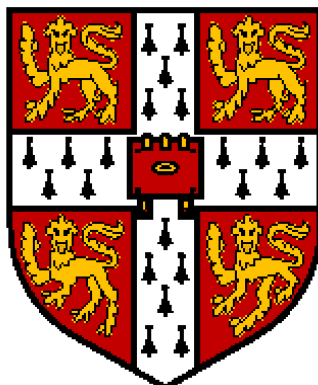


UNIVERSITY OF CAMBRIDGE



# MICROBIAL DEGRADATION OF RDX

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This thesis is submitted for the degree of Doctor of Philosophy. This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text.

“Science – that sister who, at all events, does not laugh in your face,  
but always repays you, though sometimes in rather hollow coin,  
for the attentions bestowed upon her”

Victor Hugo  
Hunchback of Notre Dame  
1831

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First of all I'd like to dedicate this work to my mother. She'd be pleased (I hope!), although she'd probably have preferred to be alive to read it. She'd probably be a bit proud of me too, and I guess I owe her a lot.

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I'll remember you all when I'm rich and famous!!!

## Abstract

Large amounts of land and groundwater have been polluted through the manufacture, detonation and disposal of explosives. Explosives are xenobiotic compounds, being toxic to biological systems, and their recalcitrance leads to persistence in the environment. The methods currently used for the remediation of explosive contaminated sites are expensive and can result in the formation of toxic products. Bioremediation, using characterized bacteria or isolated enzymes, holds potential for explosive remediation. Enzymes which can degrade or transform two of the three classes of explosives, nitrate esters and nitroaromatics, have been identified, but no enzymes with activity against the nitramine explosives have been characterized. Of the nitramines, RDX is currently the most widely used military explosive and is of particular environmental concern because of its mobility in soil.

The aim of this project is to isolate bacteria able to degrade RDX aerobically, and to characterize the basis of this ability. Nineteen bacteria which could degrade RDX as a sole source of nitrogen were isolated. All the isolates were identified as unique strains of rhodococci and their ability to degrade RDX was compared. *Rhodococcus rhodochrous* strain 11Y was chosen for further characterization.

*Rhodococcus rhodochrous* strain 11Y can remove RDX from culture at a rate comparable to the best characterized strains to date. The activity against RDX is present in cells grown in the absence of RDX, and is increased threefold in its presence. Three of the six nitrogens from RDX are utilized for growth, but related nitramine explosives cannot be used by strain 11Y as sources of nitrogen. Products of RDX degradation by strain 11Y were identified as nitrite, formate and formaldehyde, which do not correlate entirely with the proposed mechanisms of aerobic RDX breakdown.

A genomic library from strain 11Y was transformed into a non RDX degrading strain of *R. rhodochrous*, and RDX degrading clones were selected for by enrichment using RDX as a sole source of nitrogen. A fragment of DNA which was able to confer upon this strain the ability to degrade RDX was isolated and found to contain three open reading frames. Subcloning identified the fragment necessary for activity against RDX, containing a cytochrome P450-like gene with a flavodoxin domain at the N-terminal end, designated *xplA*. A reductase-like gene, designated *xplB*, was found immediately upstream. The use of a P450 in RDX degradation was proved functionally using a P450 specific inhibitor. This is the first time that a gene responsible for the degradation of RDX has been cloned and identified. P450s are often responsible for the degradation or detoxification of xenobiotic compounds, and commonly operate through hydroxylation of the substrate. The function of the fused flavodoxin domain in electron transport has not been demonstrated, but would represent a novel class of P450 system. The expression of XplA and XplB has been achieved in *E. coli*, although active recombinant proteins were not obtained.

## Abbreviations

bp	base pair(s)
cDNA	complementary DNA
CL20	2,4,6,8,10,12-hexanitrohexazaisowurtzitane
DEPC	diethyl pyrocarbonate
DNX	hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
dH <sub>2</sub> O	ultra high pressure purified water
DMF	N,N-dimethylformamide
DNA	deoxyribose nucleic acid
dNTP	dinucleotide triphosphate
Dstl	Defence science and technology laboratory
EDTA	ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GCG	Genetics Computer Group
GTN	glycerol trinitrate, nitroglycerine
HMX	high melting explosive, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
HPLC	high performance liquid chromatography
IPTG	isopropyl- $\beta$ -D-thiogalactopyranoside
kb	kilobase pair(s)
kDa	kilodalton
LA	Luria Bertani agar
LB	Luria Bertani broth
MXN	hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
NED	N-(1-naphthyl)ethylenediamine
OD <sub>600</sub>	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAH	polyaromatic hydrocarbon
PCR	polymerase chain reaction
PEG	polyethylene glycol
PETN	pentaerithritol tetranitrate
PVDF	polyvinyl difluoride
rDNA	ribosomal DNA
RDX	royal demolition explosive, hexahydro-1,3,5-trinitro-1,3,5-triazine
Rf	retardation factor
RNA	ribose nucleic acid
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
sp.	species
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TES	N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid
TLC	thin layer chromatography
T <sub>m</sub>	melting temperature
TNT	2,4,6-trinitrotoluene
TNX	hexahydro-1,3,5-trinitroso -1,3,5-triazine
tRNA	transfer RNA
X-gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside

## **Publication arising from this work**

**Seth-Smith, H.M.B., Rosser, S.J., Basran, A., Travis, E.R., Dabbs, E.R., Nicklin, S. and Bruce, N.C.** (2002) Cloning, Sequencing and Characterization of the Hexahydro-1,3,5-trinitro-1,3,5-triazine Degradation Gene Cluster from *Rhodococcus rhodochrous*. *Appl. Environ. Microbiol.* **68**:4764-4771.

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## Chapter 1. Introduction

### 1.1 Explosives

Explosives are materials which, when suitably initiated, result in the rapid release of energy. Detonation of the solid explosive generates expanding hot gases. This expansion creates a shock wave which exerts high pressures on the surroundings, causing an explosion. Explosives generally have high nitrogen and oxygen contents which aid the formation of the gaseous products, typically including carbon dioxide, carbon monoxide, oxygen, nitrogen and water vapour.

The development of explosives has sought to provide both greater power and greater control. Mass production of some of these compounds over the last century has led to extensive contamination of land, which now requires remediation.

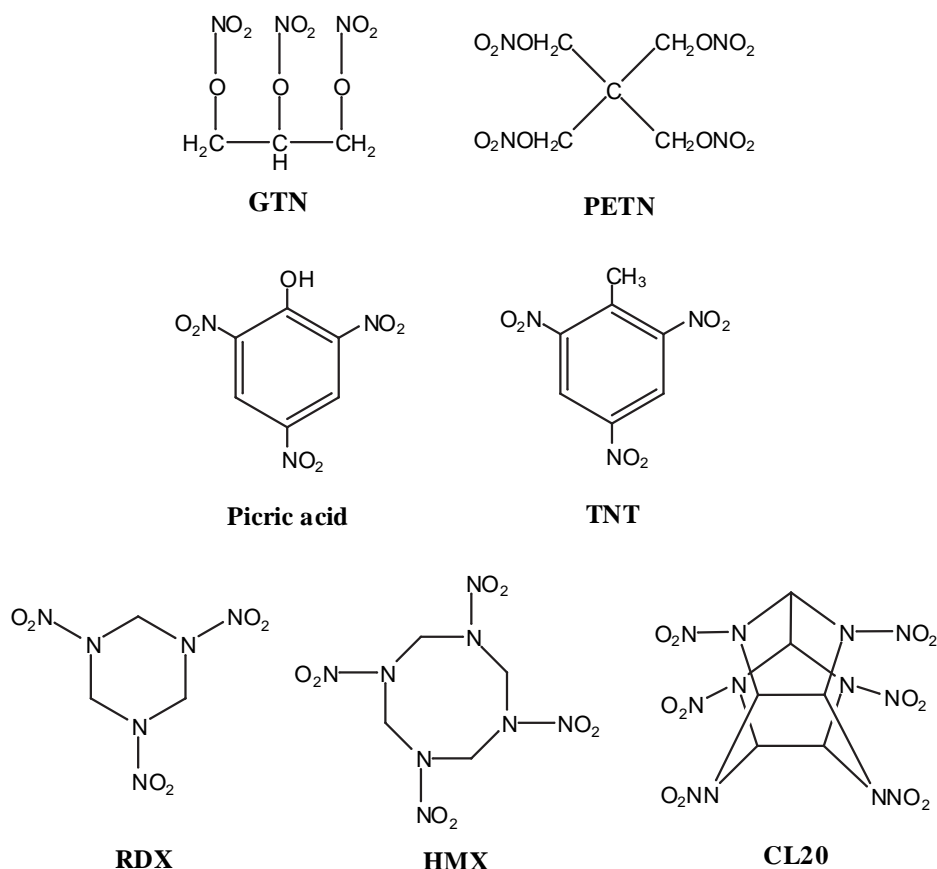
### 1.2 Development of explosives

The history of explosives discussed here extends from the mixture of gunpowder to the industrialization of the explosive developing and manufacturing processes, and is presented in more detail by Brown <sup>42</sup>.

Gunpowder is the first explosive known to be formulated, a combination of potassium nitrate (saltpetre), sulphur and charcoal discovered by the Chinese in the mid ninth century. News of this “black powder” travelled to the West and the recipe was revealed to the public by Roger Bacon in 1260. Although used initially in what are now known as fireworks, gunpowder was also instrumental in the development of the first gun, from China in 1280. Gunpowder’s destructive power has since been harnessed for more productive purposes; the first instance of its use in mining is recorded in Hungary in 1627.

