, it may simply be that microorganism do not have the capability of dealing with compounds containing foreign structures because they have not been exposed to these structures before and allowed to adapt. This adaptive response to xenobiotic compounds have been demonstrated for microorganisms under laboratory conditions (Aelion *et al.*, 1987; Madsen *et al.*, 1991).

This project will focus on three different aspects in which microorganisms could potentially be used for the biodegradation or bioremediation of environmental pollutants. The first is the decolorization and degradation of sulfonated azo dyes. The second is the degradation of nylon monomers and the third the desulfurization of coal.

1.2.1 Degradation of sulfonated azo dyes

Azo dyes belong to the class of dyes containing an azo-group (-N=N-) linked to a carbon atom and are commercially the most important class of dyes widely used in the textile, leather, printing, paper and food industries (Meyer, 1981). These groups are bound mainly to an aromatic ring which in some cases may also carry sulfonic acid groups (SO_3^-) and since both sulfo and azo groups are not naturally occurring, these compounds are recalcitrant to oxidative biodegradation (Paszczynski *et al.*, 1992).

There are currently at least 3000 azo dyes in use. A number are used in consumer goods such as mouthwashes, drugs, cosmetics and pharmaceutical products. The textile industry use a variety of azo dyes to color wool, cotton and polyester clothing (Anliker, 1979). Another big use user of azo dyes is the food industry and food colorants such as Amaranth, Orange I and Ponceau 3R have been certified in the United States since 1906 (Chung and Cerniglia, 1992). Three major azo dyes certified by the U.S. Food and Drug Administration in 1987 were: Tartrazine, 685 000 kg; Sunset Yellow, 483 000 kg; Allura Red, 946 000 kg (Prival *et al.* 1988). Because of the large amounts of azo dyes consumed every year, a number of studies have been performed to determine the human health risk since epidemiological evidence suggests that there may be link between high consumption and incidence of intestinal cancer (Hill, 1971). Some of the dyes have

been shown to be carcinogenic and mutagenic to animals and man (Combes *et al.*, 1982). Although some of the dyes are not mutagenic *per se* they may become mutagenic through intestinal microflora metabolism and/or mammalian azo reduction and chemical reduction (Chung, 1983) and in many cases this may be due to the aromatic amines which are formed by the enzymatic cleavage of the azo bond with the subsequent *N*-ring hydroxylation and *N*-acetylation of the aromatic amine (Chung and Cerniglia, 1992). A recent study by Chung and Cerniglia (1992) identified common structures such as *p*-phenylenediamine and benzidine moieties of azo dyes that seem to be linked to their mutagenicity. For the phenylenediamine moiety, methylation or substitution of nitro group for an amino group did not decrease mutagenicity. However, sulfonation, carboxylation, deamination or substitution of an ethyl alcohol or an acetyl group for the hydrogen in the amino groups resulted in decreased mutagenicity. Methylation, methoxylation, halogenation or substitution of an acetyl alcohol or an acetyl group for hydrogen in the amino group did not affect mutagenicity for the benzidine group. Mutagenicity of azo dyes can therefore be predicted by this structure - activity relationship.

A more practical problem associated with the manufacturing and processing industry of azo dyes concerns the discharge of azo dye containing effluent. A dye concentration of less than 1ppm, below that of other chemicals found in waste water, is visible in water (Zollinger, 1987). The discharge of highly colored effluent from untreated waste water of dyeing mills is an environmental eyesore and as a result a number of methods for decolorizing these dyes have been investigated including adsorption, precipitation, chemical degradation, photodegradation and biodegradation (Zollinger, 1987).

Adsorption by activated charcoal, silica gel, peat, wood and ion-exchange resins is not economically feasible (Zollinger, 1987). Viable chemical methods include oxidative degradation by chlorine and ozone. Chlorine treatment works well only with specific azo dyes while ozone treatment is more effective but more expensive (Zollinger, 1987).

Complete mineralization of azo dyes to CO_2, H_2O, NO_3^-, SO_4^{2-}, Cl^- would be ideal but because of the xenobiotic azo linkage these dyes are not readily degraded. However, a

Table 1. Organisms capable of mineralizing or decolorizing azo dyes

Organism	Azo dye	Reference
Algae		Liu and Liu (1992)
Phanerochaete chrysosporium	Orange II, Tropeolin O, Congo Red, Acid Red, Direct Blue, Chrysophenine, Biebrich Scarlet, Tetrazine, Yellow 9, Basic Green 4, crystal violet, brilliant green, cresol red, bromophenol blue	Glenn and Gold (1983), Cripps *et al.* (1990), Paszczynski *et al.* (1991), Paszczynski and Crawford (1991)
Bacterial consortium	Mordant Yellow 3	Haug *et al.* (1991)
Bacillus subtilis	*p*-aminoazobenzene	Horitsu *et al.* (1977)
Clostridium spp.		Rafii *et al.* (1990)
Pseudomonas KF46	Orange II	Zimmermann *et al.* (1984)
Pseudomonas cepacia	*p*-aminobenzene	Idaka *et al.* (1987)
Streptococcus faecalis	Acid Yellow	Scheline *et al.* (1970)
Streptomyces spp.	sulfonated azo dyes	Paszczynski *et al.* (1992)
Proteus vulgaris	azo food dyes	Dubin *et al.* (1975)

number of organisms capable of decolorizing or mineralizing azo dyes have been discovered including algae, fungi and bacteria (Table 1). *Pseudomonas* species capable of mineralizing selected monoazo dyes of the type Naphthalene Orange G have been grown after selective pressure in a chemostat. The first step is a reductive cleavage of the azo bond by the enzyme, Orange II azoreductase (Meyer, 1981). This enzyme is, however, very specific in its ability to degrade azo compounds (Zimmermann *et al.*, 1982). Haug *et al.* (1991) have shown that a bacterial consortium is capable of mineralizing the sulfonated azo dye Mordant Yellow 3. More recently, the lignin degrading white-rot fungus *Phanerochaete chrysosporium* in addition to degrading a number of pollutants including chloropolyphenols (Valli *et al.*, 1991), nitrotoluenes (Fernando *et al.*, 1990), polycyclic aromatic hydrocarbons (Bumpus, 1989) and dioxin (Bumpus *et al.*, 1985), is capable of mineralizing the azo dyes Disperse Yellow 3, Disperse Orange 3 and Solvent Yellow 14 (Sparado *et al.*, 1992). This fungus is also able to decolorize the azo dyes Orange II, Tropeolin O, Congo Red, Acid Red 114, Acid Red 88, Biebrich Scarlet, Direct Blue 15, Chrysophenine, Tetrazine (Cripps *et al.*, 1990; Paszczynski and Crawford, 1991; Paszczynski *et al.*, 1991) and the triphenylmethane dyes Basic Green 4, crystal violet, brilliant green, cresol red, bromophenol blue and pararosa-anilines (Bumpus and Brock, 1988). An initial study implicated the fungal

lignin degrading system since a crude lignin peroxidase was required for the initial step of Orange II and Tropeolin O decolorization (Cripps *et al.*, 1990). Subsequent work has shown that a Mn(II) peroxidase was also responsible for azo dye decolorization (Paszczynski and Crawford, 1991). In a study performed by Paszczynski *et al.* (1992), the ability of *P. chrysosporium* and *Streptomyces chromofuscus* to mineralize several sulfonated azo dyes were examined. The relationship between azo substitutions with sulfo groups and biodegradation was also examined. They found that *P. chrysosporium* mineralized all the sulfonated azo dyes and that the substitution pattern did not significantly influence the susceptibility of the dyes to biodegradation. On the other hand, *S. chromofuscus* was not able to mineralize dyes with a sulfo group or both sulfo and azo groups. Pasti-Grigsby *et al.* (1992) implicated peroxidase in the initial azo dye biodegradation by *P. chrysosporium* and *S. chromofuscus*. The manganese peroxidase of *P. chrysosporium* and the extracellular peroxidase of *Streptomyces* spp. showed a similar substrate specificity.

1.2.2 Degradation of nylon monomers

Nylons are polyamide step-growth polymers. Nylon-6,6 was first made by W.H Carothers at the Du Pont company in 1933 and is so called because each of its monomers, diaminohexane and adipic acid, consist of six carbon atoms (Hart, 1991). Another type of nylon known as nylon-6 was first produced in Germany in 1940 and is manufactured by polymerizing caprolactam, a cyclic amide, in the presence of a catalytic amount of water under the same conditions used in nylon-6,6 manufacture. The water hydrolyzes the small amount of caprolactam to ε-aminocaproic acid and then a combination of ring opening reactions involving amino groups and condensation reactions between amino and carbonyl groups results in the formation of the polyamide (Wiseman, 1986).The properties of nylon-6 is very similar to that of nylon-6,6 and the two are virtually interchangeable in their applications.

All these xenobiotic compounds are found in the effluent from industrial manufacturing plants and therefore constitute a pollution problem. For this reason, the microbiological degradation of these monomeric compounds is of great interest since bioremediation offers a potential solution in the treatment of these industrial xenobiotic pollutants.

Limited research into organisms capable of degrading nylon monomers and oligomers has been done. *Flavobacterium* sp. K172 and *Pseudomonas* sp. NK87 are able to grow on 6-aminohexanoate cyclic dimer and the enzymes 6-aminohexanoate cyclic dimer hydrolase encoded by the *nylA* gene and 6-aminohexanoate dimer hydrolase encoded by *nylB* gene are responsible for their breakdown (Kanagawa *et al.*, 1989; Kinoshita *et al.*, 1975). The genes for the enzymes are plasmid encoded in both of the strains (Negoro *et al.*, 1983). Okada *et al.* (1983) showed that a second 6-aminohexanoic acid linear oligomer hydrolase enzyme, encoded by the *nylB'* gene, was present on the plasmid. This enzyme was 88% identical to the enzyme encoded by the *nylB* gene and the authors suggested that the second enzyme arose by gene duplication followed by mutation under selective pressure. More recently a new nylon oligomer degradation gene, *nylC*, has also been found on plasmid pOAD2 from this *Flavobacterium* strain (Negoro *et al.*, 1992). A number of organisms capable of degrading ε-caprolactam have been identified including *Corynebacterium aurantiacum* (Fukumura and Teramura, 1982), *Flavobacterium* sp. (Negoro *et al.*, 1980) and *Pseudomonas* spp. (Boronin *et al.*, 1986). A number of wild-type natural strains of *Pseudomonas* capable of degrading ε-caprolactam via the ε-aminocaproate, adipic hemialdehyde, adipate, succinyl-CoA, CO_2, H_2O pathway have been discovered (Boronin *et al.*, 1984). The degradation of this compound has been shown to be under the control of large 300-MD plasmids that have been well characterized (Naumova *et al.*, 1988; Esikova *et al.*, 1990).

1.2.3 Desulfurization of coal

Combustion of sulfur containing fossil fuels such as coal is primarily responsible for the release of SO_2, the cause of acid rain, into the atmosphere. Legislation in the United States, therefore, requires the removal of about 90% of the sulfur in coal to meet the Clean Air Act standards for sulfur emissions (Kilbane, 1990). The source of this problem is the presence of both organic and inorganic forms of sulfur in coal. Pyrite, the main inorganic sulfur containing compound in coal, is effectively removed from coal by physical, chemical and microbiological means prior to combustion. Sulfur is currently removed during or after combustion by processes such as fluidized-bed combustion or flue gas desulfurization (Kilbane, 1991). These processes employ both physical and chemical methods and require costly equipment that is expensive to maintain. There is

also a further problem with the disposal of sulfurous wastes generated by these processes (Maka and Cork, 1989). The methods involved in microbial desulfurization: (a) require low capital and operating costs, (b) are more energy efficient, and (c) can remove finely distributed pyrite without any loss of coal (Kargi and Robinson, 1982). Therefore the microbial desulfurization of coal offers an attractive economically viable alternative to the more conventional chemical and physical desulfurization methods.

Desulfurization of inorganic sulfur by organisms such as *Thiobacillus ferrooxidans, Thiobacillus thiooxidans* and *Sulfolobus acidocaldarius* can remove 90% or more of the inorganic sulfur within a few days (Kilbane, 1989). Inorganic sulfur, however, is not the only form of sulfur present in coal and a large amount is present in the organic form. This form of sulfur is not as easily removed as the inorganic sulfur form because it forms an integral part of the carbon matrix and desulfurization would therefore specifically require cleavage of the carbon-sulfur bonds. In addition, the removal of sulfur should not be coupled to the removal of carbon since this will result in a reduction of the calorific value of the coal. The goal is, therefore, to find an organism or group of organisms capable of removing both inorganic and organic sulfur from coal without breaking down the carbon matrix and reducing the fuel value of the coal.

A number of different organic sulfur containing compounds are present in coal as thiol, sulfide, disulfide and thiophene functional groups (Maka and Cork, 1989). It has generally been accepted that thiophene is the main sulfur containing functional group in coal (Nishioka *et al.*, 1986; Spiro *et al.*, 1984) and the compound dibenzothiophene (DBT) is therefore regarded as a good model compound representative of the organic sulfur forms found in coal (Kilbane, 1989). Efforts have recently focused on finding microorganisms capable of desulfurizing DBT (Monticello and Finnerty, 1985; Krawiec, 1989; Kilbane, 1989).

An attempt to mutagenize *Escherichia coli* to degrade thiophene succeeded in the isolation of *thyA* mutants that expressed a novel enzyme with the capability of oxidizing a variety of substrates containing the sulfone moiety (Juhl and Clark, 1990). These mutants were also more resistant than wild-type strains to aromatic sulfone antibiotics

such as dapsone. Although DBT is often chosen as the model compound of the organic sulfur forms found in coal, researchers have also examined the desulfurization of alternative organic sulfur containing compounds such as dibenzyldisulfide (Miller, 1992). In this report, a methanogenic mixed culture derived from a sewage digester was able to desulfurize dibenzyldisulfide releasing toluene and benzyl mercaptan as intermediates.

The majority of research has, however, focused on the compound DBT and a number of organism capable of using this compound as a sole carbon or sulfur source have been reported (Table 2). It has previously been considered that DBT degradation generally proceeds via two pathways. In the first pathway outlined in figure 1, first shown by Kodama *et al.* (1970) in a *Pseudomonas* species, DBT is degraded by hydroxylating the benzoid component of the compound to a dihydroxy-dihydro-derivative and after a series of steps producing 3-hydroxy-2-formylbenzothiophene (Figure 1a). This pathway

Table 2. Organisms capable of using dibenzothiophene as sole C- or S-source

Organism	Description	Reference/s
Mixed bacterial culture	desulfurization of DBT, benzothiophene, dibenzylsulfide	Kohler *et al.* (1984)
Beijerinckia sp.		Laborde *et al.* (1977)
Brevibacterium sp. DO	Mineralization of DBT via benzoate	van Afferden *et al.* (1990)
Corynebacterium sp. SY1	'4S' pathway	Omori *et al.* (1992)
Pseudomonas sp.	Plasmid mediated	Monticello *et al.* (1985)
		Fortnagel *et al.* (1989)
Sulfolobus acidocaldarius		Kargi *et al.* (1986)
Rhodococcus rhodochrous IGTS8	'4S' pathway	Kayser *et al.* (1993)

has subsequently been established in a number of other genera including *Acinetobacter, Beijerinckia* and *Rhizobia* (Krawiec, 1989). From figure 1 it can be seen that one of the benzene rings is degraded and that no actual desulfurization occurs since the final product, 3-hydroxy-2-formylbenzothiophene, still contains the sulfur containing thiophene group.