Glycerol trinitrate (nitroglycerine or GTN) was developed by Ascanio Sobrero in 1847. It was first characterized as a medicine and is still used in the treatment of angina pectoris. Today GTN is better known as a powerful explosive. It is an example of a nitrate ester explosive, characterized by the O-NO<sub>2</sub> bond. Structures of examples from all classes of important explosives are shown in Figure 1.1. GTN was found to be a very unpredictable explosive, capable of either exploding when not desired, or of not exploding when detonated. Part of the problem was due to the unreliable methods used to initiate detonation, namely by fire or with

fuses. Alfred Nobel invented the detonator, or blasting cap, in 1863, which made use of gunpowder to trigger the explosion of GTN. The invention for which Nobel is most well known followed just four years later, namely dynamite. In dynamite, GTN is stabilized through absorption into a solid (kieselguhr), which greatly reduces its erratic behaviour. GTN in this form proved much safer to use and easier to detonate.



**Figure 1.1: Structures of some important explosive compounds.** GTN and PETN are nitrate esters, picric acid and TNT are nitroaromatics and RDX, HMX and CL20 are nitramines.

The development of shells for warfare required a less sensitive explosive than GTN, which had a tendency to detonate on firing. 2,4,6-Trinitrophenol (picric acid), a nitroaromatic compound characterized by  $\text{NO}_2$  groups attached to an aromatic ring, had been developed in 1771. It was used as a dye until 1871, when its explosive properties were discovered, leading to its use in shells by 1885. Although being more stable than GTN, the drawback of picric acid was that it reacted with the metals used in the shell casings. The resulting picrates made the shells susceptible to undesired detonation by shock or friction. 2,4,6-Trinitrotoluene (TNT) is another example of a nitroaromatic explosive, first synthesized by Joseph Wilbrand in 1863, although its

explosive properties were not discovered until 1891. By 1902 the Germans were using TNT instead of picric acid in shells, and it had become commonly used in Britain by 1916. The advantages of TNT over the structurally similar picric acid included its lower shock sensitivity and lower acidity, although it can require the use of stronger detonators. Over 275,000 tonnes of picric acid and TNT were produced in Britain during World War I, and TNT has the dubious honour of being the most used military explosive of the twentieth century. Relative powers of explosives can be calculated using a power index, in which explosives are compared to picric acid, which has a power index of 100. Power indices of GTN and TNT are 159 and 117 respectively <sup>11</sup>.

A second nitrate ester explosive, pentaerythritol tetranitrate (PETN), was developed in Germany in 1894, and came into use during World War II. This very powerful explosive (power index of 161 <sup>11</sup>) proved to be too sensitive and too easily detonated to be used alone. Consequently, it is often used in detonators, or combination with other explosives; for example, it is a major component of the plastic explosive SEMTEX (which also contains RDX and plasticizer).

The first nitramine explosive (characterized by N-NO<sub>2</sub> groups) to be developed was hexahydro-1,3,5-trinitro-1,3,5-triazine, synthesized by Hans Hemming in 1899. In 1920 it was patented as an explosive, and its further development at the War Department in Woolwich, U.K. led to its naming as Royal Demolition Explosive or RDX. It is as powerful as PETN and GTN (power index of 159 <sup>11</sup>), but much less sensitive. It is commonly used in explosive mixtures including cyclotol, which comprises 60 % RDX and 40 % TNT, and composition C-4, which comprises 91 % RDX with plasticizers. RDX is currently the most widely used military explosive <sup>251</sup>.

Synthesis of a second, and even more stable, nitramine explosive, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (High Melting Explosive or HMX), followed in 1930. HMX has been in military use since the 1950s and has a power index of 160 <sup>11</sup>. A new generation of nitramine explosive, 2,4,6,8,10,12-hexanitrohexazaisowurtzitane (CL20 or HNIW), has since been developed and has recently undergone initial testing, where it was found to be even more powerful than HMX (power index unavailable).

The desire for more powerful, yet stable explosives has driven research over the last century. The majority of explosives in current use are nitramines, and of these RDX is the most important due to the extent of its use.

### 1.3 Toxicity of explosives

In addition to their destructive capacity, explosives commonly have toxic effects on biological systems. The nitrate esters, including PETN and GTN, are toxic to mammals mainly through their vasodilatory effects and ability to cause methaemoglobinaemia, which affects the ability of red blood cells to transport oxygen<sup>296</sup>. GTN has been used medically, in small doses, as a vasodilator for over 100 years<sup>296</sup>; the effects are thought to be due to the action of nitric oxide (NO) produced by metabolism of GTN<sup>269</sup>. Low levels of exposure in humans lead to headaches and nausea and occasionally to vomiting and abdominal pains<sup>15</sup>. Throughout the industrial production of these compounds, no fatalities or chronic effects have been reported through exposure to nitrate esters, although there have been some cases of dermatitis<sup>296</sup>. Symptoms of nitrate ester exposure in animal studies include decreases in blood pressure and respiratory problems<sup>296</sup>. Acute exposure can lead to death as a result of respiratory or cardiac arrest<sup>296</sup>, and GTN has an acute oral LD<sub>50</sub> (dose lethal to 50 % of test animals) of 0.5-0.9 g/kg body weight in rats<sup>207</sup>. GTN has been designated a class C carcinogen by the U.S. Environmental Protection Agency (EPA), indicating potential carcinogenicity, although evidence from both animal models and humans is limited<sup>251</sup>. There is very little data on the toxic effects of PETN, and no LD<sub>50</sub> is referred to in the literature. PETN is a less potent vasodilator than GTN<sup>296</sup> and appears to be relatively non-toxic<sup>251</sup>.

TNT is a mutagen and causes liver damage. In humans, TNT can cause dermatitis, vomiting, toxic hepatitis and liver damage, methaemoglobinaemia and aplastic anaemia, which affects blood cell production<sup>15, 196, 251</sup>. Those who worked with TNT during World War I were known as “canaries” due to the yellowing of the skin from jaundice caused by this “TNT poisoning”. Ninety-six workers in the U.K. died from exposure to TNT<sup>42</sup>. Picric acid, a similar nitroaromatic explosive, can cause dermatitis in low doses, with higher doses affecting the kidneys and liver<sup>15</sup>. Oral LD<sub>50</sub> values for TNT and picric acid in rats are 0.8 – 1.3 g/kg and 0.2 g/kg respectively<sup>1, 156</sup>. TNT has toxic effects as determined using earthworm reproduction tests<sup>248</sup>, and work on the luminescent bacterium *Vibrio fischeri* has deemed TNT to be “very toxic” to aquatic organisms<sup>82</sup>. Several mutagenicity studies have been carried out using TNT and its prominent metabolites on both *Salmonella* strains and mammalian cell lines<sup>182, 106, 300, 331</sup>. These studies have found TNT to be mutagenic, some of the metabolites more so than the TNT itself. However, the evidence is limited, there is no epidemiological evidence of cancer among TNT workers, and consequently it has also been given a carcinogen classification of C<sup>251</sup>.

The effects of RDX on mammals are generally characterized by convulsions. Supplying RDX to both dogs and rats results in irritability and convulsions as symptoms of chronic toxicity, and death in the rats was associated with congestion in the gastro-intestinal tract and lungs<sup>47, 318</sup> (oral rat LD<sub>50</sub> of 0.07 – 0.12 g/kg<sup>286</sup>). RDX toxicity can also cause weight loss associated with a reduction of food intake in rats<sup>191</sup>, and RDX has been used as a rat poison<sup>225</sup>. There have been several reported cases of RDX toxicity in humans. Workers in RDX factories in Germany, Italy and U.S.A. have been seen to suffer symptoms including convulsions, unconsciousness, vertigo and vomiting after exposure, usually through the inhalation of RDX powder<sup>161</sup>. A study on a child who ingested plasticized RDX and developed seizures found that RDX can transport easily into the central nervous system (CNS)<sup>335</sup>. More recent reports of men purposefully chewing the plastic explosives C-4 or SEMTEX, which contain high levels of RDX, show them to develop grand mal seizures with associated headaches or amnesia<sup>109, 131</sup>. Recovery from these episodes is complete and no recurrence of symptoms is seen in the absence of further exposure. Tests using freshwater invertebrates, green algae, fathead minnow, earthworm reproduction and luminescent bacterium *Vibrio fischeri* have found RDX to be toxic, but less so than TNT<sup>49, 50, 82, 228, 248</sup>. Although studies testing RDX on both *Salmonella* and mammalian cell lines have shown that it is not mutagenic<sup>106, 182</sup>, it is designated a class C carcinogen<sup>251</sup>.

Limited data shows that HMX is likely to be less toxic than RDX. Some effects on the CNS have been demonstrated in rats, but at significantly higher doses than for RDX<sup>251</sup> (oral rat LD<sub>50</sub> of 6.5-7.6 g/kg<sup>150</sup>). Some toxic effects of HMX have been seen using aquatic organisms, bacteria and the earthworm reproduction test<sup>82, 249</sup>. HMX has a class D carcinogen designation, meaning that there is no evidence of carcinogenicity from animal studies<sup>251</sup>. Toxicological exposure limits have not been determined for all explosives, as working practice which avoids skin contact or inhalation appears to be sufficient to prevent harm to explosive workers<sup>296</sup>.

All explosives are toxic to varying degrees. Most are classed as potential carcinogens and other toxic effects have been seen in munitions workers and animal studies. The dangers associated with exposure to explosives should not be underestimated.

## 1.4 Explosives as environmental pollutants

Explosives are present as contaminants on land as a result of their manufacture, their deployment, and from weapons decommissioning. Explosives are highly recalcitrant compounds, resistant to degradation *in situ*, meaning that the contamination persists. Most countries have not

yet addressed the problem of explosive contamination, but where it has been catalogued, the problem is significant. Two studies sponsored by the U.S. Army Environmental Center have listed the installations at which problems exist in the U.S., and the extent of the problem<sup>41, 177</sup>. The German government has also begun to characterize its explosive contaminated sites, although many of these are now residential and industrial areas<sup>291</sup>. The U.K., Canada and Australia have begun site characterization, but the problem does not appear to have been appreciated in the rest of the world. Explosives of concern as environmental pollutants have been listed as TNT, RDX and HMX<sup>177</sup>. Of these, HMX is generally found at concentrations far lower than those of RDX and TNT in contaminated soil and groundwater<sup>279, 293</sup>. RDX and TNT are found in similar concentrations on average, with HMX at concentrations at least an order of magnitude lower. Several soil studies have therefore concentrated on RDX and TNT as the major pollutants<sup>14, 267, 337</sup>. The nitrate esters are rarely found in the environment at concentrations high enough to require treatment<sup>155</sup>.

In the U.S., 115 sites at 25 installations have been identified where explosives contamination exists, and the amount of soil affected has been estimated at 669,000 cubic yards (511,517 cubic metres), equivalent to approx. 45,000 tonnes<sup>41</sup>. The amount of explosive contamination present in the soil is also enormous. At the Nebraska Ordnance Plant (NOP), concentrations of up to 5.2 g TNT per kg soil and 27 g RDX per kg soil have been found. These data show that contamination massively exceeds the clean up levels recommended by the U.S. Environmental Protection Agency of 17.2 mg TNT and 5.2 mg RDX per kg soil<sup>151</sup>.

The contamination problem worsens with the effect of leaching, as the water beneath the soil (groundwater) becomes polluted, which can lead to the spreading of the explosives. The degree to which the explosive remains in the soil or is mobilized and taken through to groundwater depends on its solubility and the degree to which it sorbs to the soil. TNT and RDX have low aqueous solubility (maximum 100 mg/l and 38 mg/l respectively at 20 °C<sup>195</sup>) meaning that groundwater is often saturated with the explosives. TNT sorbs to soil quite strongly, but RDX binds less tightly<sup>267, 281, 337</sup> (TNT  $K_d$  (dissociation constant) = 6.4 – 12.0 l/kg, RDX  $K_d$  = 0.8 l/kg<sup>277</sup>). RDX contamination is therefore less easily contained than TNT, and RDX has been observed to move further than TNT in groundwater<sup>226, 281</sup>. RDX is now of primary concern due to its ability to migrate quickly through the soil matrix.

The degree of pollution of soil and groundwater demonstrated here is a serious environmental problem which needs to be addressed. The two major targets for remediation are RDX and TNT, both of which have been manufactured in vast quantities over the last century. They are commonly found co-contaminating munition sites and are both toxic to mammals and aquatic organisms. The toxicity of these compounds means not only that these sites cannot be used for alternate purposes until they have been cleaned up, but also that remediation is necessary to control the movement of these compounds in groundwater, with RDX being the more urgent problem in this respect. Remediation is urgently required for these contaminated sites.

## **1.5 Methods for soil decontamination**

Limited information regarding the remediation of explosive contaminated sites is available to the public. Some data from the U.S. regarding the clean up of contaminated installations can be obtained, and the two methods currently being used are presented.

### **1.5.1 Incineration**

Incineration is the most commonly used method for the clean up of explosive contaminated soil; several sites have already been remediated using this technology<sup>155</sup>. The process involves removing soil from the site to incinerate it and the contaminating explosives<sup>313</sup>. In practice, however, complete combustion rarely occurs, with the result that explosive residues require disposal or further treatment<sup>105</sup>. Even if explosives fully combust, some harmful compounds form: nitrous oxides (NO<sub>x</sub>), carbon monoxide (CO), hydrogen chloride (HCl) and possibly dioxins<sup>313</sup>. In addition to the effects that these compounds may have on health, leading to poor public acceptance, the costs of incineration are very high: each ton of soil to be remediated has been estimated to cost \$ 800 (\$ 725 per tonne soil)<sup>103</sup>.

### **1.5.2 Composting**

Composting of contaminated soil uses resident soil microbes to degrade the contaminants. Composted soil may be supplemented with organic matter (which reduces the concentration of the contaminant and provides carbon sources for the microbes), have its moisture content controlled and be aerated at intervals<sup>350</sup>. The temperature often increases during composting as a function of microbial activity, creating better conditions for the degradation of the contaminants.

RDX and TNT levels have been found to decrease substantially during composting<sup>115, 116, 154, 333, 350</sup>. Conditions vary between studies; explosives removal has been seen in nonaerated and aerated piles<sup>116</sup>, with slightly greater removal under thermophilic conditions (55 °C) than mesophilic (35 °C)<sup>333</sup>. Very few studies address the identities of metabolites; carbon dioxide is a product, identified using <sup>14</sup>C-RDX<sup>154</sup>, and the reduction of the nitro groups to give nitroso derivatives of RDX: hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) along with methanol and formaldehyde<sup>105</sup> has been observed. TNT tends to undergo transformations rather than mineralization<sup>44</sup>. These products bind to the soil and become unextractable<sup>105</sup>, meaning that not all the compounds will be removed, which may be unacceptable for complete remediation.

The benefit of composting is that the toxicity and mutagenicity of the composted soil and the leachate is much reduced when compared to the original contaminated soil<sup>115, 116, 154</sup>. However, there have been no detailed investigations into the products formed or the specific bacteria responsible for the removal of the explosives. Indigenous bacteria will vary from site to site and may break explosives down in different ways. Composting is also an expensive process as it requires the movement of soil to form piles, amendment of the soil and possibly regular aeration. However, at an estimated cost of \$ 300 per ton (\$ 272 per tonne) soil<sup>155</sup>, it is not as expensive as incineration.

The two methods currently used for remediating explosive contaminated sites, incineration and composting, appear to remove the parent compound, but many of the products are uncharacterized and may be toxic. Both methods require moving the material, either for mixing or for *ex situ* treatment. This greatly increases the costs of the remediation, and both are expensive methods. Given the large amount of land still requiring remediation, and the costs of the existing technologies, new technologies are required for the low cost remediation of explosives.

Studies on composting do suggest, however, that soil microbial populations can break down explosive compounds, and possibly detoxify them. This provides the idea of using soil micro-organisms as a resource for remediation, but only after better characterization.

## 1.6 Biodegradation of explosives

Micro-organisms are able to degrade a wide range of compounds, including xenobiotics which have been introduced into the environment relatively recently<sup>283</sup>. Modern explosives have been used extensively over the last century, becoming known as serious environmental pollutants in the past few decades. Most explosive compounds contain chemical groups, such as the nitramine group, which were not previously found in nature. It could be proposed that the novelty of these compounds would mean that the environmental microflora would not possess enzymes which could degrade or transform them. However, composting has demonstrated that micro-organisms are able to remove explosives from soil. These micro-organisms therefore hold great potential for the effective remediation of explosives, and potentially the breakdown of these compounds to harmless products.

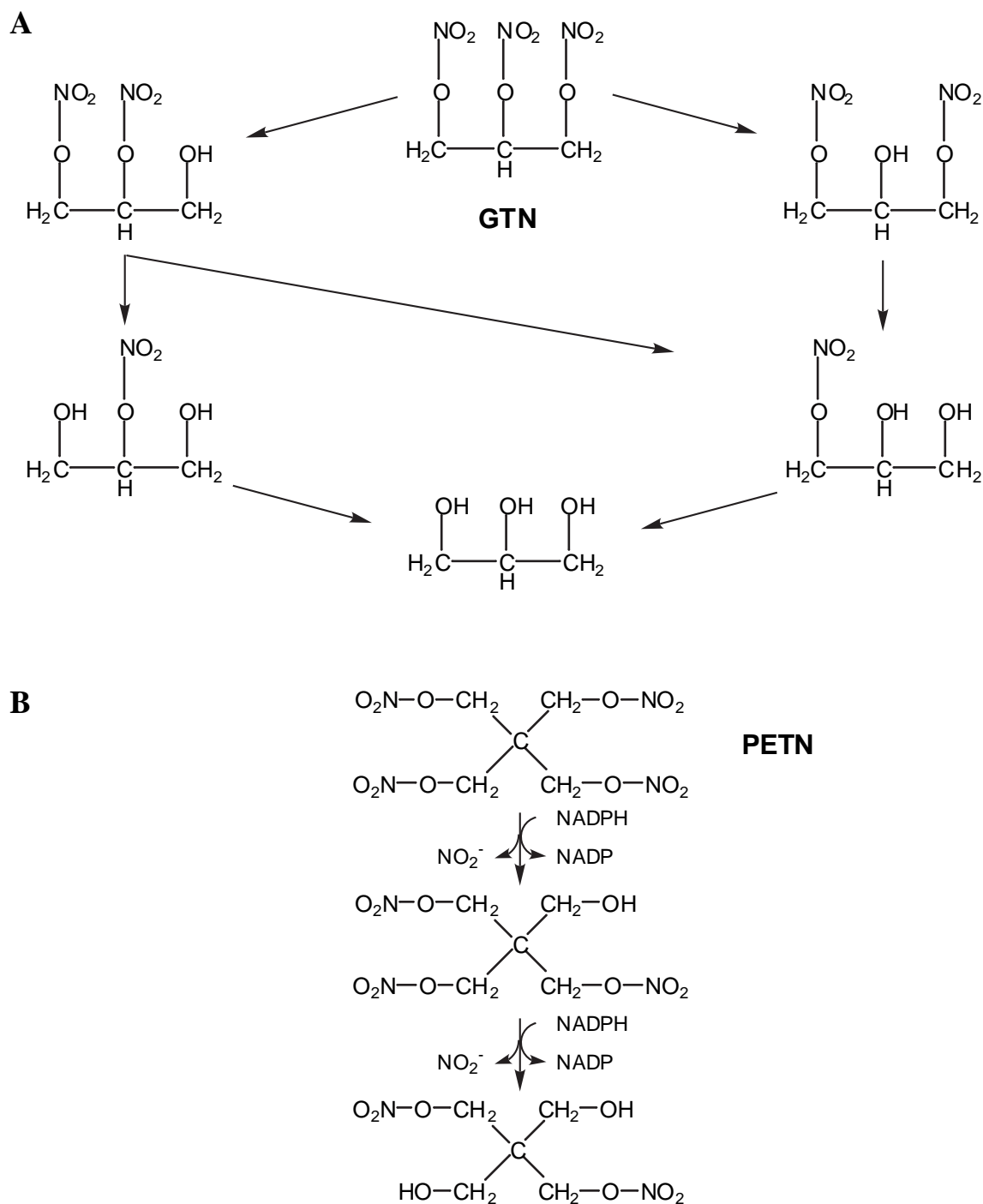
The use of specific bacterial strains to remediate explosives could be a more effective method than either incineration or composting. Using characterized bacteria, under defined conditions, the products of degradation can be determined and specific isolates which break the compound down into non-toxic products can be selected for use. Either the organisms themselves, or the relevant enzymes, can be used in bioremediation. A description of the main biotransformation and biodegradation routes of the three classes of explosives is presented, and reviews on this subject can be found<sup>88, 112, 136, 162, 252, 290, 322</sup>.