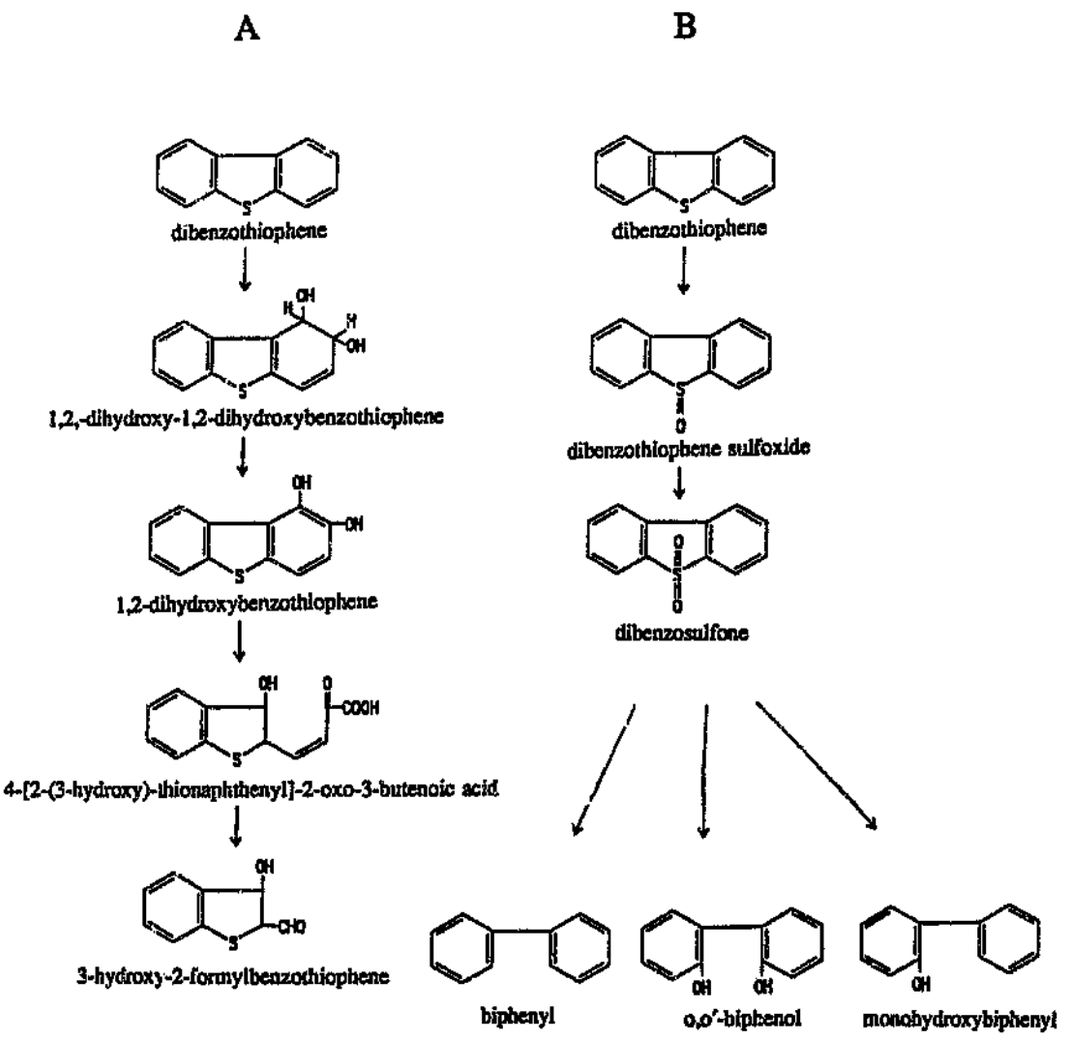


Figure 1. Postulated dibenzothiophene desulfurization pathway. (A) The so-called Kodama pathway and (B) the extended '4S' pathway.

The second or so called '4S' pathway in which DBT is degraded by progressive oxidation of sulfur to sulfoxide/sulfone/sulfonate/sulfate has recently been shown (Isbister *et al.*, 1985; Omori *et al.*, 1992; Kayser *et al.*, 1992). In this pathway, sulfur is released in the form of a sulfate ion with only a partial disruption of the carbon skeleton (Figure 1). An extended '4S' pathway has been proposed in which the end product or intermediate metabolite may either be a monohydroxyphenyl or biphenyl instead of the usual 2,2'-dihydroxybiphenyl (Krawiec, 1989). A number of organisms including *Corynebacterium* sp. SY1 (Omori *et al.*, 1992), *Pseudomonas* sp. (Isbister *et al.*, 1985) and *Rhodococcus rhodochrous* (Kayser *et al.*, 1992) seem to degrade DBT via this pathway and the end product of DBT degradation by *Corynebacterium* sp. SY1 seems in fact to be monohydroxyphenyl. More recently slightly altered pathways for the degradation of DBT have been proposed. van Afferden *et al.* (1990) proposed that *Brevibacterium* sp. DO desulfurized DBT to DBT-5-oxide to DBT-5-dioxide and finally to benzoate.

1.3 Nocardioform bacteria

Nocardioforms are aerobic Gram-positive, soil-dwelling, filamentous actinomycetes characterized by a type-IV cell wall containing *meso*-diaminopimelic acid with arabinose and galactose as the diagnostic sugars. Bergey's Manual of Systematic Bacteriology describes 9 genera including *Nocardia, Rhodococcus, Micropolyspora, Saccharopolyspora, Pseudonocardia, Oerskovia, Promicromonospora, Nocardioides* and *Intrasporangium* belonging to the nocardioforms which are closely allied to *Corynebacterium, Arthrobacter* and *Mycobacterium* (Lechavalier, 1989).

Nocardioforms are a group of bacteria that are attracting increasingly more interest because of their metabolic diversity (for review see Tárnok, 1976; Raymond and Jamison, 1977). Recent work has shown that these organisms have great biotechnological potential since they are capable of degrading a range of chemical pollutants including aromatic compounds (Janke and Ihn, 1989; Bruce and Cain, 1990), polycyclic aromatic compounds (Walter *et al.* 1991, Grund *et al.*, 1992), substituted aromatics (Dickel and Knackmuss, 1991; Lenke *et al.*, 1992, Apajalahti *et al.*, 1987;

Table 3. Metabolic diversity of *Rhodococcus*

Organism	Characteristic	Reference
R. chlorophenolicus	dechlorination of tetrachlorohydroquinone	Apajalahti *et al.* (1987)
R. rubropertinctus	terephthalate degradation	Naumova *et al.* (1987)
Rhodococcus CG-1, CG-2, CP-2	degradation and O-methylation of polychlorinated phenols	Häggblom *et al.* (1988)
R. rubropertinctus N657	fluorinated aromatic degradation	Engesser *et al.* (1988)
Rhodococcus AM 144	cometabolism of aniline, phenol and their monochlorinated derivatives	Janke and Ihn (1989)
R. rhodochrous	*n*-alkanes degradation	Sorkhoh *et al.* (1990)
R. rhodochrous CP-2	metabolism of propane	Woods and Murrell (1989)
R. rhodochrous	quinate and shikimate degradation	Bruce and Cain (1990)
R. erythropolis Y2	haloalkanes halidohydrolase	Sallis *et al.* (1990)
Rhodococcus sp. QT-1	1,3-dinitrobenzene	Dickel and Knackmuss (1991)
R. rhodochrous CTM	2-methylaniline degradation	Fuchs *et al.* (1991)
Rhodococcus strain	dioxane, tetrahydrofuran and cyclic ether degradation	Bernhardt and Dickmann (1991)
Rhodococcus sp. UW1	pyrene degradation	Walter *et al.* (1991)
R. equi	methylperhydroindanedione catabolism	Miclo and Germain (1990)
Rhodococcus C1	1,8-cineole catabolism	Roger Williams *et al.* (1989)
Rhodococcus sp. B4	naphthalene degradation	Grund *et al.* (1992)
R. erythropolis HL 24-1, HL 24-2	2,4-dinitrophenol, picric acid catabolism	Lenke *et al.* (1992), Lenke and Knackmuss (1992)
Nocardia sp. DSM 1069	degradation of coniferyl alcohol and other lignin-related compounds	Eggeling and Sahm (1980)
R. rhodochrous	metabolism of lignin-related compounds	Andreoni *et al.* (1991)
Rhodococcus	enantiomer selective amidase	Mayaux *et al.* (1991)
R. rhodochrous J1	nitrilase	Kobayashi *et al.* (1988)
R. rhodochrous NCIB 11216	conversion of dinitriles	Bengis-Garber *et al.* (1989)
Rhodococcus H13-A	biosurfactant production	Vogt Singer and Finnerty (1990)

Engeser *et al.*, 1988), aliphatic compounds (Ashraf and Murrell, 1990; Bernhardt and Dickmann, 1991; Sallis *et al.*, 1990), lignin-related compounds (Eggeling and Sahm, 1980; Andreoni *et al.*, 1991). In addition, they are also able to produce products of commercial importance such as biosurfactants (McDonald *et al.*, 1981; Vogt Singer and

Finnerty, 1990) and are capable of performing bioconversions used in the production of acrylamide (Watanabe, 1987; Nagasawa, 1988) and steroid compounds (Ferreira *et al.*, 1984).

1.3.1 Cloning in *Rhodococcus*

Due to the interest in this organism a number of cloning vectors have been developed. Vogt Singer and Finnerty (1988) developed a *Rhodococcus-E. coli* shuttle vector by joining a cryptic *Rhodococcus* plasmid to pIJ30, a pBR322 derivative containing an *E. coli* origin of replication, an ampicillin resistance determinant and a thiostrepton resistance determinant from *Streptomycetes*. Hashimoto *et al.* (1992) developed an *Rhodococcus-E.coli* shuttle plasmid by joining cryptic plasmids from *R. rhodochrous* to derivatives of the *E. coli* plasmid pUC19. Ampicillin and kanamycin were the resistance determinants present on these plasmids. In this laboratory, a *Rhodococcus-E. coli* shuttle vector was constructed by joining the *E. coli* positive selection vector, pEcoR251, to a nocardioform arsenic resistance plasmid pDA30 (Dabbs *et al.* 1990). This resulted in a vector, pDA37, which was functional in both *E. coli* and *Rhodococcus*. An ampicillin resistance gene ensured maintenance in *E. coli* and an arsenic resistance served the same function in *Rhodococcus*. In addition, the vector had an EcoRI gene that could be used as a positive selection function in *E. coli* and this allowed *Rhodococcus* libraries to be constructed with this vector. A number of genes have since been successfully identified from libraries constructed with this vector (Goldman, 1991; Heiss *et al.*, 1992; Andersen and Dabbs, 1991).

Because of problems with toxicity associated with the use of arsenate and arsenite in the laboratory, second generation cloning vectors that use chloramphenicol rather than arsenic resistance for maintenance have been developed (Quan and Dabbs, 1993). These vectors employ the chloramphenicol resistance gene excised from the *E. coli* cloning vector pACYC184. They have an advantage not only in that chloramphenicol is less toxic than arsenate and arsenite, but that they are also smaller and selection in the presence of chloramphenicol rather then arsenate and arsenite allows transformants to grow up twice as fast.

1.4 Aims of this project

There are three essentially different aspects that this project will deal with all concerned with microbial removal of environmental pollution. The first part will be a continuation

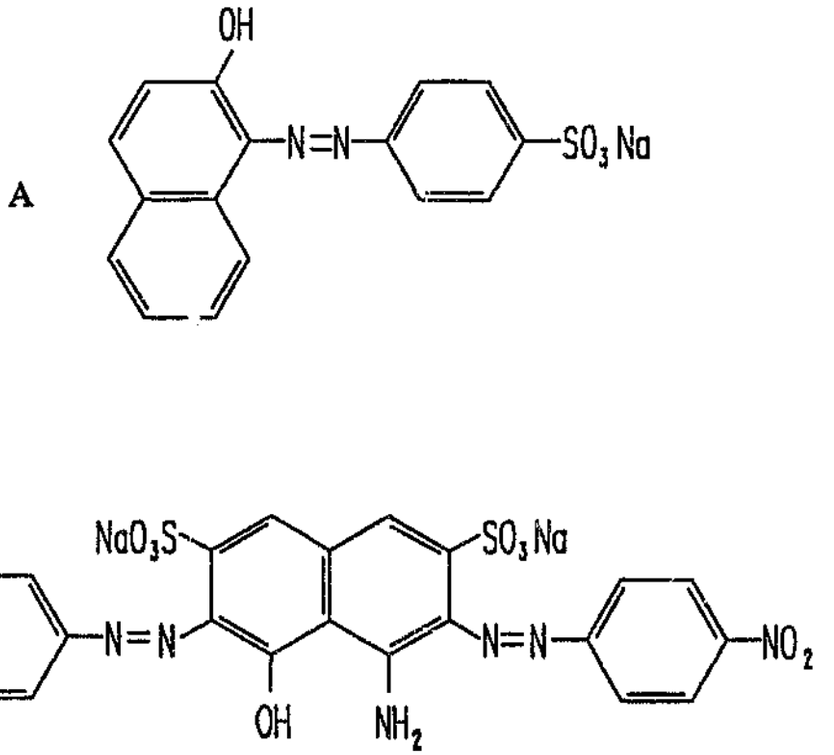


Figure 2. Chemical structure of the sulfonated azo dyes Orange II (A) and Amido Black (B).

of the work done by a former student concerned with decolorization of azo dyes (Heiss, 1992). Many of the laboratory *Rhodococcus* strains have the ability to decolorize the azo dyes Amido Black and Orange II (Figure 2). The gene/s involved in decolorization were cloned by the complementation of non-decolorizing mutants of *R. erythropolis* ATCC 4277 and *R. equi* ATCC 14887 (Heiss *et al.* 1992). The DNA restoring decolorization capacity in GH1, the non-decolorizing mutant of *R. erythropolis* ATCC 4277, was cloned onto an 6.4-kb fragment (Figure 3). A second 50-bp fragment restored

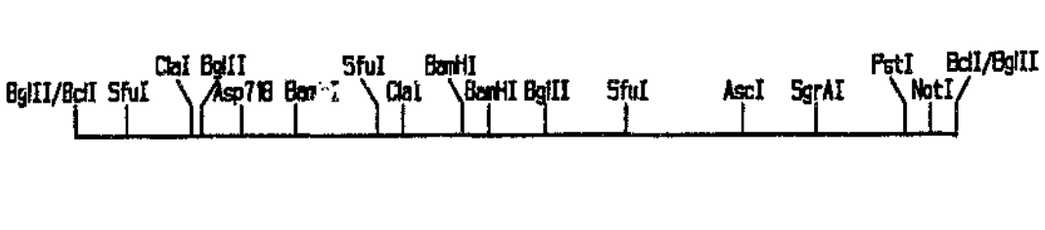


Figure 3. Restriction map of 6.4-kb insert of pGSH1 from BclI library of ATCC 4277 restoring decolorizing capacity in GH1.

decolorization in GH2, the non-decolorizing mutant of *R. equi* ATCC 14887. This part of project will therefore involve subcloning the 6.4-kb fragment complementing GH1 with a view to sequencing the gene/s involved. Since the 50-bp fragment complementing GH2 cannot code for an intact polypeptide, this part of the project will also attempt to retrieve an intact gene from one of nocardioform libraries available using this small fragment as a probe. More mutants of decolorizing strains of *Rhodococcus* will also be made to see whether one or more genes is involved in the decolorization process.

The second part of this project will involve enriching and screening using a method developed by Goldman (1991) soil samples for nocardioforms that can use either of the nylon monomeric compounds caprolactam and diaminohexane as a sole carbon and energy source. This method essentially employs three different selective steps. The first selection involves incubation of the soil sample in a defined media in which the steroid compounds sodium deoxycholate and sodium taurocholate have been supplied as the sole carbon source. Most of the laboratory strains of *Rhodococcus* can use these two compounds as a sole carbon source (Goldman, 1991). The second step is an antibiotic selection with naladixic acid which inhibits DNA gyrase and seems to be preferentially active against Gram negative organisms. The final selection is for organisms that can use the compounds of interest as the sole carbon source. Since there are laboratory *Rhodococcus* strains that can use the nylon monomers adipic acid and aminocaproic acid as a sole carbon and energy source, genes involved in the degradation of caprolactam

and diaminohexane could be transferred to these strains so that a strain could be developed that would be able to degrade polymeric nylon molecules.

The final part of this project will screen laboratory *Rhodococcus* strains for their ability to use the model organic sulfur containing compound in coal, dibenzothiophene, as a sole source of sulfur. Failing this, a soil enrichment and screening procedure for nocardioforms, similar to the one used to screen for organisms capable of using caprolactam and diaminohexane as a sole carbon and energy source, will be employed. Once this organism has been found, the genes involved in dibenzothiophene metabolism will be cloned and characterized.

2. MATERIALS AND METHODS

2.1 Strains

The strains employed in this work are listed in table 4. Strains used routinely were made rifampicin resistant by selecting for spontaneous rifampicin resistant mutations on LA plates supplemented with 100µg/ml rifampicin.

2.2 Plasmids and phages

Plasmids and phages used in this work are listed in table 5. Plasmids were stored in either TE (10mM Tris.HCl, 10mM EDTA pH 8.0) or a 4mM Tris.Hcl (Ph 8.0) buffer at -20°C to prevent nicking of the DNA. Phage lysate was stored at 4°C.

2.3 Growth and maintenance of strains

For the growth of *Escherichia coli*, *Rhodococcus* and *Bacillus subtilis* strains, LB media was used and when necessary this was supplemented with 1.5% agar when solid media was required. Defined media was prepared from 10× stock solutions of either A-N buffer for *E. coli* or stock 3 for *Rhodococcus* and supplemented with the necessary amino acid or vitamin requirements. Strains SQ4 and SQ5 both grew well in LB and stock 3 minimal media and did not require any nutritional supplements although initially SQ5 grew poorly in LB and seemed to require phosphate for good growth. This, however, changed after several subcultures on LA plates.

E. coli, *B. subtilis* and SQ4 strains were incubated at 37°C overnight while nocardioform strains and SQ5 were incubated at 28°C for 3 days before growth was visible.

For short term storage, strains were kept on agar plates at -4°C. For long term storage freshly grown strains were kept in 33% glycerol at -70°C.

2.4 Media composition and buffers

A detailed description of the composition of the media, the buffers and the various reagents used in this work is given in appendix A.