### 1.6.1 Nitrate ester explosives

Biodegradation of nitrate esters occurs through successive denitrations, each nitro group reacting more slowly than the previous one<sup>23, 55, 328</sup>. The degradation of GTN can eventually lead to the production of glycerol in some cases<sup>55, 203</sup>, which can then be used as a carbon source<sup>2, 55, 284</sup> (Figure 1.2). This type of activity has been observed under both aerobic and anaerobic conditions, using mixed cultures or pure strains including *Pseudomonas* sp., *Agrobacterium radiobacter* and *Bacillus* sp.<sup>2, 23, 55, 203, 328-330</sup>. Enzymes which catalyse this reaction have been isolated from nitrate ester degrading organisms, and all have been found to be similar reductases<sup>29, 99, 287</sup>.

There is very little information on microbial activity towards PETN. *Enterobacter cloacae* strain PB2 was isolated from contaminated soil through its ability to use PETN as a sole source of nitrogen; this strain can also obtain nitrogen from GTN<sup>28</sup>. The enzyme responsible for this activity is PETN reductase, a homologue of old yellow enzyme<sup>99</sup>. It can catalyse the

reduction of two of the four nitro groups of PETN to alcohol groups, and performs a similar reaction on GTN (Figure 1.2).



**Figure 1.2: Microbial transformation of nitrate esters.** Nitrate ester reductase enzymes catalyse the removal of nitrite ( $\text{NO}_2^-$ ) from nitrate esters. A. GTN can be transformed to glycerol in three steps<sup>203</sup>. Each step requires the oxidation of NAD(P)H and releases nitrite. B. PETN has two of its nitro groups reduced<sup>28</sup>.

### 1.6.2 Nitroaromatic explosives

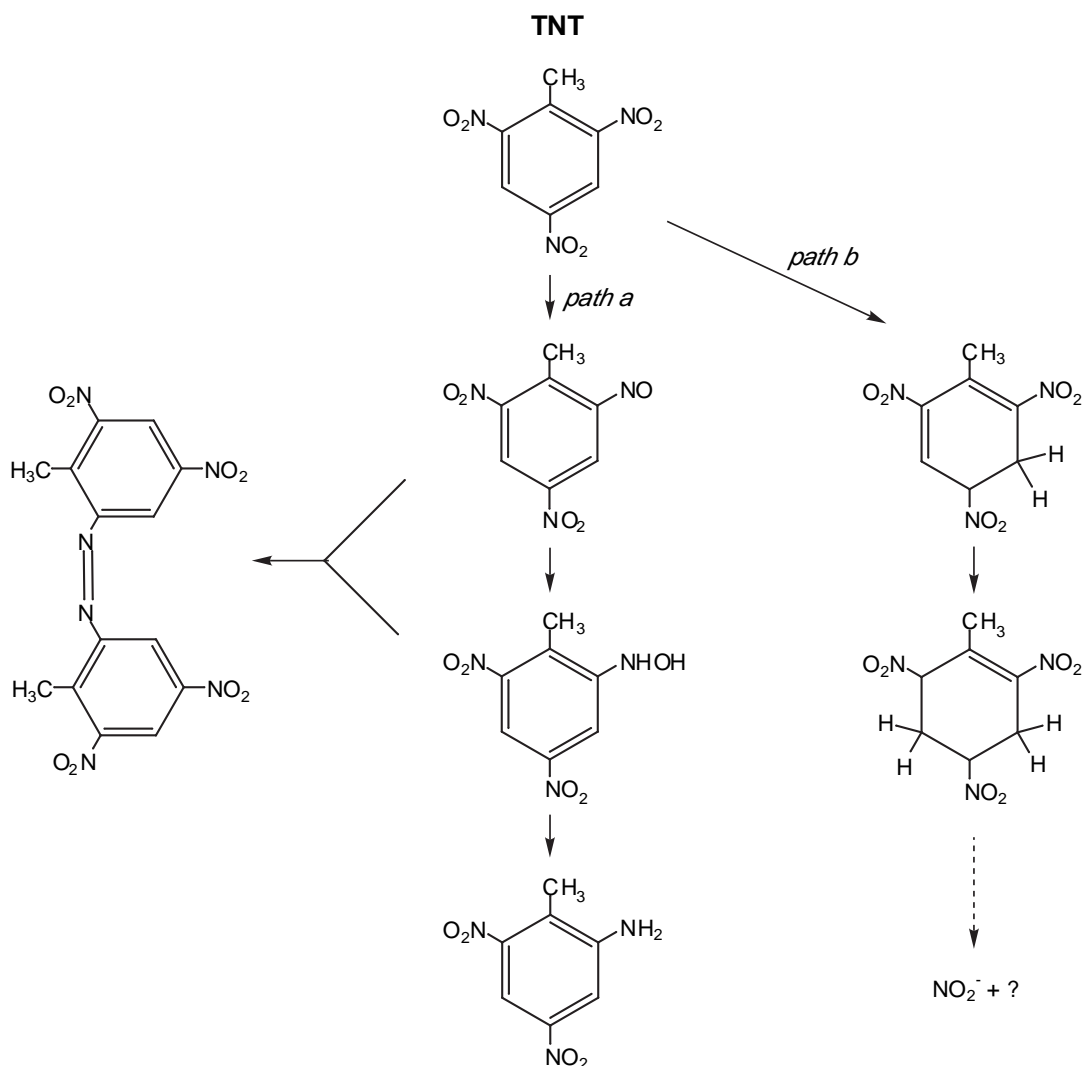
Due to the stability of the aromatic ring, and the electron-withdrawing properties of the nitro groups in nitroaromatics, microbial action on compounds such as TNT and picric acid generally proceeds via reduction of the nitro groups, reducing them successively to nitroso, hydroxylamino and amino groups<sup>290</sup> (Figure 1.3). The amino derivatives in particular are very stable and adsorb very strongly to soil<sup>244</sup>, factors which strongly hinder further breakdown in the environment. In addition, the hydroxylamino and amino derivatives can dimerize to form azo and azoxy dimers, which are resistant to further metabolism<sup>84, 192</sup>. These reduction reactions are catalysed by nitroreductases, examples of which have been cloned from *Enterobacter cloacae* and *Escherichia coli*<sup>45, 347</sup>.

There are reports of hydride attack on the ring systems of TNT and picric acid by species of *Rhodococcus*, *Nocardioides* and *Mycobacterium*<sup>17, 190, 320</sup>, which can lead to the production of nitrite and hypothesized mineralization, but no other products have been identified<sup>100</sup> (Figure 1.3). PETN reductase from *Enterobacter cloacae* (§1.6.1) is able to catalyse this reaction, as well as the reduction of the nitro groups of TNT described above<sup>100</sup>. A *Pseudomonas* sp. was reported to have been engineered to be able to utilize TNT as a source of both carbon and nitrogen, indicative of ring cleavage and mineralization. However, several products of the nitroreductase-type pathway were also seen, including azoxy dimers, indicating that more than one reaction was occurring, with undesirable dead end products<sup>84</sup>.

The fungus *Phanaerochaete chrysosporium* can liberate carbon dioxide from TNT during its metabolism, indicating ring cleavage and mineralization<sup>89</sup>. The ligninolytic fungal enzymes are thought to be responsible, but a pathway has not been elucidated<sup>136</sup>.

### 1.6.3 Nitramine explosives

RDX is much more amenable to biodegradation than its co-contaminating explosive, TNT. With no aromaticity it appears to be able to undergo several types of reaction. Under anaerobic conditions, reduction of the nitro groups forms nitroso intermediates which subsequently break down further<sup>199</sup>. Under aerobic conditions, nitrite has been seen to accumulate<sup>59, 95</sup>. These studies are discussed in more detail later (§1.7.2). Unlike the nitrate ester and nitroaromatic explosives, there has been no identification of enzymes responsible for any of the reactions that RDX undergoes. This interesting and fundamental area remains to be investigated.



**Figure 1.3: Microbial transformation of TNT.** *Path a.* TNT can undergo reduction of the nitro groups through nitroso, hydroxylamino and amino derivatives<sup>290</sup>. Each of the nitro groups may undergo reduction, although triaminotoluene only forms under anaerobic conditions. Azo and azoxy dimers can form from nitroso and hydroxylamino dimerization<sup>192</sup>. *Path b.* TNT can also undergo hydride addition to form hydride- and dihydride-Meisenheimer complexes. Nitrite is produced from this reaction, but other products are unidentified<sup>100</sup>.

There are very few reports of microbial activity against HMX, and no literature concerning CL20 biodegradation. HMX removal during composting has been observed, with it generally disappearing more slowly than RDX<sup>115, 116, 121, 333</sup>. Products of the anaerobic degradation of HMX include nitroso derivatives, indicating a mechanism similar to the anaerobic degradation of RDX, again requiring longer to achieve a similar percentage removal<sup>33, 139</sup>. Aerobic removal of HMX from solution using a microbial consortium from horse manure also produced nitroso-HMX compounds<sup>130</sup>. No pure strain responsible for the degradation of HMX has been isolated.

The reduced microbial activities against these compounds may be due to their solubilities, which are lower than that of RDX, the greater steric effects within transition states, and the higher bond dissociation energy of the N-NO<sub>2</sub> bond which results from this<sup>138</sup>, or perhaps greater resistance to transport across bacterial membranes. If the mechanism by which the nitramines are degraded are different, it may be that micro-organisms have not been sufficiently exposed to either HMX or CL20 to date, and that the numbers of bacteria able to degrade the explosives will increase over the coming decades.

Enzymes able to break down or transform two classes of explosives, nitrate esters and nitroaromatics, have been characterized. Transgenic plants containing these enzymes have been created and found to be able to degrade these explosive compounds or remove them from medium (§1.8). This represents a major advance in explosive remediation. No such system exists for the nitramine explosives, and work in this area is urgently required. The degradation of nitramines, especially that of the widely used and common pollutant RDX, clearly requires further investigation.

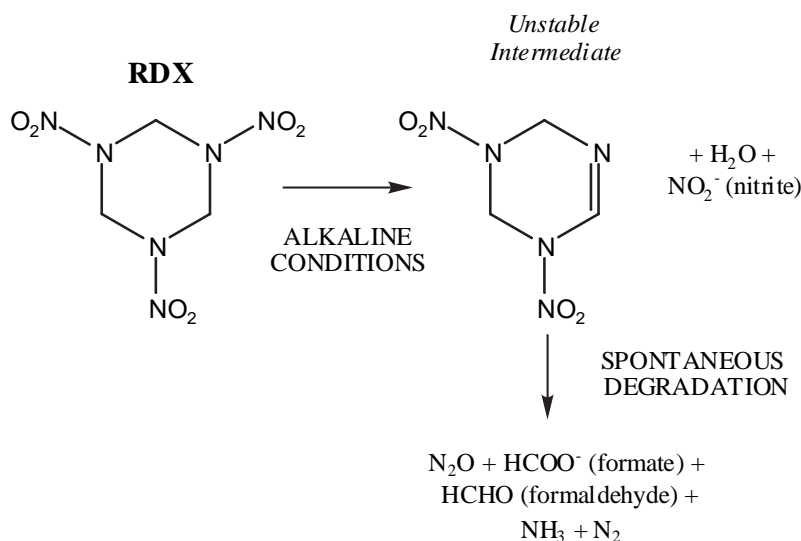
## 1.7 RDX degradation

The products formed from the various methods of breakdown of a compound can give useful information on the weakest bonds, the groups most likely to be attacked and the most likely mechanisms underlying its degradation. Despite the amount of work that has been done on RDX breakdown, surprisingly few pathways to explain the degradation have been proposed. A survey of the postulated mechanisms of RDX breakdown is given by Hawari<sup>138</sup> who proposes that when the first bond in the molecule is broken, the molecule is destabilized to such an extent that it undergoes spontaneous decomposition. The N-N bond is described as the most likely target for degradation, as it is relatively weak compared to the rest of the molecule, with a bond dissociation energy of 48 kcal/mol compared to the C-N and C-H bond energies of 85 and 94 kcal/mol respectively<sup>138</sup>.

## 1.7.1 Physico-chemical breakdown of RDX

### 1.7.1.1 Alkaline hydrolysis

RDX can be broken down by alkaline hydrolysis. The mechanism by which this reaction is proposed to occur involves the abstraction of a proton by the alkali, with the simultaneous loss of nitrite ( $\text{NO}_2^-$ ), to form an unstable intermediate<sup>148</sup>. This then degrades to form nitrous oxide ( $\text{N}_2\text{O}$ ), ammonia ( $\text{NH}_3$ ), nitrogen gas ( $\text{N}_2$ ), formaldehyde ( $\text{HCHO}$ ) and formate ( $\text{HCOO}^-/\text{HCOOH}$ ) (Figure 1.4), with the product ratios dependent on the strength of the alkali.



**Figure 1.4: Proposed mechanism for the alkaline hydrolysis of RDX.** Proton abstraction followed by nitrite loss creates an unstable intermediate which hydrolyses spontaneously to the products shown. The final degradation products were all identified<sup>148</sup>. Formate appears to be produced both from direct ring cleavage and from formaldehyde at the elevated pH<sup>142</sup>.

### 1.7.1.2 Thermal decomposition

The thermal decomposition of RDX occurring at temperatures above its melting point results in a host of products: nitrogen gas, nitrous oxide, nitric oxide ( $\text{NO}$ ), carbon dioxide ( $\text{CO}_2$ ), formaldehyde, formate, ammonia, nitrate ( $\text{NO}_3^-$ ) and nitrite<sup>62</sup>. Hydroxy-s-triazine and MNX, the mononitroso derivative of RDX, have also been identified during thermal decomposition<sup>18</sup>, as have water, nitrogen dioxide ( $\text{NO}_2$ ), hydrogen gas ( $\text{H}_2$ ) and hydrogen cyanide ( $\text{HCN}$ ), which is often observed as an intermediate rather than a final product<sup>18, 90, 202, 280</sup>. A survey of the literature shows that the specific products formed and their ratios depend on the conditions of heating and pressure<sup>3, 201</sup>, and whether the reaction occurs in the gas or condensed phase<sup>332</sup>. Consequently, no definite reaction mechanism has been determined. Two mechanisms by which the first bond in RDX is broken have been proposed: either N-N bond scission as the first step<sup>62, 63, 332</sup>, or



RDX/kg was remediated using 10 % Fe<sup>0</sup> to within EPA recommended limits (§1.4)<sup>151</sup>. The only product detected was ammonium, with no nitrate or nitrite present. However, both these latter nitrogen containing compounds can be reduced to ammonium by Fe<sup>0</sup> and nitrite is seen when RDX is rapidly passed through a column containing Fe<sup>0</sup><sup>282</sup>, indicating that nitrite is an initial product. A more detailed study of RDX removal by Fe<sup>0</sup> under aqueous conditions showed low levels of the nitroso derivatives: MNX, DNX and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) being produced<sup>282</sup>. These intermediates were lost after 96 h and ammonium was seen being produced throughout the course of the experiment. This may indicate that two mechanisms are at work, one allowing the release of ammonium and another reducing the nitro groups.

Fenton oxidation uses Fe<sup>2+</sup> with H<sub>2</sub>O<sub>2</sub> to produce hydroxyl radicals which carry out the oxidation of target compounds. Products of the treatment of RDX with Fenton's reagent include formate, nitrate, ammonium, the tentatively identified methylenedinitramine (C(NHNO<sub>2</sub>)<sub>2</sub>), nitrogen and formaldehyde<sup>24,353</sup>. No nitrite was seen, but it is suggested to be the product of the first reaction step, being oxidized to nitrate immediately<sup>353</sup>. The formate is proposed to be a result of the action of Fenton's reagent on formaldehyde, rather than an independent ring cleavage product<sup>353</sup>.