Table 4. Strains used in this work

Strain	Characteristics	Source
E. coli		
MM294-1	*supE44 hsdR17 endA1 pro thi* rif^R	E. Dabbs
GM161	*supE44 hsdR1 thr-1 leuB6 dam-4 thi-1 lacY1 tonA21* λ$^-$	E. Dabbs
DH1	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	E. Dabbs
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	M. Kitakawa
KL98-1	prototroph rif^R	E. Dabbs
RK4904	*proC32 trpE38 lysA23 metE70 argH1 zij-602::Tn10 rpoB208 thi-1 lac236 xyl-5 mtl-1 rpsL109 cyc-19 tsx-67 supE44* λ$^-$	E. Dabbs
Rhodococcus		
ATCC 12674 *R. erythropolis*	Azo dye decolorizer	E. Dabbs
ATCC 4277 *R. erythropolis*	Azo dye decolorizer	E. Dabbs
DSM 1069 *R. erythropolis*	Azo dye decolorizer	E. Dabbs
ATCC 14887 *R. equi*	Azo dye decolorizer	E. Dabbs
ATCC 25593 *R. rubropertinctus*		E. Dabbs
DSM 20131 *R. fascians*		E. Dabbs
SQ1	Highly transformable mutant of ATCC 4277-1	Quan (1991)
SQ2	Non-decolorizing mutant of SQ1	This work
SQ3	Non-decolorizing mutant of SQ1	This work
SQ6	Non-decolorizing mutant of ATCC 1069 sm^R	This work
GH1	Non-decolorizing mutant of ATCC 4277	Heiss (1992)
GH2	Non-decolorizing mutant of ATCC 14887	Heiss (1992)
GH2-1	Rif^R of GH2	This work
B. subtilis		
1A2	Prototroph	BGSC[a]
Other		
SQ4	*Pseudomonas* sp. isolated from caprolactam enrichment	This work
SQ4-1	Rif^R of SQ4	This work
SQ5	Actinomycete isolated from dibenzothiophene enrichment	This work
SQ5-1	Rif^R of SQ5	This work

[a] Bacillus Genetic Stock Centre, Ohio University

Table 5. Plasmids and phages used in this work

	Description	Source/reference
Plasmids		
pBR322	amp^R tet^R	E. Dabbs
pACYC184	cm^R tet^R	E. Dabbs
pUC18, pUC19	amp^R *lacZ*	M. Kitakawa
pEcoR251	amp^R EcoRI	E. Dabbs
pEcoR251-*lysA1*	plasmid from BglII library of SC4 complementing *lysA* auxotrophy in RK4904	This work
pEcoR251-*lysA2*	plasmid from BglII library of SC4 complementing *lysA* auxotrophy in RK4904	This work
pIGTS1	cm^R	K. Kayser
pDA37	asa^R asl^R amp^R EcoRI	E. Dabbs
pGSH1	pDA37 containing insert complementing GH1 (BclI library)	Heiss (1992)
pGSH2	pDA37 containing insert complementing GH2 (BamHI library)	Heiss (1992)
pGSH10	pDA37 containing BglII fragment of pGSH1	This work
pGSH11	pDA37 containing BglII fragment of pGSH1	This work
Bcl2	Derivative of pDA37, cm^R	E. Dabbs
22	Derivative of pDA37, cm^R	E. Dabbs
pC194	cm^R	E. Dabbs
pDA52	*Bacillus-E. coli* shuttle vector	S. Andersen
Phage		
C4	plaquing on *R. erythropolis* ATCC 12674	E. Dabbs
C5	plaquing on *R. erythropolis* ATCC 12674, ATCC 4277, DSM 1069	E. Dabbs
C	plaquing on *R. erythropolis* ATCC 4277	E. Dabbs
H	plaquing on *R. erythropolis* ATCC 4277	E. Dabbs

2.5 DNA manipulations and cloning techniques

2.5.1 Phenol-chloroform extractions

About 80µl of TE-saturated phenol was usually added to about 200µl of DNA sample. This was mixed by inversion and then centrifuged at 4°C for 5 minutes to separate the organic and aqueous phases. The upper aqueous layer was removed and if necessary a further phenol step was performed until the there was no visible protein at the interface. The aqueous layer was then mixed with 80µl of chloroform and the two layers were

separated by centrifugation for 15 seconds at room temperature. The upper aqueous phase was then removed into a sterile tube.

2.5.2 DNA precipitation

DNA was usually precipitated with 1/10 volume 1M NaCl and 2.5 volumes of 96% ethanol by microfuging the sample for 20 minutes at 4°C. Dig-labelled DNA was precipitated with 1/10 volume 4M LiCl and 2.5 volumes ethanol at -70°C for 30 minutes. DNA was recovered by centrifugation of the sample at 4°C for 10 minutes.

2.5.3 DNA preparation from CsCl density gradients

Ethidium bromide (EtBr) was removed by extraction with 1/10 volume butanol. This procedure was usually performed about 3 times to remove all traces of EtBr. The CsCl was removed by adding 2 volumes of TE and 2.5 volumes of ethanol and precipitating the DNA by centrifugation at room temperature for 20 minutes. The DNA sample was then dried and resuspended in the appropriate buffer.

2.5.4 Restriction enzymes

Enzymes were obtained from either Boeringer Mannheim or New England BioLabs and used according to the manufacturer's recommendations.

2.5.5 Ligation

T4 DNA ligase (Boeringer) was used for all ligation reactions. Ligation was generally performed at 14°C for 18 hours in a minimal volume (10 - 20µl).

2.5.6 Alkaline phosphatase

Calf intestinal alkaline phosphatase (Boeringer) was used. DNA was resuspended in 90µl of a 10mM Tris.HCl (pH 8.0) buffer to which was added 10µl of 10× phosphatase buffer. DNA with a 5'-overhang was incubated at 37°C for 1 hour with 1 unit of enzyme. Blunt or recessed ends were first incubated at 37°C for 15 minutes and then for a further 45 minutes at 55°C with another unit of phosphatase. The enzyme was inactivated by heating the sample at 75°C for 10 minutes in the presence of 5mM EDTA followed by a phenol-chloroform extraction.

2.5.7 Agarose gel electrophoresis

A stock solution of agarose (SeaKem HGT, FMC) was usually prepared in 0.5× TBE buffer (0.089M Tris.HCl, 0.089M boric acid, 2mM EDTA pH 8.5) and sterilized by autoclaving. Gels were prepared by melting the agarose stock solution in a microwave oven and then pouring the gel at 4°C. Ethidium bromide (1µg/ml) was added to both the gel and buffer and gels were allowed to polymerize for 2 hours. Samples were loaded with bromophenol blue as the dye marker and the gel was generally run between 8 and 9 V cm^{-1} until the dye front reached the bottom of the gel.

Gels with an agarose concentration greater than 1.2% were prepared immediately before use and were autoclaved and then poured at room temperature. Well-formers were removed before the gel had completely set.

2.5.8 Low-gelling agarose

Low-gelling temperature agarose (SeaPlaque, FMC) was prepared in the same manner as normal agarose. These gels were, however, run at 4°C at about 12 V cm^{-1} for 2 hours. The bands of interest were examined under long wavelength UV light (366nm) to prevent nicking of the DNA and then removed with a scalpel blade and placed into Eppendorf tubes. DNA was extracted by melting the gel at 65°C for 10 minutes and then performing a phenol extraction at room temperature. A further 3 phenol extractions were performed at 4°C followed by a chloroform extraction at room temperature. DNA was precipitated with 1/10 volume NaCl and 2.5 volumes ethanol by centrifugation at room temperature.

Alternatively, if the DNA did not require purification, digestion was performed in the low-gelling agarose since it remains liquid at 37°C.

2.5.9 Electroelution

The DNA was run in low-gelling temperature agarose and bands of interest were excised. The gel was placed in dialysis tubing sealed at one end and filled with 0.5× TBE so that no air bubbles remained in the tubing. The tubing was clipped at the other end to retain the buffer and then placed in a submersible electrophoresis unit so that the

current flowed along the length of the tubing. The unit was run at 40V overnight at 4°C. The gel was then removed and viewed under UV light to ensure that the DNA was eluted. The buffer was then transferred to Eppendorf tubes and the DNA purified by two phenol and chloroform extractions.

2.5.10 Screening for pUC18 recombinants

A chromogenic detection system was used to screen for pUC18 recombinants that had lost the function of the *lacZ* gene in *E. coli* strain JM109. This method made use of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) and a nonmetabolizable inducer isopropylthio-ß-D-galactoside (IPTG). Before the cells were spread on the plates, 40µl X-gal (20mg/ml) and 10µl IPTG (100mM) was added to 100µl water and spread on the plates. Plates were incubated overnight at 37°C before formation of the blue precipitate became apparent.

2.6 Plasmid preparations

2.6.1 *E. coli* bulk plasmid preparation

Cultures were grown overnight in 100ml of LB with the appropriate selective agent to maintain the plasmid. Cells were spun down in a JA-10 rotor at 6000rpm for 10 minutes, resuspended in 5ml of solution 1 (50mM glucose, 25mM Tris.HCl, 10mM EDTA, pH 8.0) and then left for 20 minutes at room temperature. Afterwards, 10ml of solution 2 (0.2M NaOH, 1% SDS) was added and the tube was mixed gently by inversion and left at room temperature for 15 minutes. A further 7.5ml chilled solution 3 (5M KAc, pH 6.0) was then added and the tube shaken vigorously and left in an ice/water slurry for 15 minutes. The cells were spun down in a chilled JA-20 rotor at 18000rpm for 15 minutes at 4°C. The supernatant was decanted into a clean Sorvall tube and 0.6 volumes of isopropanol was added to precipitate the DNA. The tube was left on the bench for 15 minutes and then centrifuged in a prewarmed rotor at 10000rpm for 15 minutes at 25°C. The supernatant was decanted and ethanol was added to rehydrate the pellet. The ethanol was gently poured off and the pellet dried under vacuum for 20 minutes. The DNA was then resuspended in 4.2ml TE for 2 hours at 42°C whereafter 400µl EtBr and 4.4g CsCl was added and the refractive index adjusted between 1.389

and 1.390. This was then loaded into a Beckman Quick-seal tube and spun overnight at 45000rpm.

2.6.2 SQ4 total DNA preparation

A large volume of SQ4 (500ml) was necessary to provide a satisfactory yield of DNA. The culture was incubated in a baffled flask on a rotary shaker to provide aeration since the organism was an obligate aerobe. LB proved adequate as the culture media and incubation was performed at 30°C for 2 days until the culture was turbid. To assist the adherence of the pellet to the walls of the container, $CaCl_2$ was added to a 5mM concentration. Cells were spun down at 8000rpm for 15 minutes in a Beckman JA-10 rotor. The supernatant was decanted and the cells resuspended in 4ml of TE containing freshly added lysozyme (5mg/ml) and then incubated at 37°C for 30 minutes. A solution of 0.4ml solution B (TE + 10% SDS) was added to lyse the cells and the mixture was incubated at 55°C for a further 30 minutes. The cellular debris was removed by centrifuging the solution in a 50Ti tube at 45000rpm for 2 hours. The supernatant was transferred to a clean Sorvall tube and 4.4g CsCl was added. After dissolving the CsCl by gentle inversion, the tube was spun at 18000rpm for 15 minutes. The liquid was carefully removed from under the scum and transferred to a clean Sorvall tube. Ethidium bromide (500µl) was added and the refractive index was adjusted between 1.391 and 1.392. The CsCl gradient was generated in a Beckman VTi65.2 rotor at 45000rpm for 15 to 18 hours.

2.6.3 *Rhodococcus* total DNA preparation

A culture was grown to stationary phase in 600ml TYG on a shaker. Cells were collected by centrifugation in a Beckman JA-10 rotor at 6000rpm for 10 minutes. The cells were resuspended in 5ml of solution A (50mM sucrose, 10mM Tris.HCl, pH 8.0) with 5mg/ml freshly added lysozyme and then transferred to a Sorvall tube and incubated at 37°C for 30 minutes. Cells were then spun down at 8000rpm for 5 minutes in a Beckman JA-20 rotor and resuspended in 4ml of TE to which a minute quantity of proteinase-K had been added. One-tenth volume solution B (TE + 10% SDS) was added and the tube was incubated at 37°C for 30 minutes. The viscous solution was transferred to a 50Ti tube and spun at 40000rpm for 30 minutes in a Beckman L5-50

ultracentrifuge. The supernatant was transferred to a clean Sorvall tube and 4.4g CsCl was added. Thr solution was mixed thoroughly by inverting the tube several times and then centrifuged at 15000rpm for 15 minutes. The liquid was decanted from under the scum. EtBr (400µl) was added and the refractive index adjusted between 1.391 and 1.392. The solution was transferred to a Beckman Quick-seal tube and spun in VTi65.2 vertical rotor in a Beckman L7-55 ultracentrifuge at 45000rpm overnight.

2.6.4 *E. coli* mini plasmid preparation

The mini-plasmid prep is based on the alkaline lysis method of Birnboim and Doly (1979). Bacterial cultures were grown overnight in test tubes containing 2ml of LB and incubated at 37°C on a rotary wheel. Half of this culture was transferred to a sterile Eppendorf tube and the cells were pelleted by microfuging for 1 minute at room temperature. The supernatant was decanted and the pellet was resuspended in 100µl of solution 1 by vortexing and left to stand at room temperature for at least 5 minutes. Solution 2 (200µl) was added and the tube was mixed gently by inversion and left to stand for 15 minutes at room temperature. Solution 3 (150µl) was added and the tube was mixed vigorously by vortexing and placed on ice for 15 minutes. To remove the precipitate, the tube was centrifuged for 2 minute at 4°C. The supernatant was removed to a sterile Eppendorf tube. To precipitate the DNA, one-sixth volume isopropanol was added and the tube mixed by inversion. The tube was incubated at 42°C for 2 minutes to allow the contents to reach room temperature. The DNA was pelleted by centrifuging the tube for 10 minutes at room temperature. The pellet was washed in 96% ethanol and then dried in a speed vacuum for 20 minutes. The DNA was resuspended in 40µl of a 4mM Tris.HCl (pH 8.0) solution containing freshly boiled ribonuclease (10µg/ml) and a small aliquot (10µl) was then analyzed on an agarose gel.

2.6.5 *B. subtilis* mini-plasmid preparation

Bacillus mini-plasmid preparations were performed using the *E. coli* mini-plasmid preparation method outlined above. Cultures were, however, not incubated longer than 10 hours because this decreased the yield of plasmid. Plasmid screens were usually performed on 1ml of culture and resuspended in 30µl 4mM Tris.HCl (pH 8.0). An aliquot of 15µl run on a gel gave a clearly visible plasmid band.

2.6.6 *Rhodococcus* mini-plasmid preparation

A culture was grown in a Bijou bottle in 1ml TYG at 28°C with the appropriate selective agent in a rotary shaker overnight. A small aliquot of cells (200µl) was transferred to a sterile Eppendorf tube and 800µl of solution A containing freshly added lysozyme (5mg/ml) was added and incubated at 37°C with shaking for 1 hour. The resulting protoplasts were pelleted by microfuging for 1 minute and then resuspended in 280µl TE. Solution B (40µl) was added and the sample was mixed gently and left at room temperature for 10 minutes. A further 40µl of a chilled solution C (4.5M NaAc, pH 6.0) was added and the tube was mixed vigorously and placed in an ice/water slurry for 30 minutes. The tube was then microfuged for 20 minutes and the supernatant decanted into a sterile Eppendorf tube and further purified with a phenol and chloroform extraction. The DNA was ethanol precipitated and dried under vacuum for 20 minutes. The pellet was resuspended at 42°C for 2 hours in 20µl of a 4mM Tris.HCl (pH 8.0) solution to which freshly boiled RNAse had been added (10µg/ml). A small aliquot (10µl) was analyzed on a gel.

2.7 Transformations

2.7.1 *E. coli* transformation (Adapted from Sambrook *et al.*, 1989)

Generally a 50ml culture of prewarmed LB in a side-arm flask was inoculated with 1/10th volume of an overnight *E. coli* culture. The culture was incubated at 37°C on a rotary shaker until the O.D. (600nm) was 0.2. The flask was then chilled in an ice-water slurry for 1 minute and the cells spun down in a prechilled Beckman JA-20 rotor at 6000rpm for 10 minutes at 4°C. The supernatant was discarded and the cells resuspended in half the original volume of ice-cold transformation buffer (50mM $CaCl_2$, 10mM Tris.HCl, pH 8.0). The cells were then placed on ice for 15 minutes and then centrifuged at 6000rpm for 10 minutes. The supernatant was discarded and the cells resuspended in 1/15 of the original volume of transformation buffer. Aliquots of 0.2ml were put into chilled Eppendorf tubes and stored at 4°C for 2 hrs. Plasmid DNA was added to the Eppendorf tubes and the mixture was left for 30 minutes at 4°C. The solution was mixed every 10 minutes by bubbling air through with a Gilson pipette. The cells were then heat-shocked at 42°C for 90 seconds. Prewarmed LB (0.5ml) was added

to each tube which were then incubated at 37°C for 1 hr to allow for phenotypic expression of the resistance gene. The cells were aliquoted onto dried plates and spread with a sterile 1ml pipette and then incubated at 37°C overnight. Colonies were visible the following day.

2.7.2 *B. subtilis* transformation

2.7.2.1 Competent cell method (Adapted from Anagnostopoulos and Spizizen, 1961)
Cells taken from a freshly streaked plate were inoculated into 2.5ml prewarmed SPI media in a 100ml flask at an O.D 600nm of between 0.2 and 0.3. The flask was incubated with shaking at 37°C for 4 hours. Culture tubes for the second growth period were prepared by adding 0.1ml culture into 0.9ml prewarmed SPII media. DNA diluted in MG was added to the tubes which were then incubated at 37°C for 90 minutes. Aliquots of 200µl were spread onto selective plates which were incubated at 37°C for 48 hours.