## 1.7.2 Biodegradation of RDX

### 1.7.2.1 Anaerobic biodegradation of RDX

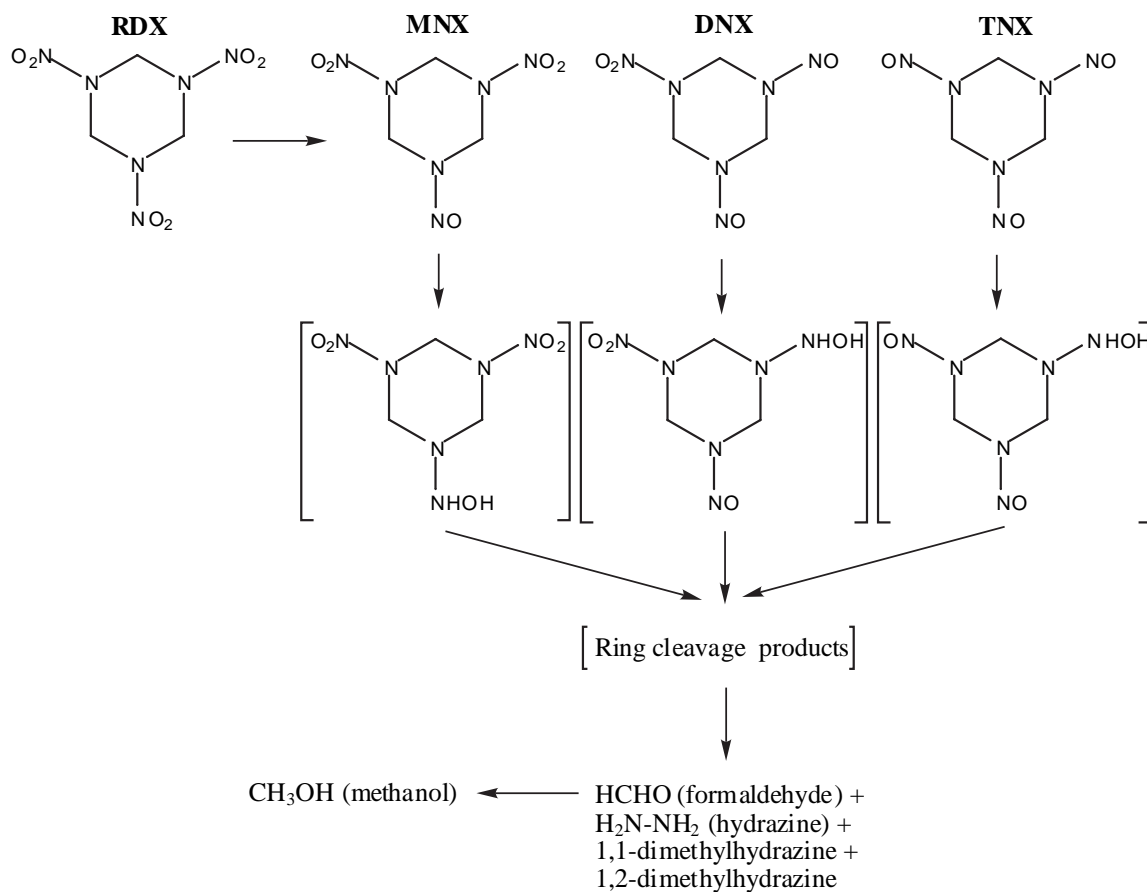
Biodegradation of RDX was initially studied under anaerobic conditions, and it was thought for a long time that RDX removal could only occur anaerobically<sup>199</sup>. RDX removal from culture was first observed in 1973 using a system containing purple photosynthetic bacteria<sup>289</sup>; the anaerobic photosynthetic activity was thought to be responsible for a possible reduction of the compound. Since then, anaerobic RDX degradation has been observed using microbial consortia from contaminated material and sewage sludge<sup>103, 137, 199, 274, 275, 308</sup>, and has also been performed under nitrate reducing<sup>98</sup> and sulfate reducing<sup>30, 31</sup> conditions. These cultures generally take between one week and two months to degrade RDX, when supplied at concentrations ranging from 0.015 mM to 0.17 mM. The most rapid degradation of RDX using anaerobic sludge reported 90 % removal of 0.27 mM RDX within 2 days<sup>137</sup>.

In addition to this use of mixed cultures, the vast majority being uncharacterized in terms of the microbes present, some investigations have concentrated on anaerobic pure cultures. *Clostridium bifermentans* was the first pure strain capable of the anaerobic degradation of RDX

to be isolated<sup>243</sup>. It was purified from an anaerobic consortium and found to be able to remove 0.23 mM RDX to 25 % of its original concentration within 24 h. *Morganella morganii*, which fully removed 0.33 mM RDX within 27 days, was chosen as the most efficient isolate of three from the family *Enterobacteriaceae*, which were found to transform RDX under oxygen-depleted conditions<sup>167</sup>. Several strains which could biotransform RDX anaerobically were isolated from horse manure, the most effective being *Serratia marcescens* which removed 0.23 mM RDX over 10 days<sup>345</sup>. During this work on RDX degrading anaerobes, several intermediates and products have been identified, from which pathways of RDX degradation have been put forward.

Using sewage sludge as a source of microbes, 0.23 mM RDX was removed from anaerobically incubated nutrient broth over a period of 7 days<sup>199</sup>. Analysis of the compounds formed led to the proposal of a pathway involving the production of nitroso intermediates from RDX through sequential reductions of the nitro groups (Figure 1.6). MNX is produced first, followed by DNX and TNX, all of which were isolated by high performance liquid chromatography (HPLC) and identified using gas chromatography-mass spectrometry (GC-MS). Further reduction is hypothesized to create hydroxylamino-substituted derivatives, with subsequent ring cleavage resulting in the observed products: formaldehyde, methanol (CH<sub>3</sub>OH), hydrazine (H<sub>2</sub>N-NH<sub>2</sub>), 1,1-dimethylhydrazine and 1,2-dimethylhydrazine. The hydrazines are known mutagens, but were present only in very low quantities and have not been identified since in anaerobic systems<sup>137</sup>. There have also been queries as to whether the hydrazines were correctly identified, and subsequent studies have found them to be unstable<sup>138</sup>. The nitroso intermediates have been identified in several other studies during anaerobic RDX degradation<sup>98, 137, 167, 345</sup>. The enzyme responsible may be similar to a type I nitroreductase from *Enterobacter cloacae*, which has been found to oxidize NADPH in the presence of RDX, albeit at a very low rate (68.7 nmol/min/mg protein), indicating that the RDX is being reduced<sup>168</sup>.

Further elucidation of intermediates produced from anaerobic RDX degradation with sewage sludge has led to the proposal of a second mechanism of RDX breakdown, which may occur in parallel with the reductive pathway<sup>137</sup>. This involves cleavage of the ring directly to methylenedinitramine and bis(hydroxymethyl)nitramine, which break down to formaldehyde and nitramine (NO<sub>2</sub>-NH<sub>2</sub>). Bacteria present in the sludge are then thought to convert these to the end products carbon dioxide, methane (CH<sub>4</sub>) and nitrous oxide (Figure 1.7)



**Figure 1.6: Putative pathway for the anaerobic biodegradation of RDX via nitroso intermediates**<sup>199</sup>. Compounds identified include the three nitroso derivatives, formaldehyde, methanol, hydrazine and the two dimethylhydrazines. The hydroxylamino derivatives and ring cleavage products are hypothetical intermediates and are shown in brackets.

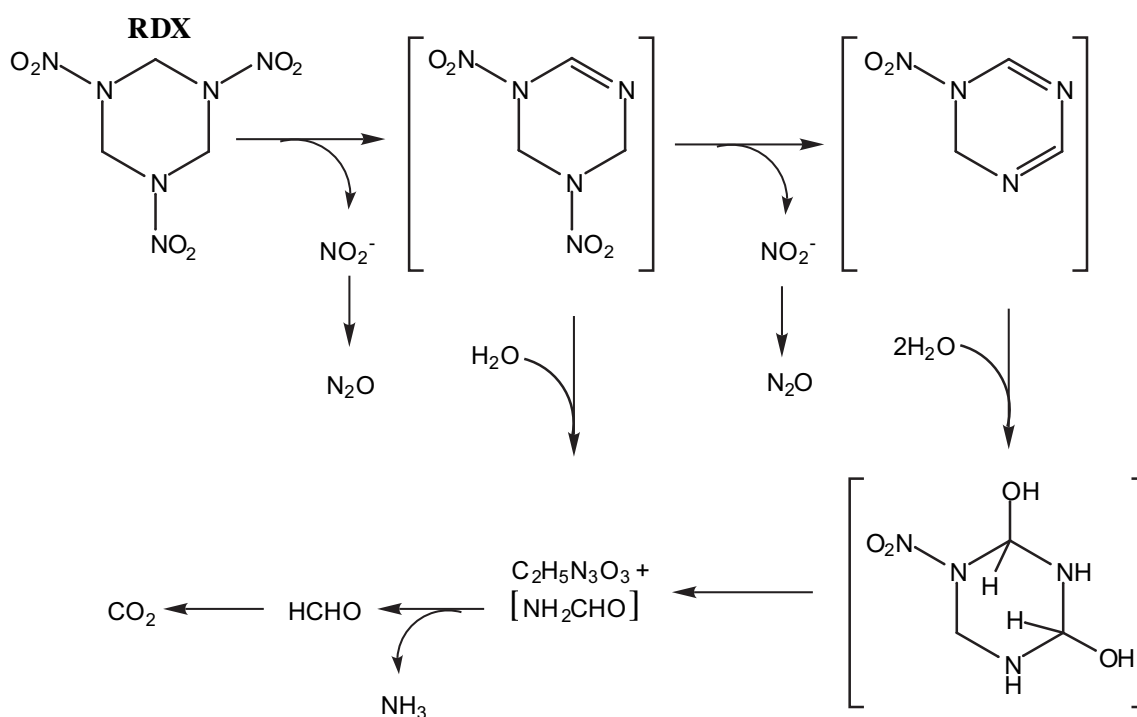


RDX could be fully degraded over 50 days, with metabolites including nitrous oxide and traces of MNX and methanol<sup>276</sup>.

### 1.7.2.3 Aerobic biodegradation of RDX

The first reported aerobic degradation of RDX was published in 1983 and identified three pure strains of *Corynebacterium* capable of growing on RDX as a sole nitrogen source<sup>338</sup>. The fastest of these strains removed 0.18 mM RDX from culture over 32 h. Since then, a consortium of bacteria from contaminated soil was reported to degrade 38 % of 100 mM RDX over 5 days<sup>303</sup>, and a pure aerobic bacterial strain, also isolated from contaminated soil, was found to be able to remove 0.23 mM RDX from culture over 40 h<sup>157</sup>. Using this pure strain, the accumulation of an unidentified metabolite was detected using HPLC<sup>157</sup>, and it has since been tentatively identified as NO<sub>2</sub>-NH-NH-CHO, which would indicate that ring cleavage had occurred<sup>138</sup>. *Stenotrophomonas maltophilia* strain PB1 removed 0.27 mM RDX over 7 days<sup>27</sup>, during which two metabolites were identified: C<sub>3</sub>H<sub>9</sub>N<sub>3</sub>O<sub>5</sub> and methylenedinitramine<sup>138</sup>. The activity was reported to be inducible and to require reducing power provided by sugars<sup>27</sup>. All these strains grew on RDX when it was supplied as a sole source of nitrogen, and none of them were able to use RDX as a source of carbon.

The most thoroughly described aerobic RDX degrading bacterium is *Rhodococcus* sp., strain DN22<sup>59</sup>. This strain was isolated from explosive contaminated soil, and uses RDX as sole nitrogen source, degrading 0.16 mM within 20 h. The activity is repressed by growth on ammonium as a nitrogen source and is thought to be plasmid-borne<sup>60</sup>. Characterization of the metabolites produced from RDX by this strain has been performed recently, leading to the proposal of the first pathway for aerobic RDX biodegradation<sup>95</sup>. No nitroso intermediates were identified, strongly suggesting that aerobic RDX biodegradation follows a different path to anaerobic biodegradation. The mechanism proposed (Figure 1.8) involves denitration as a first step to form a hypothetical intermediate identical to that postulated in the alkaline hydrolysis pathway. After a series of hypothetical intermediates, two compounds are formed; one, the hypothetical NH<sub>2</sub>CHO, which breaks down further to ammonium and formaldehyde resulting in the liberation of carbon dioxide, and the second, C<sub>2</sub>H<sub>5</sub>N<sub>3</sub>O<sub>3</sub>, which accumulates as a dead end product.



**Figure 1.8: Proposed mechanism of RDX biodegradation by *Rhodococcus* sp. strain DN22<sup>95</sup>.** Denitration as an enzymatic first step creates unstable intermediates which undergo ring cleavage.  $\text{NO}_2^-$ ,  $\text{N}_2\text{O}$ ,  $\text{NH}_3$ ,  $\text{HCHO}$  and  $\text{CO}_2$  were identified as products of RDX degradation, as well as the dead end product  $\text{C}_2\text{H}_5\text{N}_3\text{O}_3$ . Hypothetical components of the pathway are shown in brackets.

It is apparent that the biodegradation of RDX generally occurs more rapidly using aerobic bacteria than anaerobic micro-organisms. In addition, no toxic compounds such as the nitroso derivatives or proposed hydrazines found during anaerobic RDX degradation have been identified during aerobic degradation, indicating that this may prove a safer method for remediating RDX. As yet, no determinant underlying the ability to degrade RDX has been identified from either aerobic or anaerobic sources, at either the genetic or biochemical level. In order to realize the potential of microbial based bioremediation, further investigation into aerobic RDX degrading organisms, and the genetic basis for their ability, is necessary.

### 1.7.3 Products of RDX breakdown

A limited set of final breakdown products appear to be produced from RDX degradation, regardless of the mechanism responsible. These are: nitrite, nitrate, ammonium (or ammonia), nitrogen, nitrous oxide, nitric oxide, carbon dioxide, carbon monoxide, formate, formaldehyde, water, hydrogen and hydrogen cyanide. The first action on the RDX molecule includes the

following: proton abstraction liberating nitrite, N-N bond scission, concerted breakdown to monomer, reduction of nitro groups to nitroso and C-N bond cleavage. Some of the intermediates from some of the pathways have been determined, and the end products identified indicate that the molecule is eventually mineralized after the breakage of any bond. Competing chemical or biological reactions appear to be responsible for the diversity of products seen.

## 1.8 Applications of explosive degrading enzymes

Once the enzyme responsible for a particular reaction has been identified and purified, it is possible to characterize the mechanism by which the enzyme performs that reaction, without the complications of side reactions that occur using whole cells or cell extract. The products of the reaction can be determined which, for the purposes of bioremediation, should be non toxic and preferably indicate the mineralization of the compound. Once characterized, possible further uses for an enzyme can be investigated.

There is considerable interest in phytoremediation, using plants to decontaminate soil and groundwater. In particular, plants engineered with bacterial enzymes have generated a great deal of interest. Bacteria have the metabolic capabilities to break down xenobiotic compounds, such as explosives, where plants generate large amounts of biomass, penetrating deep into soil, are self-sustaining and have the potential to encourage public acceptance in the area of bioremediation.

PETN reductase from *Enterobacter cloacae* (§1.6.1) has been engineered into tobacco plants. These transgenic plants have enhanced tolerance to both GTN and TNT, compared to wildtype plants which exhibit a range of toxic effects under the same conditions. The transgenic plants are also able to denitrate GTN much more efficiently than the wildtype<sup>101</sup>. More recently, tobacco plants have been engineered with another bacterial enzyme, nitroreductase<sup>45</sup>. These plants have enhanced resistance to TNT toxicity and enhanced uptake of the explosive, effectively remediating the medium in which they are grown<sup>127</sup>.

## **1.9 Aims of this study**

The aim of this project is to isolate aerobic bacterial strains capable of degrading RDX. Bacteria from explosive contaminated areas in particular may have developed the ability to break down RDX in order to use it as a nutrient source. Comparisons of these bacteria may identify a strain suitable for study at the genetic level. The isolation of the gene(s) responsible for RDX degrading activity will be attempted.

Isolation and characterization of a gene encoding a protein which is able to degrade RDX would represent a new stage in explosive bioremediation. As RDX is such a major contaminant of land and groundwater, this technology is greatly needed as a step towards the biodegradation of this pollution.

## Chapter 2. General materials and methods

### 2.1 Reagents

The reagents used in this study were purchased from Anachem (Luton, Bedfordshire, U.K.), Fisher (Loughborough, Leicestershire, U.K.), Life Technologies (Paisley, U.K.) or Sigma (Dorset, U.K.) unless otherwise stated. All reagents were of analytical grade or above. DNA modifying enzymes and restriction endonucleases were purchased from New England Biolabs (Hitchin, Hertfordshire, U.K.), Roche (Lewes, East Sussex, U.K.) or Stratagene (Amsterdam, The Netherlands) unless specified otherwise. RDX, TNT, HMX and CL20 (> 95 % purity) were kindly provided by the Defence Science and Technology Laboratory (Dstl), Fort Halstead, U.K. Oligonucleotide primers were synthesized by Sigma-Genosys Ltd. (Cambridge, U.K.). All aqueous buffers and solutions were prepared using Elga (High Wycombe, Buckinghamshire, U.K.) purelab maxima ultra high pressure purified water (dH<sub>2</sub>O).

### 2.2 Organisms, plasmids and growth conditions

#### 2.2.1 Bacterial strains

Bacterial isolates were obtained through selective enrichments on minimal medium with RDX as a sole nitrogen source from explosive contaminated soil, supplied by Dstl (§3.2.1). Strain 11Y was provided by Dr. Amrik Basran (Institute of Biotechnology). *Rhodococcus rhodochrous* CW25, described in Quan and Dabbs<sup>238</sup>, was kindly provided by Professor Eric R. Dabbs (University of Witwatersrand, South Africa) for use as a rhodococcal cloning host. *Escherichia coli* One Shot<sup>®</sup> TOP10 strain, chemically competent (Invitrogen, Paisley, U.K.), was used as a cloning host. *E. coli* expression strains used were: BL21(DE3) (Stratagene), B834(DE3) (Novagen, Madison, Wisconsin, U.S.A.) and Rosetta(DE3) (Novagen).

#### 2.2.2 Plasmids

The *Rhodococcus* - *E. coli* shuttle vector pDA71 was provided as a kind gift by Professor Eric R. Dabbs and was used in the construction of rhodococcal genomic libraries. PCR products containing A-overhangs were routinely cloned into pCR-2.1 TOPO<sup>®</sup> vector according to the manufacturer's protocol (Invitrogen, Paisley, U.K.) before sequencing. The vectors pGEM<sup>®</sup>-5Zf+

and pGEM<sup>®</sup>-7Zf+ (Promega, Southampton, U.K.) were used in cloning and sequencing. Vectors pET-11a and pET-16b (Novagen) were used for expression.

### 2.2.3 Media

Isolates were grown on defined liquid minimal medium which consisted of 40 mM potassium phosphate buffer (pH 7.2) containing 10 mM glycerol, 5 mM glucose, 5 mM succinate, trace elements and RDX or NH<sub>4</sub>Cl as a sole nitrogen source at concentrations mentioned in relevant sections. Stock 80 mM potassium phosphate buffer contained 3.1 g KH<sub>2</sub>PO<sub>4</sub> and 9.9 g K<sub>2</sub>HPO<sub>4</sub> per litre dH<sub>2</sub>O. All components were sterilized individually for 20 min at 15 psi and 121 °C in a SAL autoclave (SAL, Bradford, U.K.) and added aseptically. Trace elements<sup>250</sup> were added aseptically and RDX stock (1 M in N,N-dimethylformamide (DMF)) was diluted appropriately. The medium was supplemented with 40 µg/ml chloramphenicol when required for specific experiments. Cultures were grown at 30 °C using a rotary shaker at 110 rpm.

Solid media (RDX zone of clearance plates) were prepared in order to visualize utilization of RDX. Electrophoresis grade agarose (Life Technologies, Paisley, U.K.) was added to the medium at 2 % w/v and RDX was added at a concentration of 5 mM. This was poured over a layer of 0.6 % w/v agarose in 40 mM potassium phosphate buffer, which was used in order to provide support and improve visualization.

Strains were also grown in Luria Bertani broth (LB)<sup>257</sup> at 37 °C using a rotary shaker at 180 rpm. The medium was supplemented with 40 µg/ml chloramphenicol, 100 µg/ml carbenicillin, 200 µg/ml isopropyl-β-D-thiogalactopyranoside (IPTG) and 25 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as required for specific experiments. Solid media (Luria Bertani agar, LA) were derived from LB by the addition of 15 g/l Bacto-agar (Difco, Oxford, U.K.).

Bacterial isolates and recombinant strains were maintained by regular subculture onto minimal medium or LA plates and stored at 4 °C. Stocks of the organisms were also stored in 15-30 % v/v glycerol at –80 °C.