2.7.2.2 Protoplast method (Adapted from Chang and Cohen, 1979)
A *Bacillus* starter culture was grown in 10ml LB overnight with shaking. This culture was used to inoculate (1/100) 50ml of prewarmed LB in a sidearm flask. This flask was then incubated at 37°C until an O.D. 600nm of between 0.4 and 0.5 was reached. The culture was then pelleted in Sorvall tubes at 6000rpm for 10 minutes. The supernatant was removed and the pellet resuspended in 5ml SMMLB containing freshly added lysozyme (2mg/ml). The cells were incubated at 37°C for 90 minutes with gentle rocking and then pelleted by centrifugation at 5000rpm for 10 minutes at 4°C. The supernatant was decanted and the cells gently resuspended in 2.5ml SMMP. Plasmid DNA was mixed with 2× SMM. Generally, 0.5ml protoplasts and 1.5ml of a PEG solution were then added. After 2 minutes, 5ml SMMLB was added. The cells were again pelleted at 5000rpm for 10 minutes at 4°C and resuspended in 1ml SMMLB. To allow for phenotypic expression, cells were incubated at 37°C for 90 minutes with gentle shaking. Aliquots of 0.1ml were plated onto DM3 regeneration plates which were then incubated at 37°C for about 48 hours before colonies became visible.

2.7.3 *Rhodococcus* transformation

Recipient cells were grown to pre-stationary phase in LBSG medium and 1ml of the cells was pelleted by microfuging for 15 seconds. The supernatant was decanted and the cells were resuspended in 1ml protoplast buffer containing freshly added lysozyme (5mg/ml). The cells were incubated in a 37°C waterbath for 1 hour and mixed occasional by inverting the Eppendorf tube several times. During this time, a 50% PEG 4000 (Merck) solution was prepared by adding 0.5g PEG 4000 to 1ml protoplast buffer. The PEG was dissolved by microwaving for a few seconds. The cells were pelleted for 10 seconds, washed in 1ml protoplast buffer and then resuspended in the required volume of buffer. Generally 100µl of protoplasts was added to an Eppendorf tube containing the DNA to be transformed. PEG was added to a final concentration of 25% and the solution was left for at least 4 minutes at room temperature. The contents of the tube was spread onto a chilled regeneration plate with a sterile pipette and after allowing enough time for phenotypic expression, the plate was underlayed with the appropriate selective agent. With pDA37 and related plasmids this was usually 0.5ml of a 3M arsenate, 0.5M arsenite solution to give a final concentration of 60mM arsenate and 10mM arsenite. Strain ATCC 14887, however, was underlayed with 60mM arsenate and 20mM arsenite. For the chloramphenicol resistance plasmids, plates were underlayed with 0.25ml of a 4mg/ml stock to give a final chloramphenicol concentration of 40µg/ml. The underlay time for strain ATCC 12674 and derivative was 10 hours, for ATCC 4277 and derivatives, 12 hours, and for ATCC 14887, 14 hours. Regeneration plates were incubated at 28°C until colonies were visible.

2.8 Nocardioform mutagenesis

2.8.1 NTG mutagenesis

A 1ml aliquot of a stationary phase culture grown in LB with 0.4% Tween-80 was briefly spun down in a microfuge for 1 minute. The supernatant was removed and the cells were resuspended in 0.9ml of a 10mM Tris.HCl (pH 8.2) solution. To this was added 0.1ml of a 2.5mg/ml NTG solution dissolved in 10mM Tris.HCl (pH 8.2). The cells were dispersed by vortexing and then incubated at 37°C for 2 hours. After washing the cells in 1ml of a 10mM Tris.HCl solution, the cells were outgrown in LB containing

0.4% Tween-80 and 40µg/ml rifampicin. A suitable dilution giving about 150 colonies single colonies per plate was spread on non-selective media and mutants were then screened by patching onto appropriate plates.

2.8.2 Plasmid curing with ethidium bromide

Plasmid curing was generally performed with ethidium bromide. Curing of ATCC 4277 strains including GH1 and SQ1 was done by growing the strains in LB containing 20µg/ml ethidium bromide. The culture was then streaked to single colonies on non-selective medium and individual colonies were patched onto appropriate plates to screen for the loss of the plasmid encoded resistance function. With ATCC 14887 strains it was necessary to use a 30µg/ml ethidium bromide concentration since this proved to be the retardatory concentration. To verify that the plasmid was lost a mini-preparation was done and compared with a plasmid containing sample.

2.9 Sequence analysis

2.9.1 DNA sequence information

DNA sequence information was obtained from the EMBL DNA sequence database in Heidelberg, Germany via a WITS VMA e-mail request to the network file-server at the following bitnet address: Netserv@EMBL-Heidelberg.DE. The command GET NUC:x was used to retrieve sequences from the database with the accession number substituted for x. Sequences were obtained in mail notebook format and transferred from the WITS mainframe to IBM-PC compatible format using the Shine-Link file transfer facility. MS-DOS 5.0 EDIT facility was used to removed extraneous information and the search and replace facility used to change the ASCII characters (e.g. ╒ to [and σ to]) that had been corrupted in the transfer of information. Decoding software was also obtained from the EMBL library.

2.9.2 Sequence analysis

DNA sequences were analyzed for restriction sites using the SEQAID II ver. 3.81 package developed by D.D. Rhoads and D.J. Roufa of the University of Kansas and was obtained as a freeware package from the EMBL library.

2.10 Phage work

2.10.1 Increasing phage titre

The phage of interest was obtained from a plaque by removing the top agar into a sterile Sorvall tube and centrifuging at 12000rpm for 10 minutes. Phage was propagated by adding cells to the supernatant and allowing to stand for 30 minutes. Top agar (2ml) was added and the tube was mixed by swirling between the hands. The mixture was then poured onto plates and then incubated for several days at room temperature. The phage was serially passaged a number of times till an adequate titre was reached. To prevent propagation of lysogenic phage, strains with different resistances were used alternately so that cells from the previous passage did not survive subsequent selections. Phage lysate was sterilized by filtration through a 0.22µm filter (Millipore) and kept at 4°C.

2.10.2 Phage typing

Phage lysate was spotted onto a freshly poured lawn of the test organism in top agar and incubated at room temperature for 2 to 3 days. A clearing of the lawn in the area at which lysate was added indicated the ability of the phage to infect and lyse the organism.

2.11 Nocardioform enrichment

2.11.1 Selective enrichment technique

A nocardioform selective enrichment technique based on the method developed by Goldman (1991) was used. This technique was developed to isolate nocardioforms from soil their natural habitat. Essentially this technique used three different selective steps. The first step involves incubation of 1g of soil in about 5ml MM with either of the steroid compounds, sodium taurocholate (0.02%) or sodium deoxycholate (0.02%) as the sole carbon source. Incubation was performed on a rotary wheel at room temperature for 2 weeks. Cycloheximide (200µg/ml) was also added to keep fungal contamination to a minimum. The antibiotic nalidixic acid (10µg/ml) which is preferentially effective against Gram negative organisms was also added. The original method also used

nystatin as an additional antifungal agent but cycloheximide was sufficient to prevent most fungal contamination. Supernatant from this culture was used to seed another round of selection. In the final selection step, the supernatant from the second selection round was used to inoculate MM containing the compound of interest as the only carbon source. A loopful of this culture was then spread on a MM plate containing the compound of interest as the sole carbon source. Single colonies were then selected and spotted onto MM plates to confirm whether the compound was used as a carbon source. Alternatively this was also tested by using liquid culture experiments.

2.11.2 Direct selection

The direct selection technique involved incubation of about 1g of soil in about 5ml of a basal salts media. The compound of interest was added as the only carbon or sulfur source with no antifungal agents. The test tubes were incubated at room temperature for about 9 days and about 10µl of the supernatant used to inoculate a second round of test tubes containing the same media. After incubation for another 9 days, a loopful of culture was streaked to single colonies on an agar plate. Individual colonies were removed and growth rates were compared by spot testing on plates with and without the compound of interest.

2.12 Characterization of unknown organisms

2.12.1 Gram stain

The test organism was taken from either a fresh broth culture or an agar plate and was heat fixed onto a glass slide. The organism was first stained with crystal violet for 1 minute and then washed under running tap water. The stain was fixed by flooding the slide with iodine for 1 minute. The stain was then washed off with running tap water. The organism was decolorized with an iodine-acetone solution and then counterstained with safranin for 30 seconds. The slide was again washed under running tap water and blotted dry prior to viewing.

2.12.2 Oxidation/Fermentation test

For the oxidation/fermentation test the media of Hugh and Leifson (1953) was used with the one-tube adaptation of Porres and Stanyon (1974). Semi-solid basal media (10ml) supplemented with a 10% glucose concentration was added to 16 × 125mm screwcap tubes. The media was allowed to solidify at 4°C before inoculating with an inoculating needle. The tubes were incubated at 37°C. Fermentation of the glucose resulted in a colour change of the media from green to yellow extending throughout the tube. Oxidation of the media, on the other hand, changed the colour from green to yellow in the upper half of the tube only.

2.12.3 Denitrification test

Semi-solid denitrification media (10ml) was added to 16 × 125mm screwtop tubes. To ensure anaerobic conditions, 3ml of a 1% water agar solution was added to the semi-solid medium. The media was allowed to solidify at 4°C. Tubes were inoculated with an inoculating needle and incubated at 37°C for 2 days. The nitrate source used was KNO_3 and comparison of growth in media in which the KNO_3 had been omitted served as a negative control. Growth of the organism with KNO_3 as the terminal electron acceptor and the evolution of bubbles in the media indicated the nitrate was being used as the terminal electron acceptor and consequently reduced to N_2.

2.12.4 Acid from glucose

The oxidation/fermentation test media was used to test acid production from glucose. Bromothymol blue was used as the pH indicator and glucose was added to a 10% concentration. Acidification of the media resulted in a colour change from green (pH 7.2) to yellow (pH < 6.0).

2.12.5 Utilization of carbon sources

Carbon sources were generally made up with distilled water and sterilized briefly by autoclaving at 121°C for 1 minute. Organic acids were first neutralized with 10M NaOH before autoclaving. Testing for carbon source utilization was generally performed in a minimal salts liquid media by comparing the growth in media with and without any added carbon source. The ability to utilize a carbon source was scored as positive if the

turbidity of the sample containing the added carbon source was greater than the sample without any added carbon source. Compounds were generally tested at a concentration of 0.05% except for compounds which were toxic at this concentration (e.g. catechol, phenol, diaminohexane).

2.12.6 Catalase test

An inoculating needle was used to pick the centre of a pure colony onto a clean glass slide. A drop of 30% H_2O_2 was added over the organism with a Pasteur pipette. Immediate bubbling indicated the release of O_2 and this was scored as a positive result.

2.12.7 Electron microscopy

Copper grids (200 mesh) coated with Colloidin were used to sample the bacteria. The grids were placed onto a drop of bacterial culture for 15 minutes to allow for bacterial attachment. They were then rinsed with sterile distilled water and placed onto a droplet of 2% neutralized phosphotungstic acid (pH 7.0) and stained for 30 minutes. After rinsing in sterile distilled water, the grids were air dried overnight and then viewed with a JEM-100S transmission electron microscope at 80eV the following day.

2.13 Chromogenic detection of catechol and protocatechuate

This technique was based on the method described by Parke (1992) and relies on the compound *p*-toluidine for the chromogenic detection of protocatechuate and catechol. These two compounds are key intermediates in the breakdown of a large number of aromatic compounds and a chromogenic detection system for either of these compounds will facilitate the screening of a number of libraries for genes involved in the degradation of aromatic compounds such as phenol, *p*-hydroxybenzoate and many of the ligninolytic compounds. To screen for these enzymes *p*-toluidine (100µg/ml) was added to MM with glucose as the carbon source (0.1%) and an aromatic compound of interest (2.5mM). In an initial test as little as 2.5nmol of catechol applied directly to the plate in a volume of 2.5µl could be detected. A similar concentration of protocatechuic acid was not detected. $FeCl_3$ added to the media at a concentration of 1.5mM enhanced the detection of catechol but not of protocatechuic acid.

2.14 Southern hybridization

Hybridization was performed with the nonradioactive DNA labelling and detection kit supplied by Boeringer Mannheim. DNA used as the probe was labelled by random primed incorporation of digoxigenin-labelled deoxyuridine triphosphate. This labelled sequence was then detected by immunological methods using an antibody-conjugate (antidigoxigenin alkaline phosphatase conjugate). A enzyme catalysed colour reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT) allowed visual detection of the probe.

2.14.1 Blotting

The blotting of DNA onto the nitrocellulose membrane was performed as described by Sambrook *et al.* (1989). After electrophoresis, the gel was soaked in 100ml of denaturing solution (1.5M NaCl, 0.5N NaOH) for 45 minutes on a rotary platform. The gel was rinsed briefly in sterile distilled water and then placed in 100ml neutralizing solution (1M Tris.HCl pH 7.4, 1.5M NaCl) for 30 minutes with constant agitation. The neutralizing solution was then replaced with a fresh solution and the gel was soaked for a further 15 minutes. While the gel was soaking the transfer apparatus was prepared. This consisted of a piece of Plexiglass, wider and longer than the gel, supported on a plastic dish. A piece of Whatman 3MM paper folded over the Plexiglass served as the wick. The dish was filled with transfer buffer (10× SSC) in which the ends of the Whatman 3MM paper was suspended. Once the paper was thoroughly soaked, the 3MM paper on top of the support was smoothed out with a pipette. A piece of nitrocellulose filter (Amersham, Hybond-C) was cut to the size of the gel. The filter was then transferred to a dish of sterile distilled water with a pair of forceps, taking care not to touch the surface. The gel was removed from the neutralizing solution and placed on the support in an inverted position so that its underside was uppermost. Air-bubbles between the gel and the support were removed. The wet nitrocellulose filter was placed on the gel and air bubbles were removed. Two pieces of 3MM paper cut to same size as the gel and wet in 2× SSC were then placed on the nitrocellulose filter. Air bubbles were smoothed out with a glass pipette. A stack of paper towels, cut slightly smaller than the gel, were then placed on top of the wet 3MM papers. A Plexiglass plate was put on the towels and a 300g weight was balanced on the plate. The DNA transfer was

allowed to proceed overnight. The paper towels and the 3MM papers were removed and the positions of the gel slots were marked on the filter with a ballpoint pen. The gel was removed from the filter and then viewed under UV light to ensure that all the DNA had been transferred. The filter was soaked in 6× SSC for 5 minutes at room temperature to remove any residual agarose and then dried on a paper towel for at least 30 minutes at room temperature. Thereafter, it was sandwiched between two dry pieces of 3MM paper and the DNA was fixed to the filter by baking at 80°C for 2 hours in a vacuum oven.

2.14.2 Probe labelling

DNA was linearized with an appropriate restriction endonuclease and purified with a phenol/chloroform extraction and an ethanol precipitation. The DNA was denatured by heating in a water bath for 10 minutes at 95°C and then quickly chilled in an ice/water slurry. The hexanucleotide mixture (2µl) and 2µl of dNTP labelling mixture was added to 1µg of freshly denatured DNA. The volume was made up to 19µl with sterile distilled water and 1µl of Klenow enzyme was added. The mixture was incubated at 37°C overnight and the reaction was stopped by adding 2µl of 0.2M EDTA (pH 8.0). The labelled DNA was precipitated with 2.5µl 4M LiCl and 75µl prechilled ethanol at -70°C for 30 minutes, dried under vacuum, and then resuspended in 50µl TE.

2.14.3 Hybridization

The filters were prehybridized in 20ml of hybridization solution at 68°C for 2 hours distributing the solution from time to time. Hybridization was performed overnight with 5ml of hybridization solution containing 2µg of labelled DNA. The hybridization solution could be reused by freezing the solution and reboiling when it was needed again.

2.14.4 Washing

Filters were washed twice with at least 50ml of washing solution 1 (2× SSC, 0.1% SDS) for 5 minutes at room temperature and twice with washing solution 2 (0.1× SSC, 0.1% SDS) for 15 minutes at 68°C.

2.14.5 Immunological detection

The filters were washed for 1 minute in buffer 1 (100mM Tris.HCl, 150mM NaCl, pH 7.5). Antibody-conjugate was diluted to 150mU/ml in buffer 1 and the filter was incubated in 10ml of the diluted antibody-conjugate solution. Unbound antibody-conjugate was removed by washing the filter twice for 15 minutes with buffer 1. The membrane was equilibrated for 2 minutes with 20ml of buffer 3 and then incubated in the dark with 10ml of the colour solution sealed in a plastic container. Bands were usually visible within a few hours and developed completely within a few days.

3. RESULTS

3.1 Soil enrichment for nocardioforms with interesting metabolic potential

3.1.1 Nocardioform enrichment method

The nocardioform soil enrichment procedure was used on a sample of soil obtained from a garden compost heap in Pretoria. After the selective and enrichment steps, samples were spread onto MM plates containing the following compounds as sole carbon source: Amido Black (0.05%), Orange II (0.05%), DBT (0.2%), indigo (0.2%), caprolactam (0.1%) and diaminohexane (0.1%). Individual colonies of interest were spotted on MM plates with and without an added carbon source and their growth rates compared. By this criterion it seemed that most of the colonies of interest were unable to utilize any of these compounds as a sole carbon and energy source. Moreover, Gram-stain results and replica spotting onto plates containing either fusidic acid (20µg/ml), thiostrepton (20µg/ml) or naladixic acid (30µg/ml) indicated that the majority of organisms isolated were Gram negative (>75%). A number of strains, Cap2, Cap4 and Cap17 showing a similar morphology were isolated from the caprolactam enrichment. These strains were all Gram negative.

3.1.2 Direct selection method

A direct selection was also performed on mushroom compost soil obtained from a garden in Pretoria for organisms capable of using caprolactam as a sole carbon source and dibenzothiophene as a sole sulfur source. An organism was isolated that showed better growth in MM containing caprolactam as carbon source than the negative control with no added carbon. This organism was called SQ4 and was further characterized (see below).

The DBT selection produced a pigmented colony that seemed to grow better on MM plates supplemented with DBT. Spot tests and liquid culture experiments seem to indicate that the organism grew better in the presence of DBT than without. This organism was called SQ5 and was also characterized further (see below).

3.2 Characterization of unknown isolates

3.2.1 Characterization of SQ4

A number of physical and biochemical tests were performed on SQ4 to further characterize this organism (Table 6). Results indicated that the organism was a Gram-negative rod and that it was motile by a single polar flagellum (see Fig. 4). The organism was an obligate aerobe that produced acid from glucose by an oxidative rather than a fermentative pathway. Under denitrifying conditions, it was also capable of anaerobic growth in the presence of KNO_3.

Nutritional characterization indicated that SQ4 used a variety of aromatic compounds in addition to the nylon monomers, adipic acid (0.05%), diaminohexane (0.03%) and aminocaproic acid (0.05%) as a sole carbon and energy source. SQ4 was also able to use aminocaproic acid (0.03%), caprolactam (0.1%) and diaminohexane (0.01%) as a sole nitrogen source. The growth curve in figure 5 indicates that adipic acid and *p*-hydroxybenzoate were the quickest metabolized carbon sources while diaminohexane was metabolized only after a lengthy incubation. This could be indicative of spontaneous diaminohexane-utilizing mutants arising. To test this hypothesis, the growth rate of an organism obtained from this culture was compared against a culture obtained from the original inoculum. The growth of the organism obtained after incubation with diaminohexane was much better than the original culture and it therefore seemed that a mutant strain of SQ4, capable of using diaminohexane better than the parental strain, arose after lengthy incubation in MM with diaminohexane as the sole carbon source. SQ4 did not use the ligninolytic compounds veratric acid, ferulic acid, syringic acid, vanillic acid and cinnamyl alcohol as a sole carbon source.

These results tentatively suggest that this organism belongs to the genus *Pseudomonas*. It is well known that *Pseudomonas* species often have naturally occurring plasmids that carry the information for the breakdown of xenobiotic compounds, for example the TOL plasmid (Burlage *et al.*, 1989). A nocardioform mini-plasmid preparation was performed on an SQ4 culture grown in LB overnight to see whether any plasmids were visible. No plasmid bands were seen with this method.

A



B



Figure 4. Transmission electron micrographs of SQ4 showing rod-shaped morphology and single polar flagellum at (A) 5000× and (B) 8000× magnification. Bar length indicates 1μm.

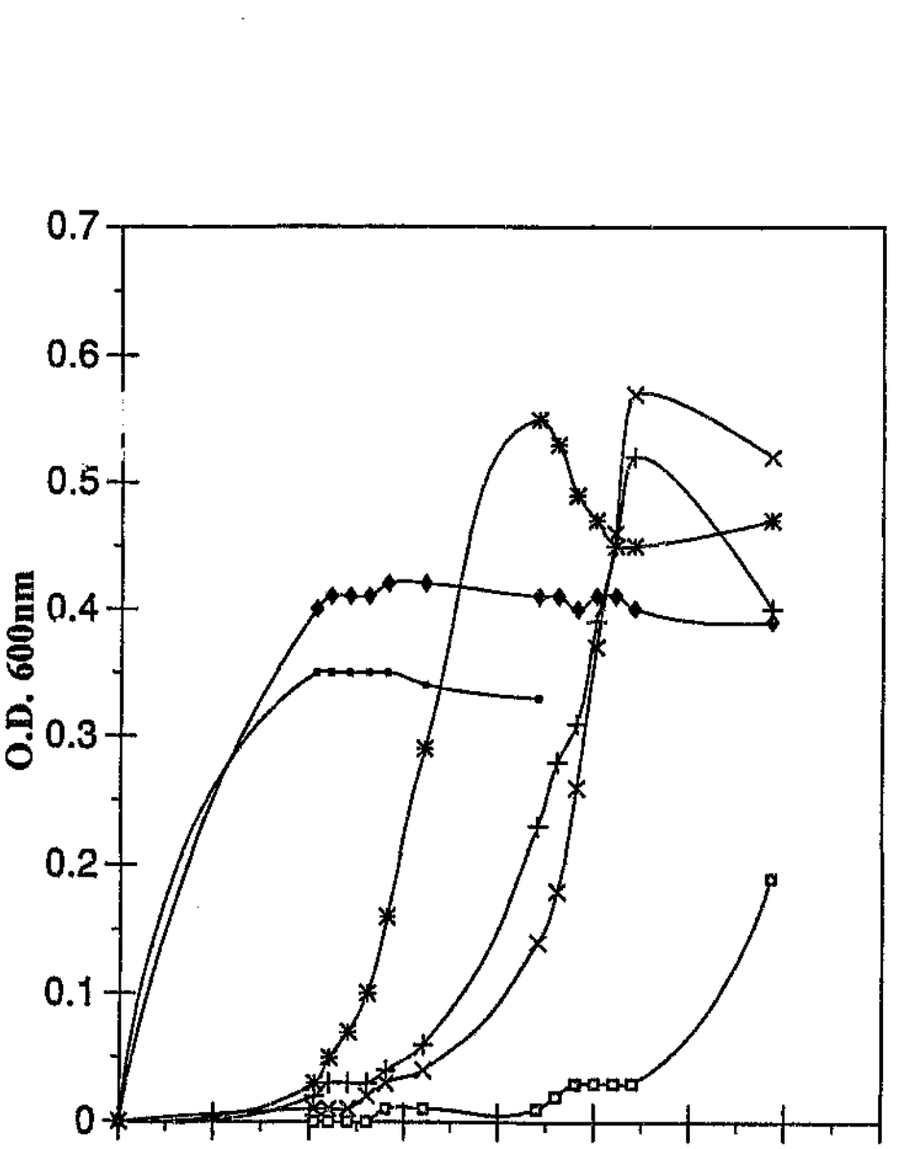


Figure 5. Growth curve of SQ4 incubated at 37°C in MM with the following compounds as sole carbon source: adipic acid (■), aminocaproic acid (+), caprolactam (*), diaminohexane (▒), glucose (×), *p*-hydroxybenzoate (♦).

3.2.2 Characterization of SQ5

SQ5 was an orange/red pigmented Gram-positive filamentous acti[illegible]te. A number of different organic compounds were tested to see whether they cou[illegible]e used as the sole carbon source. Sodium benzoate appeared to be best carbon source although glucose, phenol and cinnamyl alcohol were also used as a sole carbon source. None of the nylon monomers, adipic acid, aminocaproic acid, caprolactam and diaminohexane

Table 6. Physical and biochemical characteristics of SQ4

Test	Characteristic
Gram stain	-
Morphology	rod
Motility	+
Flagella	single, polar
Growth at 37°C	+
Flourescent pigment	-
O/F	O/-
Acid from glucose	+
Denitrification	+
Catalase	+
Utilization as C-source	
Sodium benzoate (0.05%)	+
m-Hydroxybenzoate (0.05%)	+
p-Hydroxybenzoate (0.05%)	+
Protocatechuic acid (0.05%)	+
Phenol (0.02%)	+
Catechol (0.02%)	-
Adipic acid (0.05%)	+
Aminocaproic acid (0.05%)	+
Caprolactam (0.05%)	+
Diaminohexane (0.03%)	+
Cinnamyl alcohol (0.05%)	-
Ferulic acid (0.05%)	-
Syringic acid (0.05%)	-
Vanillic acid (0.05%)	-
Veratric acid (0.05%)	-

nor the ligninolytic compounds, veratric acid, ferulic acid, syringic acid and vanillic acid were used as a sole carbon source. Additional physical and biochemical tests summarized in table 7 were performed to further characterize this organism.

The generation time of this organism was similar to that of the *Rhodococcus* strains and it was incubated at 28°C for 3 days before visible colonies appeared. A nocardioform mini-plasmid preparation was performed on a culture grown in TYG to see whether it harboured any naturally occurring plasmids. No plasmid bands were seen. An attempt to transform this organism with pDA37, plasmid 22 and pIGTS1 also failed although it seemed that spontaneous mutants to 60mM arsenate and 10mM arsenite arose fairly readily. Transformation of *R. fascians* DSM 20151 with pIGTS1 was used as the positive control since the plasmid was derived from a *R. fascians* strain. Phage typing of this organism was performed with the nocardiophages Q5 and a phage specific for *R. rubropertinctus* ATCC 25593. The ATCC 25593 specific phage infected both the positive control, ATCC 25593, and the test organism, SQ5. Phage Q5, on the other hand, infected only SQ5 suggesting that SQ5 is a *Rhodococcus* species or very closely related organism.

3.3 Construction of chromosomal library of SQ4

3.3.1 Construction of BglII chromosomal library of SQ4

SQ4 chromosomal DNA was digested with a number of enzymes to select a suitable enzyme for library construction. A total of 10 enzymes were tested including BamHI, BclI, BglII, BstYI, HindIII, HpaII, NheI, PstI, TaqI and XbaI. Of these, HpaII and TaqI were not suitable since they had 4-bp recognition sequences and therefore cut the DNA too frequently producing small fragments. The remaining enzymes digested the chromosomal DNA completely except for BglII, NheI and XbaI which appeared to have only partially digested the DNA. BglII was eventually selected because there was a unique BglII site in the vector and a partial digestion of chromosomal DNA would hopefully increase the average insert size. A BglII chromosomal library of SQ4 was therefore constructed in the positive selection vector pEcoR251. The library comprised a total of 8118 transformants with an average insert frequency of 70% and an average insert size of 5500-bp as determined from three plasmid screens performed at the beginning, the middle and the end of library construction (see Fig. 6). Assuming a genome size of 6.3×10^6-bp, the probability that a particular sequence was represented in this library was greater then 99%. With a genome size of 1.1×10^7-bp, the

Table 7. Characteristics of SQ5

Characteristic	Result
Gram stain	+
Morphology	filamentous
Pigmentation	red/orange
Catalase	+
Utilization as C–source	
Sodium benzoate (0.05%)	
m–Hydroxybenzoate (0.05%)	–
p–Hydroxybenzoate (0.05%)	–
Protocatechuic acid (0.05%)	–
Catechol (0.02%)	+
Phenol (0.02%)	+
3,4–Dimethylbenzoate (0.05%)	–
Adipic acid (0.05%)	–
Aminocaproic acid (0.05%)	–
Caprolactam (0.05%)	–
Diaminohexane (0.05%)	–
Cinnamyl alcohol (0.05%)	+
Ferulic acid (0.05%)	–
Syringic acid (0.05%)	–
Veratric acid (0.05%)	–
Vanillic acid (0.05%)	–
Dibenzothiophene (0.05%)	–
Ethanol (0.2%)	+
Tween–80 (0.3%)	+

probability changes to 94%. The library was constructed in MM294-1 and purified plasmid obtained from a CsCl gradient was transformed into the *E. coli* strain KL98-1. This *E. coli* strain was selected as the recipient because it was prototroph and was not able to use any of the nylon monomers or aromatic compounds as a sole carbon source. The spontaneous mutation of KL98-1 to Cap^+ was less than 2.6×10^9 cells/ml. The library was screened for genes enabling KL98-1 to use caprolactam, diaminohexane,



Figure 6. BglII digestions of plasmids from a BglII SQ4 genomic library construction. Lanes (1) pEcoR251, (2)-(6) and (8)-(12) transformants (7) λII molecular weight markers.

adipic acid, aminocaproic acid, benzoate, *p*-hydroxybenzoate, *m*-hydroxybenzoate, phenol, protocatechuic acid and catechol as a sole carbon source. In addition, the library was also screened for genes allowing the growth of KL98-1 on MM plates with aminocaproic acid, diaminohexane and caprolactam as the sole nitrogen source. No positive clones were recovered in these selections.

3.3.2 Screening BglII library of SQ4 for genes involved in phenol and *p*-hydroxybenzoate degradation

The BglII library of SQ4 in MM294-1 was screened for genes involved in phenol and *p*-hydroxybenzoate degradation. A dilution of the MM294-1 library was spread onto MM chromogenic detection plates containing phenol (0.02%) so that there were a total

of about 2000 colonies per plate. Five plates or a total of about 10 000 clones were screened by looking for the characteristic brown color development associated with the build-up of either protocatechuate or catechol in the medium surrounding the cells. No such color development was seen after incubating the plates for 3 days and therefore it was concluded that the complete gene sequences were not represented in the library. After leaving the plates on the bench top for a few more days, one colony produced the characteristic brown precipitate. However, after streaking this colony on plates supplemented with either rifampicin or ampicillin it was concluded that this organism was a contaminant, since it failed to grow on either of the selective plates. This organism was not characterised further but it did show that these plates could be used for the chromogenic detection of genes involved in the degradation of aromatic compounds into either catechol or protocatechuate.

3.3.3 Testing of SQ4 BglII library

To test whether a comprehensive BglII library of SQ4 had been constructed, the library was transformed into an auxotrophic *E. coli* strain, RK4904, that required the amino acids proline, lysine, tryptophan, methionine and arginine for growth (see table 4). Four of the 5 markers were selected for screening of the library by complementation since the fifth marker, *trpE*38, was a leaky mutation and would not be suitable. Of the 4 mutations tested, only the *lysA*23 mutation, was complemented. To confirm that complementation had occurred, the plasmid from the complementing clone was transformed back into RK4904 to see whether the lysine requirement was complemented.

Since the insert was fairly large it could probably encode the information for more than one gene. The chromosomal map of *E. coli* K-12 (Bachmann, 1989) was used to select a marker closest to the *lysA*23 marker. An *E. coli* strain carrying this specific marker was then transformed with pEcoR251-*lysA*1 to see whether it complemented this marker. The marker chosen, *thyA*, was not complemented by pEcoR251-*lysA*1.

A plasmid screen of a number of transformants also revealed that the inserts were of similar size. One of these plasmids, pEcoR251-*lysA*1, was used for subsequent characterization. These results suggested that the BglII library was not representative since complementation occurred only in 25% of the selected auxotrophic markers.

3.3.4 Restriction map of DNA fragment complementing *lysA*23 mutation in RK4904

A BglII digestion of pEcoR251-*lysA*1 revealed that the insert was about 8.4 kb in size. Preliminary work involved purification of this fragment by low-gelling agarose, followed by digestion with a number of restriction enzymes to determine which enzymes were suitable for mapping. Those enzymes that cut infrequently were initially mapped and

Table 8. Restriction sites of fragment from BglII library of SQ4

Enzyme	No. of sites	Enzyme	No. of sites
1) Asp700	0	14) NheI	0
2) Asp718	0	15) NruI	2
3) BamHI	0	16) NsiI	1
4) BfrI	0	17) PstI	4
5) BglI	>5	18) PvuI	?
6) BglII	0	19) PvuII	5
7) BstEII	3	20) SacI	0
8) DraI	0	21) SalI	4
9) DraIII	4	22) ScaI	0
10) EcoRI	1	23) SnaBI	1
11) EcoRV	2?	24) StuI	2
12) HindIII	0	25) XbaI	0
13) NcoI	3	26) XhoI	1

these sites were then used as references points for triangulating sites produced by other enzymes. Table 8 lists the enzymes that were screened together with the probable number of sites. Using this information a restriction map of this fragment was produced (Fig. 7).

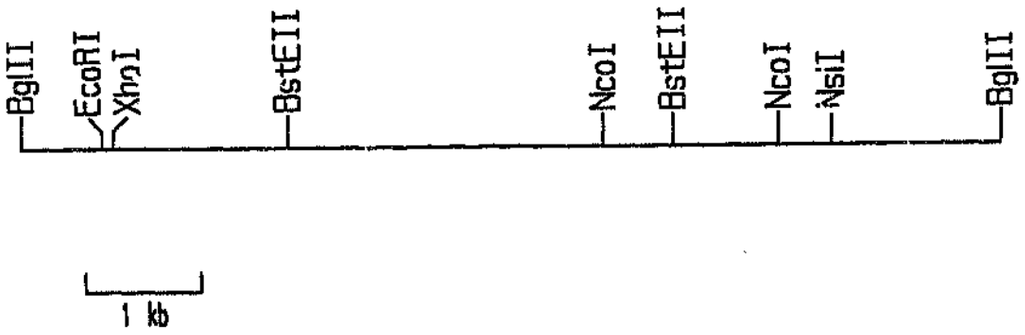


Figure 7. Restriction map of the DNA fragment from the BglII genomic library of SQ4 complementing the *lysA*23 mutation in *E. coli* RK4904.

3.3.5 Construction of pEcoR251-*lysA*2

Expression of the gene on pEcoR251-*lysA1* in the *E. coli* auxotroph resulted in complementation of the *lysA*23 mutation. To see whether expression of the gene occurred from its own promoter or as a result of read through from a promoter on pEcoR251, the BglII fragment was joined to pEcoR251 in the reverse orientation. If read through from a plasmid promoter was occurring one would not expect expression of the gene if it was joined in the reverse orientation. If expression of the gene occurred from its own promoter, then changing the orientation of the insert would not affect the final result. The BglII fragment was inserted into pEcoR251 in the other orientation and this was confirmed by an EcoRI digestion of the plasmid. *E. coli* strain RK4904 was then transformed with this construct, pEcoR251-*lysA*2, to see if it complemented the *lysA*23 mutation. Spot tests on MM plates with and without lysine revealed that both pEcoR251-*lysA*1 and pEcoR251-*lysA*2 complemented the *lysA*23 mutation of RK4904 and this, therefore, suggested that expression of this gene occurred from its own promoter.

3.3.6 Construction of second chromosomal library of SQ4

Since the BglII library was not a representative library of the entire SQ4 genome either more clones could be constructed to make the library more comprehensive or a second library could be built. The latter option was chosen and since there were unique PstI, SfuI and HindIII sites in the *EcoRI* gene these enzymes were screened first. PstI was

chosen as the initial enzyme with which to build the second library since the positive selection function of pEcoR251 seemed to work best with this enzyme and not as well with HindIII and SfuI (S. Andersen, personal communication).

Initially, fractionation of partially digested chromosomal DNA greater than 4 kb in size was done on low gelling agarose. An attempt was made to purify the DNA by the electroelution technique. The recovery efficiency of this method, however, was very low and therefore it was not used further although transformants were obtained from the purified DNA.

An attempt was then made to construct the library with partially digested chromosomal DNA without fractionation. This method however also proved to be problematic since PstI digestion seemed to produce a large number of very small fragments resulting in a large number of transformants with very small inserts.

It was then decided to use HindIII instead. Fortunately, HindIII sites were infrequent and as a result large fragments were generated. The HindIII library comprised a total of 7638 transformants with an average insert size of 6.8-kb at an insert efficiency of 90%. Assuming a genome size of 6.3×10^6-bp, the probability of any particular sequenced represented in this library is greater then 99.9%. The probability of any particular sequence being present in this library assuming a genome size of 1.1×10^7-bp is greater then 98.5%.

3.4 Subcloning of azo-dye decolorizing gene from pGSH1

Using the restriction map of pGSH1 (see Fig. 3), an attempt was made to subclone the gene/s involved in azo dye decolorization. The first and most obvious step was subcloning of the BglII fragment from pGSH1 into pDA37 since it has previously been shown that removal of this fragment resulted in a loss of decolorizing activity (Heiss, 1992). Furthermore, pDA37 was a convenient positive selection vector with a unique BglII site that could be used. The BglII fragment was inserted into pDA37 in both orientations and this was confirmed with an Asp718-HindIII double digestion. These constructs, pGSH10 and pGSH11, were both tested to see whether they restored

decolorization activity in GH1. Neither of the plasmids was able to restore this ability presumably because one or both of the BglII sites cut into an essential region of the DNA.

The next logical step was subcloning of the two SfuI fragments since the SfuI sites conveniently bracketed both of the BglII sites. This step proved to be more difficult since pDA37 did not have a unique SfuI site. A derivative of pDA37, plasmid 22, which had a unique ClaI site was obtained. Since ClaI produces compatible cohesive termini with SfuI, this plasmid could be used to clone the SfuI fragment. Unfortunately, the ClaI site was not situated within the antibiotic resistance gene nor the EcoRI gene of plasmid 22 and therefore no negative or positive selection function could be used. Alkaline phosphatase was therefore used to reduce the background of religated plasmid without much success.

Plasmid 22 has unique sites for ClaI and Asp718 both of which could be used for cloning. Since NarI produce the same compatible cohesive sites as ClaI and BsiWI the same as Asp718, these enzymes could also be used. Digestion with these enzymes revealed that there were 2 NarI sites and 3 BsiWI sites in the insert. These sites have not yet been mapped.

3.5 Isolation and characterization of non-decolorizing *Rhodococcus* mutants

3.5.1 NTG mutagenesis

Non-decolorizing mutants of ATCC 4277 and ATCC 14887 had previously been selected after mutagenesis with NTG (Heiss, 1992). These mutants were complemented by pGSH1 and pGSH2 respectively. To gain further insight into the inactivation mechanism it was necessary to select additional non-decolorizing mutants to see whether these were complemented by either of the plasmids. Strain CQ1 was therefore mutagenized with NTG and non-decolorizing mutants were selected by spreading a suitable dilution (less than 300 colonies per plate) of the culture on plates supplemented with Amido Black. Colonies characteristically were visible after about 3 days incubation at 28°C. Initially colonies were dark in color but after 4 days incubation decolorization

started and colonies changed from a blackish to a salmon color. Concomitant with this change was a change in the color of the media surrounding the decolorizing colonies. In contrast, non-decolorizing mutants retained their dark blue appearance on media supplied with Amido Black and were prominent against a background of salmon-colored colonies. These black colonies occurred at a frequency of about 6×10^{-3} and were subjected to a spot test to verify that they were non-decolorizers. The majority of mutants showed some decolorization of AB while others decolorized the dye more slowly than the SQ1 control. One of the slower decolorizing mutants, SQ2, was retained together with a second non-decolorizing mutant, SQ3. Interestingly, SQ3 appeared to be a phenotypically distinct from GH1 when grown in MM media supplemented with Amido Black. Although both mutants did not decolorize Amido Black, GH1 appears not to accumulate the Amido Black and retains a translucent appearance in its presence. SQ3, on the hand, seemed to accumulate the Amido Black and therefore had a very dark appearance on Amido Black plates. This would seem to suggest that different mutations were responsible for disabling the azo dye decolorization function.

A third non-decolorizing mutant of DSM 1069 was obtained by NTG mutagenesis performed by E.R. Dabbs. This mutant, SQ6, was obtained at a frequency of 1×10^{-4} and, like SQ3, it had a black appearance on MM supplemented with Amido Black suggesting that Amido Black was accumulated.

To ensure that SQ2 and SQ3 were genuine mutants and not contaminants, phage typing of these mutants was performed by E. Dabbs. Phages specific for ATCC 4277, C and H, were tested with SQ1 as a positive control. Phage C appeared to infect SQ1, SQ2 and SQ3 whereas phage H only infected SQ1 and SQ3. From these results, it seemed that SQ3 was a genuine mutant. Although, SQ2 did not give a clear result with phage H it was infected by phage C.

Further confirmation was obtained by transforming both SQ2 and SQ3 with pDA37 and comparing it to an SQ1 control transformation. Both these strains maintained the plasmid verifying that they were both genuine mutants.

3.5.2 Transformation of non-decolorizing mutants with pGSH1 and pGSH2

To see whether pGSH1 or pGSH2 complemented these mutants, they were transformed with both plasmids and transformants were spot tested on AB plates. Curiously, SQ2 or SQ3 transformed with pGSH2 appeared to grow more quickly on regeneration plates than when transformed with pGSH1 suggesting that pGSH2 complemented some growth function. SQ2 showed some a degree of azo dye decolorization was not tested further. Spot tests revealed that pGSH1 complemented azo dye decolorization in SQ3. This was not the case for pGSH2. The same experiment was repeated with SQ6.

3.5.3 Selection of decolorizing transformants directly on regeneration media

To see whether decolorizing transformants could be selected directly on regeneration media, an experiment was conducted by transforming GH1 and GH2 with plasmids pGSH1 and pGSH2 and selecting transformants on various media with Amido Black. Variations of the standard regeneration media (RM) were tested including RM without yeast extract and RM without yeast extract plus amido black. Yeast extract was selected because decolorization in the presence of yeast extract was not visualized as easily due to the color of the yeast extract. Omission of the yeast extract resulted in transformants growing up in twice the time taken to grow up in standard regeneration media. Decolorization of the azo dyes was also observed with the negative controls (GH1 and GH2 transformed with pDA37). A spot test of non-decolorizing mutants on RM plus Amido Black without yeast extract revealed that these mutants were decolorizing. This method could therefore not be used to screen directly for decolorizing transformants on RM.

3.6 Use of small pGSH2 fragment as a probe to obtain a full-length clone

The complementing fragment of the non-decolorizing mutant of ATCC 14887, GH2, was obtained from a BamHI library of ATCC 14887. The inserted fragment was about 50-bp in size and since it is too small to code for an intact polypeptide, it was decided to use this small fragment as a probe to pull out the intact gene. Chromosomal DNA from strain ATCC 4277 was digested to completion with Asp718, BamHI, BclI, ClaI and SfuI. The plasmids pDA37 and pGSH2 were labelled with digoxigenin and used to

probe the digested chromosomal DNA. Unfortunately, similar bands were detected in both cases suggesting that complementary sequences from the vector were hybridizing to the chromosomal DNA.

As an alternative, a smaller fragment of DNA including the insert was used as a probe since it was more specific. Because the library had been constructed by joining BamHI digested chromosomal DNA to the unique BglII site of pDA37, this result in a hybrid site not recognized by any restriction enzyme. The inserted fragment could not simply be removed with a single digestion, therefore, a two step procedure was required. The first step involved the purification of the largest PstI fragment which brackets the unique BglII site of pDA37. The second step involved digestion of this PstI fragment with HindIII to produce a fragment of about 450-bp in size including the insert. This fragment was purified by low-gelling agarose and, after labelling, was then used to probe digested chromosomal DNA. Unfortunately no bands of hybridization were observed.

3.7 Insertion of small insert of pGSH2 into pUC18

As preliminary work to sequencing experiments, it was decided to construct a plasmid with a small insert that could easily be sequenced. The small fragment from pGSH2 proved ideal and it was decided to join this fragment to pUC18. Since DNA from a BamHI digestion was joined to the BglII site of pDA37, this small fragment could be removed with a Sau3A digestion since the Sau3A recognition sequence is a subset of both the BamHI and the BglII recognition sites. A Sau3A digestion of the PstI-HindIII fragment of pGSH2 was performed and the resulting fragments were ligated to the BamHI site of pUC18. Transformants were recovered with inserts of three different size classes (Fig. 8). An examination of the sequence of the EcoRI gene in figure 9 revealed there were a total of 4 Sau3A site at positions 22, 193, 230 and 400 in the sequence between the PstI site and the HindIII site. Theoretically, digestion of the PstI-HindIII fragment with Sau3A should produce fragments of 16-, 22-, 37-, 170- and 171-bp plus the insert of about 50-bp. Only the 37-, 170-, 171- and 50-bp insert can be ligated to the BamHI site since only these fragments have BamHI compatible sequences on either end. This would therefore predict inserts of three different size classes, since the 170-

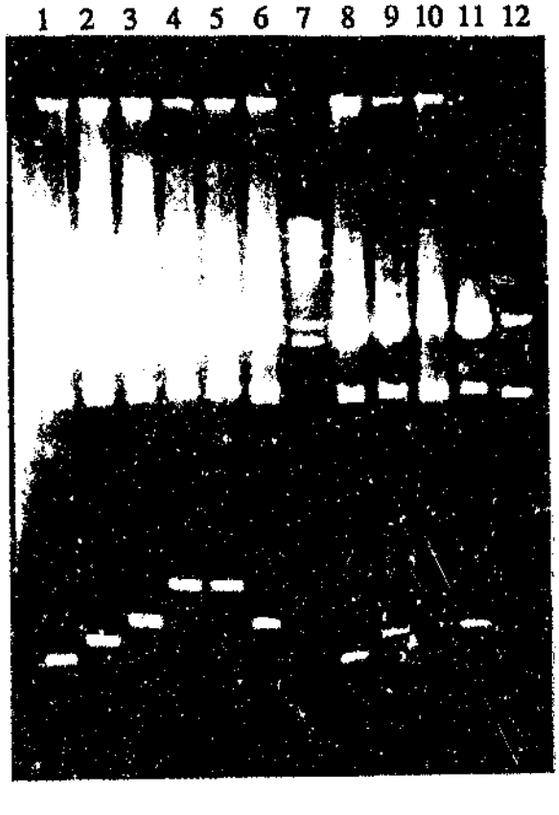


Figure 8. Plasmid screen of pUC18 transformants doubly digested with PstI and HindIII and run on a 1.2% agarose gel. Lane (1) pUC18, (2)-(6) and (8)-(12) transformants, (7) λII molecular weight markers.

and 171-bp fragments would be indistinguishable on an agarose gel. A BamHI digestion of the plasmids was done since it was predicted that this site should be restored at the point of insertion of the fragment of interest since none of the other Sau3A fragments had the correct flanking base pair to produce a recleavable BamHI site.

Results indicated that only plasmids with inserts of an intermediate size were linearized with BamHI suggesting that these plasmids probably contained the fragment of interest. Experimental evidence correlates well with the theoretical predictions suggesting that the 50-bp insert of pGSH2 had been inserted into pUC18 vector at the BamHI site.

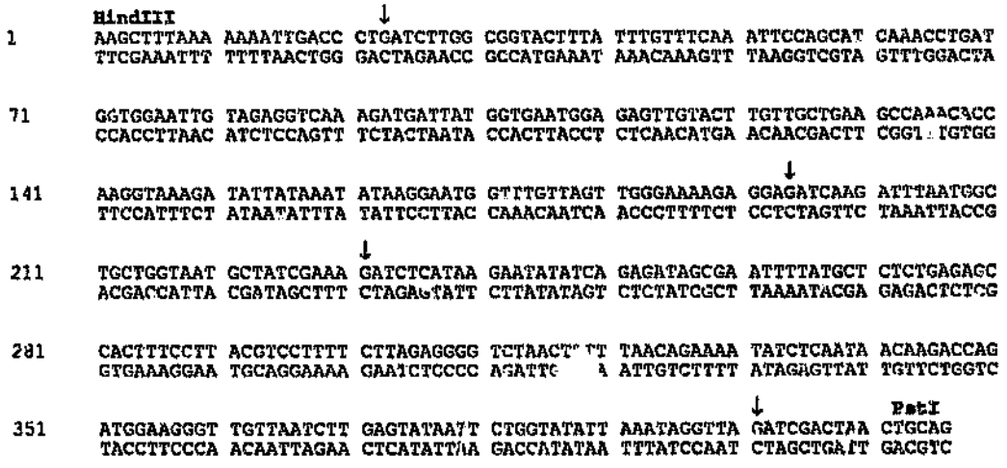


Figure 9. Sequence of *EcoRI* gene from PstI to the HindIII site (see Appendix B for map of pEcoR251). PstI and HindIII sites are named while Sau3A sites are indicated by vertical arrows.

As a further check, either the 50bp BamHI fragment could be removed from the pUC18 clone and inserted into pDA37 or the intact plasmid could be joined to a suitable vector to transform GH2 and see whether azo dye decolorization was restored. The latter option was chosen. Since pDA37 does not have any suitable sites an alternative vector derived from pDA37, plasmid 22, was used. Because the pUC18 derivatives already had an insert, inactivation of the *lacZ* gene could not be used to test for recombinants. These vectors were therefore phosphatased and joined at the Asp718 site to plasmid 22 and then transformed into GH2-1 to see whether they restored decolorization activity.

3.8 Optimization of conditions for dibenzothiophene utilization

SQ5 was isolated from an enrichment in which DBT was supplied as the only source of sulfur. To see whether this organism could indeed use DBT as a source of sulfur, growth conditions had to be optimized. Since sulfur is not a nutrient required at such high concentrations many organisms can grow in media without an additional sulfur supplement presumably obtaining their sulfur requirements from impurities in the media and glassware. Conditions, however, can be optimized so that the difference in growth requirements is highlighted.

Initially, *R. erythropolis* SQ1, *R. equi* ATCC 14887 and *R. rubropertinctus* ATCC 25593 were tested to see whether they could use DBT as a sulfur source. The growth of strains in BSM liquid culture containing DBT (0.1%) were compared against a negative control with no sulfur and a positive control with $MgSO_4$ (0.1%) as the sulfur source. Results after 3 days incubation at room temperature showed that while the positive control samples showed good growth, there was no significant difference between the test and the negative control samples indicating that DBT was not being used as a sulfur source.

When the growth rate of SQ5 was originally tested in BSM media supplemented with 0.01% DBT against a negative control without any sulfur supplement and a positive control with 0.01% $MgSO_4$ the difference was quite pronounced after 2 days incubation at room temperature. Because the DBT was added from a stock made up in ethanol, an additional experiment was required to make sure that the difference in growth rate was not due to the presence of ethanol. Subsequent experiments indicated that ethanol was a good carbon source. The experiment was repeated with ethanol (0.05%) as a carbon source and, in addition, sodium benzoate (0.05%) and Tween-80 (0.1%) were also tested as possible carbon sources (Table 9). Not much difference was seen in the samples with Tween-80 and sodium benzoate as the carbon sources. However, in the ethanol samples

Table 9. Growth of SQ5 with different carbon and sulfur sources.

	Carbon source		
Sulfur source	Tween-80	Ethanol	Sodium benzoate
$MgSO_4$ (0.01%)	+++	+++	+++
DBT (0.0025%)	+++	++	+++
no sulfur	+++	+	+++

\+ poor growth
++ intermediate growth
+++ good growth

there was a significant difference in growth rates between the negative and positive samples. The DBT sample showed an intermediate growth rate between the two controls suggesting that although DBT was not as good a sulfur source as $MgSO_4$, it was still being used as a source of sulfur.

3.9 *Bacillus* transformation

Since most of the work in this laboratory is concerned with Gram positive *Rhodococcus*, an alternative expression system to the more commonly used Gram negative organism, *E. coli*, would be more appropriate since it is known that not all Gram positive genes are expressed in Gram negative organisms. *Bacillus* was chosen because it is a commonly used Gram positive organism with a number of well characterized plasmid vectors and its growth rate is comparable to that of *E. coli* thereby ensuring that results are rapidly achieved. Also, *Bacillus*, is a well known industrial organism that has been exploited because of its ability to secrete foreign proteins. This preliminary work was concerned with the transformation of *B. subtilis* with plasmid DNA by either the competent cell method or the protoplast method.

3.9.1 Competent cell transformation

The competent cell method is reliant on the cells reaching a particular stage in their growth phase before they become competent. Initially an attempt was made to transform the plasmid pC194 of *Staphylococcus aureus* origin into a *Bacillus* prototroph 1A2. This method was, however, not successful. Literature describing *Bacillus* transformations have indicated that monomeric plasmid molecules transform *Bacillus* very poorly. An attempt was made to generate multimeric plasmid molecules by a cut and ligate process and then to transform these molecules into competent cells. This also failed and this work was not pursued further.

3.9.2 Protoplast transformation

An alternative to the competent cell method is the protoplast method which does not require polymeric circular plasmid molecules. The method previously described in section 2.6.2 was used. The first transformation attempt with this method failed because a mistake had been made in the formulation of DM3 regeneration media resulting in

media that did not support the growth of *Bacillus* protoplasts. For the second attempt, nocardioform regeneration media was substituted for DM3 regeneration media. The same nocardioform transformation protocol was followed except that nocardioform RM was used and that the chloramphenicol underlay was done at a concentration of 40µg/ml after 2 hours incubation at 37° as for nocardioform transformations. This procedure resulted in transformants growing up after 2 days incubation at 37°C. The transformants all had a watery translucent appearance which may either have been due to the effect of chloramphenicol or to levan production from the sucrose in the media.

Individual transformants were grown at 37°C in LB containing 20µg/ml chloramphenicol for 6 hours. The presence of plasmids were confirmed by performing standard *E. coli* mini-plasmid preps on these cultures. Although the yield of plasmid was not comparable to the plasmid yield from *E. coli* culture, it worked sufficiently well to show that a plasmid was present.

Since the nocardioform regeneration media worked the next logical step was to see whether the nocardioform protoplast transformation procedure could also be used. A *B. subtilis* 1A2 culture was grown up overnight in LB and 1ml of this culture was used. The nocardioform protocol was followed step for step except that the lysozyme treatment was performed for 90 minutes and the underlay was done after 2 hours incubation at 37°C with 40µg/ml chloramphenicol per plate. The plasmid used in this case was pDA52 which was a chimera of a *Bacillus* plasmid pC194 and the coli positive selection vector pEcoR251. Chloramphenicol resistant transformants were recovered indicating that the shuttle vector was maintained in *B. subtilis* and that the EcoRI gene of pEcoR251 was not expressed. The transformation efficiency, however, was very low.

4. DISCUSSION

4.1 Selection of nocardioforms with interesting metabolic potential

Because *Rhodococcus* is a natural soil-dwelling organism with diverse metabolic activity, a previously described enrichment and selective method (Goldman, 1991) and a simple direct method were used to isolate *Rhodococcus* strains with unusual metabolic activity. The success of these different methods at isolating novel *Rhodococcus* strains could therefore be assessed and if they proved to be successful, could be used for obtaining novel strains with unusual metabolic activity. For this project, these two different techniques were used in an attempt to isolate actinomycetes and specifically *Rhodococcus* strains that could use any of the following compounds as a sole carbon and energy source: Amido Black, Orange II, Indigo, diaminohexane, caprolactam and dibenzothiophene.

4.1.1 Nocardioform enrichment

The method described by Goldman (1991) using the steroid compounds, sodium deoxycholate and sodium taurocholate as an initial carbon source followed by enrichment for Gram positive organisms with the addition of nalidixic acid was not successful in enriching for Gram positive organisms. More then 75% of the organisms that were subsequently screened for carbon source utilization were Gram negative. This method, however, was partially successful in isolating several Gram negative organisms that showed a similar morphology and were capable of using caprolactam as a sole carbon and energy source. Part of the reason for the failure of this method could be because the soil contains a heterogeneous population of bacteria and organisms such as *Pseudomonas* which have a doubling time similar to *E. coli* can therefore grow much faster and out compete more slowly growing organisms such as *Rhodococcus* in a closed system.

Selective and enrichment techniques have specifically been developed to deal with this problem and although any medium is essentially selective because no single medium will support the growth of all organisms, conditions can be adjusted so as to favour the

growth of a particular organism or group of organisms. An initial step might therefore be the selection of Gram positive organisms to reduce the chances of fast growing Gram negative organisms out competing slower growing strains before employing an enrichment step. Cross (1982) reviewed different methods for the isolation of specific genera and species of actinomycetes and suggested that a combination of different selective steps seemed to be the best method for isolating particular organisms. For *Rhodococcus* in particular, nutrient enrichment, pretreatment of the soil with quaternary ammonium compounds and antibiotic treatment with naladixic acid, tellurite and penicillin were some of the suggested methods. Lechevalier and Lechevalier (1985) suggested that altering the pH of the soil by drying the sample and mixing it with calcium carbonate would favour the selection of actinomycetes.

4.1.2 Direct selection

Surprisingly, the alternative direct selection method worked very well and was used successfully to isolate a Gram negative organism, SQ4, from a caprolactam enrichment. The antifungal agent cycloheximide was not added since it could also presumable be used as an alternative carbon source. Fungal contamination, however, was not such a severe problem that it prevented the growth of bacteria. In addition, this organism was also capable of using the nylon monomers adipic acid, aminocaproic acid and diaminohexane as a sole carbon source. Biochemical and physical characterization of SQ4 tentatively suggests that the organism is a *Pseudomonas* species and according to Stolp and Gadkari (1981), identification of an organism as an obligate aerobic Gram negative with a single polar flagellum, glucose oxidizing, catalase positive and cytochrome C oxidase positive, is sufficient to place it within the genus *Pseudomonas*. Even though the cytochrome C oxidase test was the only test that was not performed, the organism's capacity to denitrify KNO_3 would seem to confirm this, since only a few Gram negative obligate aerobes including *Pseudomonas, Gluconobacter, Alcaligenes, Agrobacterium* and *Rhizobium* are capable of denitrification (Jeter and Ingraham, 1981).

This method was also used to isolate a filamentous Gram positive organism, SQ5, that seemed to use DBT as a sole sulfur source. Although light microscopy indicated that the organism was a filamentous Gram positive, only detailed chemical analysis of the

constituents of the cell wall particulary the diabasic amino acid, diagnostic sugar and mycolates will help to determine the taxonomic status of this organism (Lechavalier, 1989).

4.2 Cloning genes of interest from SQ4

To extract genes of interest a chromosomal library of SQ4 had to be built. The first, a BglII library, was built in *E. coli* MM294 and transformants were screened for genes involved in the degradation of 10 different compounds including nylon monomers and aromatic compounds. No positive transformants were recovered although this was not too surprising since the pathway involved in the degradation of aromatic compounds generally proceed via the intermediates catechol or protocatechuate and from there a series of at least 5 different enzyme reactions are required before the compound enters the tricarboxylic acid cycle (Gottschalk, 1986). Since *E. coli* K-12 is known not to possess this pathway for the degradation of aromatic compounds, this would require cloning of the genes for more than 5 enzymes on a single piece of DNA. The chances of this occurring are very small even assuming that the genes involved are all clustered together.

The degradation of the nylon monomers in *Pseudomonas* has been reported to proceed via the pathway e-caprolactam to e-aminocaproate to adipic hemialdehyde to adipate to succinyl-CoA (Naumova *et al.*). Although the chances of cloning a gene involved in the degradation of one the nylon monomers seem better since it seems that only a single enzyme reaction is required to convert adipate to succinyl-CoA, a compound familiar to *E. coli*, no genes were cloned. Adipic acid seems to be the compound most closely linked to an *E. coli* pathway since both aminocaproic acid and caprolactam are broken down to this compound. One would therefore expect the gene/s involved in the degradation of this compound to be cloned before any of the other genes.

Although no genes of interest were cloned, a simple experiment was done to see whether the SQ4 BglII library was representative. Results from this experiment suggested that the library was under-represented since it only complemented 25% of the auxotrophic mutations in an *E. coli* strain. Although calculations indicated that the

probability of a particular sequence represented in this library was at least 94%, there are a number of reasons this figure could be misleading. These calculations assume a random distribution of fragments. Digestion with restriction enzymes, however, is not a random process since these enzymes cleave only at specific sequences within the DNA. To produce a random distribution of fragments, techniques such as sonication of DNA can be used. The advantage of this method, however, is offset by problems resulting in the ligation of the sonicated DNA. An alternative is partial digestion of the DNA with an enzyme such as Sau3A which has a 4-bp recognition sequence. Although this is also strictly not a random process because only specific sequences will be recognized, the distribution of fragments will be more random than that produced by an enzyme with a 6-bp recognition sequence by virtue of the fact that there are more restriction sites for the enzyme with a 4-bp recognition site.

In the test to determine whether the BglII library of SQ4 was representative, only the *lysA* auxotrophy of *E. coli* strain RK4904 was complemented. The *lysA* mutation results in cells defective for the enzyme diaminopimelate decarboxylase [E.C. 4.1.1.20] which is responsible for the conversion of the *meso*-diaminopimelate to the amino acid lysine (Bachmann, 1990). Although the *lysA* gene from *E. coli* has been sequenced (Stragier *et al.*, 1983), the number of restriction sites mapped for the *lysA* gene cloned from SQ4 was not sufficient to make a valid comparison of the similarity of the two sequences. This result does indicate that SQ4 derived genes are expressed in *E. coli* although this is not too surprising seeing that both are Gram negative organisms.

Screening for the genes of a particular enzyme are simplified if there is an easy way of detecting the presence of that enzyme. This is the reason behind the chromogenic method of Parke (1992) which detects key intermediates of the aromatic degradative pathway, catechol and protocatechuate, by the addition of the compound *p*-toluidine to the media. Therefore, instead of screening for the end result of a complete pathway, one can screen for a particular enzyme in that pathway, for example, in the aromatic degradation pathway one can screen for the enzymes responsible for the breakdown of an aromatic compounds such as phenol, *p*-hydroxybenzoate or *m*-hydroxybenzoate into either catechol or protocatechuate. Since these reactions are carried out by single

enzyme (Gottschalk, 1986), the chances of cloning the gene for that particular enzyme is much better. Another advantage of this method is that one is screening for a specific enzyme with a known function whereas when cloning by complementation one is looking for the complementation of a specific mutation in any of a whole series of enzymes. The degradation pathway for a number of aromatic compounds including benzoate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, vanillate, shikimate, benzene, phenol, salicylate, anthranilate and naphthalene have been shown to proceed via either catechol or protocatechuate. Catechol or protocatechuate is then degraded via either an *ortho*-cleavage pathway (3-oxoadipate pathway) to succinate and acetyl-CoA or a *meta*-cleavage pathway to pyruvate (Gottschalk, 1986). A slightly modified 3-oxoadipate pathway has been shown to exist in *Rhodococcus* (Bruce *et al.*, 1989).

Parke (1992) reported that *p*-toluidine did not give a colour reaction with the intermediates of the 3-oxoadipate pathway (ß-carboxy-*cis,cis*-muconolactone, ß-ketoadipate, *cis,cis*-muconate and (+)-muconolactone). Other aromatics compounds that did not give a colour reaction with *p*-toluidine were shikimate, quinate, anthranilate, benzoate, benzylamine, 4-chlorobenzoate, *p*-coumarate, ferulate, *p*-hydroxybenzoate, D-(-)-mandelate, L-(+)-mandelate, phenol, ß-resorcylate, salicylate, syringate, terephthalate, *m*-toluate, *p*-toluate and vanillate. The chromogenic detection system using *p*-toluidine could therefore be used to screen for the genes involved in the degradation of a whole range of aromatic compounds.

There have recently been several reports of *Rhodococcus* genes that are expressed in *E. coli* including a gene involved indigo pigment production (Hart *et al.*, 1992) and a nitrile hydratase gene (Hashimoto *et al.*, 1992). Since it seems that rhodococcal promoters are recognized by *E. coli*, *Rhodococcus* genomic libraries maintained in *E. coli* strains can be screened directly in their *E. coli* hosts for genes of interest. For example, screening *Rhodococcus* libraries for genes involved in the degradation of aromatic compounds can be simplified if the screening of the library in *E. coli* is performed in conjunction with the previously mentioned chromogenic detection method. This circumvents the need to construct auxotrophic mutants for complementation

analysis and not only facilitates the screening process but also reduces the time involved.

A further use of this chromogenic detection method is the isolation and characterization of strains with mutations in the pathway for degradation of aromatic compound. Mutations in the aromatic degradation pathway affecting particulary the enzymes responsible for the breakdown of either catechol or protocatechuate result in an accumulation of either of these compounds around the cells with the mutations. This accumulation can be detected with the *p*-toluidine chromogenic detection technique since these compounds are normally excreted by the bacterium and this method can therefore be used to isolate mutants with a defective aromatic degradation pathway. Most of the *Rhodococcus* strains have an aromatic degradation pathway since the majority of strains in the laboratory can use either *p*-hydroxybenzoate or phenol as a sole carbon source (E. Dabbs, personal communication). Since this pathway is involved in the degradation of most aromatic compounds including those with a pollution potential such as the substituted aromatic compounds, the ligninolytic compounds and the polycyclic aromatic hydrocarbons, the characterization of this pathway is an essential step needed to fully understand the metabolism of aromatic compounds by *Rhodococcus*. Although it seems that some of the *Rhodococcus* strains, for example DSM 1069, excrete either protocatechuate or catechol into the medium resulting in the formation of the brown precipitate in the presence of *p*-toluidine (E. Dabbs, personal communication), this method can still be used for screening of mutants if conditions are optimized to distinguish between the wild-type and mutants (Parke, 1992). This chromogenic detection system is therefore workable and should facilitate the cloning of genes involved in the degradation of aromatic compounds. Also, with conditions optimized, this system should help in the isolation of mutants blocked in the aromatic degradative pathway.

4.3 Azo dye decolorization

Previous work regarding azo dye decolorization by *Rhodococcus* species has shown that certain species are capable of decolorizing the sulfonated azo dyes Amido Black and

Orange II (Heiss *et al.*, 1992). DNA complementing a non-decolorizing mutant has been cloned and a restriction map constructed (see Fig. 3). Previous work has shown that removal of the BglII fragment resulted in a loss of decolorization activity. Exonuclease digestion of the fragment with Bal31 from the unique NotI or HindIII site of the plasmid indicated that the proximal BglII site and the 2 SfuI sites were an integral part of the gene and that the loss of these sites was concurrent with the loss of decolorization activity. This work has shown that the BglII fragment does not contain the intact gene presumably because one or both of the BglII sites cut into the gene. This correlates with previous findings (Heiss *et al.*, 1992) and suggests that either the gene is very large or that it is part of a multicistronic operon with a promoter region required for gene expression. Although an attempt to subclone the two SfuI fragments failed, this attempt should be repeated because the 2 SfuI sites conveniently bracket the BglII site and the fragment is large enough to contain the whole gene sequence.

An attempt was also made to extract a full-length clone from the ATCC 4277 library using the intact pGSH2 as a probe. Unfortunately, pDA37 sequences from pGSH1 hybridized to the ATCC 4277 chromosomal DNA and the intact plasmid could not be used as a probe. A smaller PstI-HindIII fragment was then purified and used as the probe but no hybridization was detected. This work was not pursued further.

The 50-bp fragment from pGSH2 was removed and inserted into pUC18 as a preliminary step to sequencing. Since the size of the insert is so small, this will simplify initial sequencing attempts and sequencing of this small insert can serve as a pilot run to much bigger projects. Incidentally, since the *E. coli* vector pUC18 presumably contains no nocardioform related sequences, the pUC18 plasmids containing the 50-bp inserts form pGSH2 can be used as a probe because there will not be problem with hybridization to unwanted sequences.

A number of studies have implicated peroxidase activity in the decolorization of azo-dyes (Pasti-Grigsby *et al.* 1992; Paszczynski *et al.*, 1991). Peroxidases [E.C. 1.11.1.7] are enzymes which are widely distributed in animals, plant and microorganisms and they play an important role in the oxidation of a large number of aromatic compounds

including recalcitrant substances (Kenneth and Tardone, 1988). Peroxidases of microbial origin have been identified in a number of species including *Escherichia coli* (Clairborne and Fridowich, 1979), *Comamonas compransoris* (Nies and Schlegel, 1982), *Bacillus stearothermophilus* (Loprasert *et al.*, 1988), *Rhodopseudomonas capsulata* (Hochmann and Shemesh, 1987) and various *Streptomyces* species (Van Pée and Lingens, 1985; Zeiner *et al.*, 1988; Knoch *et al.*, 1989; Ramachandra *et al.*, 1988; Mikli and Zimmermann, 1992). Peroxidases have been shown to be involved in lignin degradation (Mikli and Zimmermann, 1992; Ramachandra *et al.*, 1988) and azo dye decolorization (Pasti-Grigsby, 1992) in a number of *Streptomyces* species. No accounts of peroxidase activity in *Rhodococcus*, a genus closely allied to the *Streptomyces*, have been reported and since a number of *Rhodococcus* species are also capable of azo dye decolorization and lignin degradation, these organisms may have a similar non-specific mechanism responsible for the oxidative biodegradation of natural and xenobiotic compounds.

Another non-specific mechanism used in the oxidative degradation of a wide variety of compounds is the cytochrome P-450 enzymes. Cytochrome P-450s have been detected in over 38 bacterial strains distributed among 16 species that comprise 12 genera (Asperger and Kleber, 1991). A number of reactions are mediated by these cytochromes including hydroxylation or dealkylation of terpenes, cholesterol, alkanes and alkylaryl ethers, selective hydroxylation of steroids, subterminal hydroxylation of fatty acids, hydroxylation of macrolides and de-esterification, dealkylation or hydroxylation of rings substituents in sulfonylurea herbicides (Asperger and Kleber, 1991). Moreover, cytochrome P-450s have been detected in a number of nocardioforms including those from *Nocardia* sp. NH1 involved in the dealkylation of *p*-alkoxybenzoate (Cartwright *et al.*, 1971) and from *R. rhodochrous* involved in alkane oxidation (Cardini and Jurtshuk, 1968). Therefore, the possibility that a non-specific enzymatic mechanism such as peroxidase or cytochrome P-450 is present in some *Rhodococcus* species is very good and play an important role in explaining the metabolic diversity of some the nocardioforms. This should, as a matter of course, be investigated further.

4.4 Dibenzothiophene utilization

An organism, isolated from soil using the direct selection method, seemed to grow better on BSM plates with DBT as the sulfate source. Further physical and biochemical characterization revealed that the organism was a filamentous Gram positive with a characteristic orange/red pigment produced on either MM or LA. Like most of the other laboratory *Rhodococcus* strains, the organism was able to use phenol as a carbon and energy source. In addition, sodium benzoate, catechol, cinnamyl alcohol, ethanol and Tween-80 were also good carbon sources and in some cases seemed to be better than glucose.

This organism was initially isolated because it seemed to be using DBT as a sulfur source. Subsequent work was done with stock 3 minimal media minus $MgSO_4$ and growth with DBT as the sulfur source was compared with the positive control where $MgSO_4$ was added and a negative control that did not have a sulfur supplement. Growth in both the positive and negative controls were comparable and therefore conditions to highlight the difference between the positive and negative controls had to be optimized. When ethanol (0.2%) was used as the carbon source and NH_4Cl (0.1%) as the nitrogen source, the difference between the negative and positive controls was dramatic when 0.01% $MgSO_4$ was added to the positive control sample. Under these conditions, the DBT seemed to be used as a poor sulfur source while the $MgSO_4$ appeared to be a much better sulfur source.

Although the work presented here was preliminary, before it is continued, a fundamental question should first be answered. Is the DBT itself and not a contaminant being used as a sulfur source and if so by which pathway is it degraded? Several tec' 'ques have been developed over the last couple of years to screen for organisms capable of degrading DBT (Krawiec, 1989). The first of these involves the presence of 'reducing power' as an indication of a metabolically active state. Practically this can be done by adding a compound such as 2,3,5-triphenyltetrazolium chloride (TTC) to the medium. TTC is a colorless water-soluble compound that is reduced by metabolically active organisms into a red-colored water-insoluble formazan compound (Bochner and Savageau, 1977). Ward *et al.* (1988) have used this technique to find organisms capable

of degrading DBT. Organisms which appear white on dilute yeast extract media and red on dilute yeast extract media supplemented with DBT were characterized further.

The second method, used in this work, is the sulfur starvation assay. Sulfur is an integral requirement for microorganisms since it is constituent in cysteine, methionine, thiamine, pyrophosphate, coenzyme-A, biotin and α-lipoic acid (Gottschalk, 1988). Organisms capable of desulfurization are selected in media devoid of any sulfur source except that supplied by the compound of interest. Organism will therefore only survive the selection if they are capable of desulfurizing the organic sulfur compound. This method has been successfully used at the Institute of Gas Technology in Chicago for isolating a *Rhodococcus rhodochrous* IGTS8 strain capable of desulfurizing DBT (Kayser *et al.*, 1993).

The third method makes use of the fact that one of the breakdown products of DBT fluoresces under UV light. The compound *o,o'*-biphenol can be detected on defined media by viewing under a UV light. The drawback to this method is that breakdown products of DBT, 2-hydroxybiphenyl and dibenzosulfone, also fluoresce under UV light and this method therefore gives an indication that some intermediates in the modified '4S' pathway may be present.

All of the above techniques do not give conclusive answers and physical techniques such as UV spectrophotometry, gas chromatography and HPLC have also been studied. Both UV spectrophotometry and GC have limitations since certain breakdown products cannot be resolved. HPLC can, however, resolve the compounds dibenzosulfoxide, dibenzosulfone, *o,o'*-biphenol, monohydroxybiphenyl, biphenyl and DBT and this, therefore, is the method of choice in confirming the presence of reactants of the extended '4S' pathway (Wyza and Isbister, 1989).

Once it is known whether DBT is being used as a carbon source and whether or not the carbon skeleton is oxidized in the desulfurization process, then the pathway can be analyzed by creating mutants unable to desulfurize DBT. These mutants can also then be used for cloning the genes involved by complementation. Although the taxonomic

status of SQ5 is uncertain, an attempt was made to transform this organisms with several of the *Rhodococcus* plasmids to see whether any of the plasmids were maintained. Plasmids, pDA37 and Bcl2 derived from *R. erythropolis* and pIGTS1 derived from *R. fascians* were used without any success. Before any cloning by complementation can be done, a plasmid capable of replicating in SQ5 must first be found. If no plasmid can be found, alternatives of using a broad host-range phage to create a plasmid vector, similar to the strategy employed by Dabbs *et al.* (1990), must be considered.

4.5 *Bacillus* transformations

An attempt was made to transform *B. subtilis* via the competent cell and the protoplast method with an *E. coli-Bacillus* shuttle vector. No success was achieved with the competent cell method presumably because of the fact that monomeric plasmids transform cells very poorly with this method (Hoch, 1991). The protoplast method was also attempted with mixed success. The transformation protocol did not give a result but when the standard nocardioform protocol was used with a number of small modifications, this method seemed to work fairly well. This is not too surprising seeing that this protocol was developed from an original protocol for transforming streptomycetes. There is therefore no reason why this method should not work for any Gram positive organism.

APPENDIX A

Media

LB

Tryptone	1%
Yeast extract	0.5%
NaCl	0.5%

LA

LB

Agar	1.5%

LBSG

LB

Glycine	3%
Sucrose	10.3%

TYG

Tryptone	1%
Yeast extract	0.5%
Glycine	2%

Top agar

Tryptone	1%
Yeast extract	0.5%
Agar	0.5%

10x A-N Buffer

$K_2HPO_4.3H_2O$	91.7g
KH_2PO_4	26.8g
$MgSO_4$	1.0g

Distilled water to 1000ml

10x Stock 3

$K_2HPO_4.3H_2O$	91.7g
KH_2PO_4	26.8g
$Na_3C_6H_7O_7.2H_2O$	5.0g
$MgSO_4$	1.0g

Distilled water to 1000ml

Minimal media

For either A-N or stock 3 minimal media prepare 2× buffer and 2× agar. Autoclave separately and add together after autoclaving. If necessary, glucose can be autoclaved with the 2× agar solution and NH_4Cl with the 2× buffer solution.

Hugh and Leifson OF basal media

Tryptone	2.0g
NaCl	5.0g
K_2HPO_4	0.3g
Agar	1.5%
Bromothymol blue	0.03-0.08g
1000ml distilled water	

Denitrification media

$K_2PO_4.3H_2O$	0.8g
KH_2PO_4	0.2g
$CaCl_2$	0.1g
$MgSO_4.7H_2O$	0.5g
$(NH_4)_2SO_4$	1.5g
Yeast extract	3.0g
KNO_3	10.0g
Glycerol	1.0%
Agar	1.0g
1000ml distilled water	
pH 7.2	

Agarose gel solutions

5x TBE

Tris.HCl	54.0g
boric acid	27.5g
0.5M EDTA pH 8.0	20ml
Distilled H_2O to 1l	

Tracking dye

glycerol in TE	30%
bromophenol blue	0.025%

***E. coli* transformation solutions**

E. coli transformation buffer

$CaCl_2$	50mM
Tris.HCl	10mM
pH 7.5	

***Rhodococcus* transformation solutions**

Basal buffer

sucrose	10.3g
K_2SO_4	25mg
$MgCl_2.6H_2O$	202mg
0.25M TES pH 7.2	10ml
87.5ml sterile distilled water	

Protoplast buffer

basal buffer	4.5ml
0.5% KH_2PO_4	50µl
1M $CaCl_2.2H_2O$	125µl

Regeneration media

NaCl	0.9g
Yeast extract	1.5g
Tryptone	3.0g
Sucrose	35.0g
Agar	1.5%

distilled water to 280ml

After these compounds have been added together in a 500ml flask it is microwaved until the sucrose has dissolved. The flask is autoclaved at 121°C for 20 minutes. After the media has cooled to 60°C the following compounds are added from stock solutions:-

1M $CaCl_2$	6ml
0.5% KH_2PO_4	3ml
10mg/ml Cycloheximide	1.5ml
10mg/ml Rifampicin	0.9ml

Plates with a constant volume of about 22ml are poured and, once set, are air dried for 2 days in a 37°C incubator.

***Bacillus* transformation solutions**

MG

$(NH_4)_2SO_4$	2g
K_2HPO_4	14g
KH_2PO_4	6g
$Na_3C_6H_5O_7.2H_2O$	1g
$MgSO_4.7H_2O$	0.2g

distilled water to 1l

After autoclaving add 10ml sterile 50% glucose

SPI media

Add 0.02% casein hydrolysate to MG from a 5% stock

SPII media

Add 0.01% casein hydrolysate to MG from a 5% stock

2× SMM

sucrose	1M
Na maleate	40mM
$MgCl_2$	40mM

pH 6.5

SMMLB

Mix equal volumes of 2× SMM and 2× LB

PEG solution

Prepare 40% PEG 6000 in 1× SMM

DM3 regeneration media

Na succinate	135g
Casamino acids	5.0g
yeast extract	5.0g
KH_2PO_4	1.5g
K_2PO_4	3.5g
agar	8.0g

pH 7.2

distilled water to 950ml

After autoclaving add:

20% glucose	25ml
1M $MgCl_2$	20ml
2% BSA	5ml

Gram stain reagents

Crystal violet

0.5% in sterile distilled water

Strong iodine

iodine	10g
KI	20g

distilled water to 1l

Iodine-acetone

iodine	10g
KI	6g
distilled H_2O	10ml

90% ethanol to 100ml

Iodine-acetone decolorizer

Strong iodine	3.5ml
Acetone	96.5ml

Safranin

1% in sterile distilled water

E. coli plasmid preparation solutions

Solution 1

glucose	50mM
Tris.HCl	25mM
EDTA	10mM
pH 8.0	

Solution 2

NaOH	0.2M
SDS	1%

Solution 3

KAc	244g
distilled water to 600ml	
glacial acetic acid	115ml
distilled water	285ml
pH 5.8	

Rhodococcus plasmid preparation solutions

Solution A

[illegible]rose	50mM
[illegible]Cl	10mM
pH 8.0	

Solution B

Tris.HCl	10mM
EDTA	10mM
SDS	10%
pH 8.0	

Solution C

NaAC	4.5M
pH 6.0	

Southern blot solutions

20× SSC

NaCl	3M
sodium citrate	0.3M
pH 7.0	

Denaturing solution

NaCl	1.5M
NaOH	0.5M

Neutralizing solution

Tris.HCl	1M
NaCl	1.5M
pH 8.0	

Hybridization solution

20× SSC	20%
blocking reagent	0.5%
sodium lauroylsarcosine	0.1%
SDS	0.02%

Washing solution 1

20× SSC	10%
SDS	0.1%

Washing solution 2

20× SSC	0.5%
SDS	0.1%

Buffer 1

Tris.HCl	100mM
NaCl	150mM
pH 7.5	

Buffer 3

Tris.HCl	100mM
NaCl	100mM
$MgCl_2$	50mM
pH 9.5	

E. coli ß-galactosidase assay reagents

X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside)
Prepare 20mg/ml in dimethylformamide and store at -20°C wrapped in aluminium foil

IPTG (Isopropylthio-ß-D-galactoside)
Prepare 2g in 8ml distilled water and sterilize by filtration. Store at -20°C.

Antibiotics and antifungal agents

Ampicillin (100mg/ml) 1:1 H_2O:ethanol
Chloramphenicol (4mg/ml) ethanol
Cycloheximide (10mg/ml) water
Fusidic acid (10mg/ml) 1:1 H_2O:ethanol
Nalidixic acid (10mg/ml) 1:1 H_2O:ethanol
Rifampicin (10mg/ml) methanol
Spectinomycin (100mg/ml) ethanol

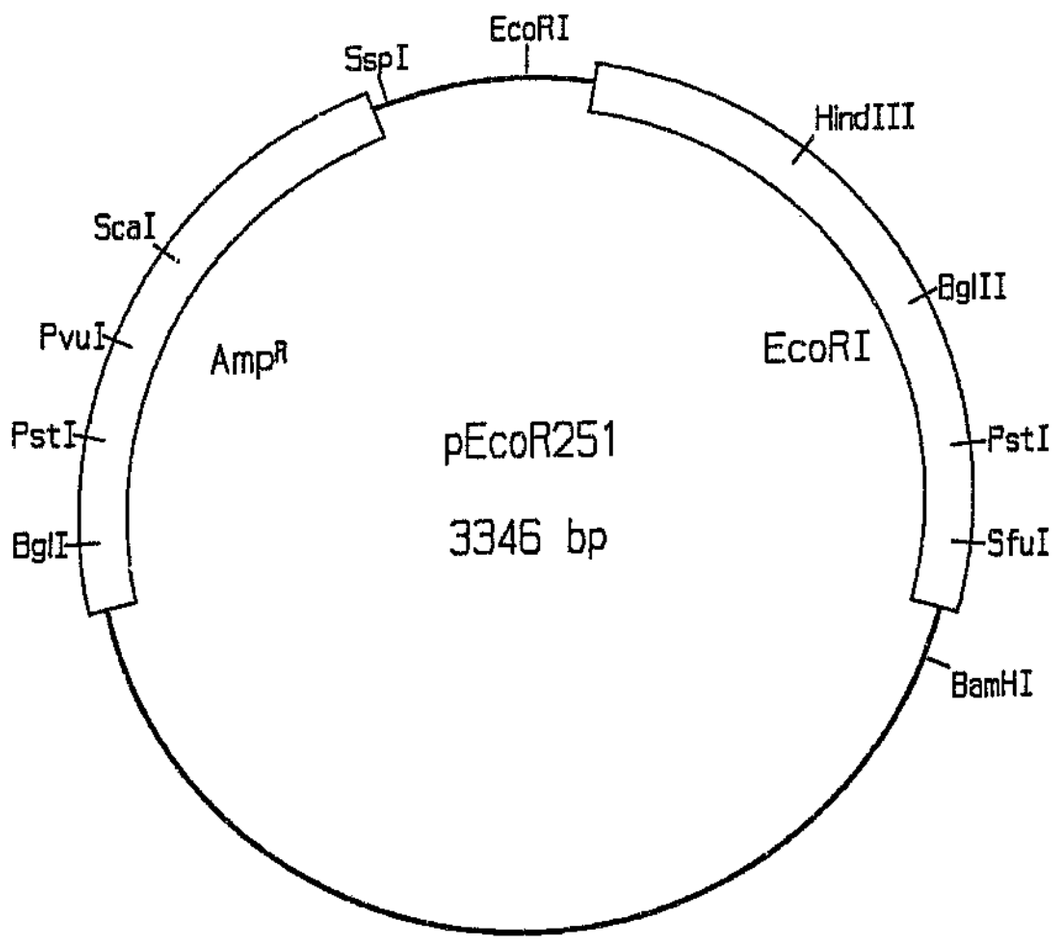
Tetracycline (10mg/ml) methanol
Thiostrepton (10mg/ml) 1:1 H_2O:ethanol

APPENDIX B

Restriction map of pDA37



Restriction map of pEcoR251



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