## 2.3 General cloning techniques

### 2.3.1 Preparation of genomic and plasmid DNA

Total DNA was prepared from rhodococcal strains by a method adapted from Ausubel et al., 1994<sup>9</sup>. Cells were resuspended in Tris-EDTA (TE) buffer containing freshly added lysozyme (from

chicken albumin) at a concentration of 10 mg/ml. This was incubated for 2 h at 37 °C to promote cell lysis prior to routine DNA extraction. The DNA was stored at –20 °C in dH<sub>2</sub>O.

Plasmid DNA was routinely prepared from *E. coli* by boiling lysis or alkaline lysis <sup>260</sup>. Plasmid DNA of high purity for sequencing applications was prepared using the QIAprep<sup>®</sup> spin Miniprep kit (Qiagen, Crawley, West Sussex, U.K.).

The DNA concentration was determined by measuring the absorbance at 260 nm, taking 1 absorbance unit to equal 50 µg/ml of double stranded DNA <sup>10</sup>. The purity of the DNA preparation was determined by calculating the ratio of the absorbance at 280 nm to 260 nm, a ratio of 1.8 or above indicating a pure sample of DNA <sup>10</sup>.

### **2.3.2 Gel electrophoresis of DNA**

DNA fragments were separated by agarose gel electrophoresis using 1 % w/v agarose in Tris-acetate-EDTA (TAE) buffer <sup>258</sup>. The loading dye consisted of 0.15 % w/v bromophenol blue, 0.5 % w/v sodium dodecyl sulphate (SDS), 0.15 M ethylenediaminetetraacetic acid (EDTA) and 50 % v/v glycerol, and was added to the sample to a final dilution of 20 % v/v. DNA was visualized by adding 10 µg/ml ethidium bromide to the gel and viewing under ultraviolet. Fragment size was determined by comparison with a 1 kb plus ladder (Gibco BRL, Paisley, U.K.) that had been run alongside the lanes of interest.

### **2.3.3 Restriction endonuclease digestion of DNA**

Restriction endonuclease digestion of DNA was routinely performed over a period of 3 h to overnight at the optimum temperature (usually 37 °C) in a volume of 10 – 20 µl, using 1 unit of enzyme per µg DNA. Double digests were performed using buffers compatible with both restriction enzymes according to the manufacturer's protocol. Complete digestion was verified using agarose gel electrophoresis.

### **2.3.4 Purification of DNA fragments from agarose**

Fragments were excised from the agarose gel, and purified using the QIAquick<sup>®</sup> gel extraction kit (Qiagen) according to the manufacturer's instructions.

### 2.3.5 DNA ligation

DNA ligations were performed using 50-100 ng vector DNA and a ratio of 1:3 vector concentration to insert ends, with the DNA heated to 45 °C for 5 min before snap chilling on ice. The ligations were performed either using T4 DNA ligase at 16-18 °C overnight or Quick ligation™ kit at room temperature for 5 min, according to the manufacturer's protocol (both New England Biolabs).

### 2.3.6 Polymerase chain reaction amplification

Polymerase chain reaction (PCR) amplification for screening purposes was performed using taq DNA polymerase (Roche), and for cloning purposes using *PfuTurbo*® DNA polymerase (Stratagene) according to the supplied protocols. The synthesized primers were dissolved in dH<sub>2</sub>O and stored at –20 °C in a stock solution of 10 pmol/μl. PCR reactions (50 μl) contained 1-100 ng DNA template, 50 pmoles of each PCR primer and dNTPs at a final concentration of 0.25 mM each. The thermocycler used was the PCR express from Hybaid (Hybaid, Ashford, U.K.). PCR conditions consisted of 95 °C for 2 min to denature the template, followed by 30 cycles of 95 °C for 30 s (denaturation of template), varied temperatures for 30 s (primer annealing) and 72 °C for 1-4 min (extension). A final cycle of 72 °C for 10 min was used for extension. Precise conditions of annealing temperature and extension time were determined based on the primer melting temperature ( $T_m$ ) and fragment length respectively, and are given in the relevant sections.

### 2.3.7 Transformation of *E. coli* hosts

Heat shock competent cells were made by CaCl<sub>2</sub> treatment as described in Sambrook et al.<sup>259</sup> or purchased from Invitrogen. *E. coli* hosts were routinely transformed by heat shock according to the method of Sambrook et al.<sup>259</sup> or the manufacturer's protocol. Recombinant plasmid-containing colonies were identified using blue-white selection where possible.

### 2.3.8 Nucleotide sequencing and analysis

Automated DNA sequencing was performed by the Protein and Nucleic Acid Chemistry Facility, Department of Biochemistry, University of Cambridge, or the Department of Genetics, University of Cambridge. The software package Sequencher™ 3.1.1 (Gene Codes Corporation, Inc.) was used to analyse the sequence. Sequences were compared to other sequences in the GenBank or Swissprot databases using the BLAST package at <http://www.ncbi.nlm.nih.gov/blast/><sup>5</sup> and the

Wisconsin Package Version 10.2 (Genetics Computer Group (GCG), Madison, Wisconsin, U.S.A.). Shading was supplied by Boxshade server ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)) and Clustal X was used for 16S rDNA sequence alignments<sup>305</sup>.

## 2.4 Analytical techniques

### 2.4.1 Spectrophotometry

Spectrophotometric work was performed using a Shimadzu UV-160A UV-Visible Recording Spectrophotometer (Shimadzu Corporation, Japan). All samples were blanked against reagent.

### 2.4.2 Thin layer chromatography

Thin layer chromatography (TLC) of RDX was performed using 200  $\mu\text{m}$  polyester plates pre-coated with ultraviolet absorbing silica gel (Machery-Nagel, Germany). Each sample (20  $\mu\text{l}$ ) was spotted a distance of 2 cm from the base of the plate and allowed to dry before separation with a mobile phase of chloroform: acetone 2:1 v/v<sup>341</sup>. The plate was sprayed with 1 M NaOH, placed at 80 °C for 15 min to promote the alkaline hydrolysis of RDX<sup>342</sup> and sprayed with freshly made Griess reagent<sup>264</sup>. The nitrite resulting from the alkaline hydrolysis was observed as pink spots. Griess reagent consisted of 0.8 g sulphanilamide, 0.04 g N-(1-naphthyl)ethylenediamine (NED) and 0.8 ml orthophosphoric acid, made up to 10 ml with dH<sub>2</sub>O. RDX migrates with an R<sub>f</sub> value of 0.80 using the conditions described<sup>341</sup>, and standards were used to compare migration distances.

### 2.4.3 High performance liquid chromatography

High performance liquid chromatography (HPLC) measurements were performed with a Waters system consisting of a 510 pump, a 7120 WISP 48-vial autosampler and a 2487 dual  $\lambda$  wavelength detector (Waters, Hertsford, U.K.). Samples (100-200  $\mu\text{l}$ ) were separated with 5- $\mu\text{m}$  C<sub>18</sub> reversed-phase column (250 x 4.6 mm; HPLC Technology, Welwyn Garden City, U.K.) with a guard column of the same packing material to protect the main column. A reverse-phase isocratic mobile phase consisting of HPLC-grade acetonitrile: water (50:50, v/v) was passed through a 0.45  $\mu\text{m}$  filter under vacuum and degassed before use and delivered at a flow rate of 0.7-1 ml/min. RDX elution was monitored at 205 nm. The RDX peak was identified by comparison of the retention time with an authentic standard. Standards of authentic RDX ranging in concentration from 2.5 to 20 nmoles were treated in an identical manner to assay samples and were run before each set of samples. Waters Millennium software was used to integrate the area under the peaks. Standard

curves of concentration versus peak area were constructed for each run of samples and used to convert peak area data of samples to concentrations of RDX.

#### 2.4.4 Griess assay

Analysis of nitrite was performed using the Griess assay<sup>264</sup>. Samples (200 µl) were added to 560 µl dH<sub>2</sub>O and 200 µl sulphanilamide solution (1 % sulphanilamide in 7 % HCl), to which 40 µl NED solution (0.5 % NED) was added, and the mixture inverted several times. The sample was assayed at 540 nm, blanked against reagent and the concentration of nitrite calculated from a standard curve of sodium nitrite which was performed with each batch of samples.

#### 2.4.5 Ion chromatography

Nitrite, nitrate, formate and ammonium were analysed by ion chromatography using a Dionex system comprising an AS40 autosampler and a DX-120 ion chromatograph (Dionex, Sunnyvale, California, U.S.A.). Integrations were performed using Dionex PeakNet software. Samples (25 µl) were assayed for anions with 8 mM Na<sub>2</sub>CO<sub>3</sub>/1 mM NaHCO<sub>3</sub> as the eluent using an AS14A column (250 x 4 mm, Dionex), and cations using a CS12A column (250 x 4 mm, Dionex) with 20 mM methane sulphonic acid as the eluent, both at a flow rate of 1 ml/min. Standards were run using the supplied anion and cation authentic standards (Dionex) diluted 1/5, 1/10, 1/50 and 1/100, with the anion standards supplemented with nitrite and formate where appropriate. Standard curves were constructed of calculated concentration versus peak area and used to convert peak area data of samples to concentrations of ions.

#### 2.4.6 Analysis of formaldehyde

Formaldehyde was analysed using the Hantzsch spectrophotometric assay<sup>214</sup>. The reagent consisted of 0.206 % v/v acetylacetone, 15.4 % w/v ammonium acetate and 0.284 % v/v acetic acid, which was added 1:1 to the sample, incubated for 10 min at 58 °C and absorbance was measured at 412 nm. Standards of formaldehyde at concentrations of 0, 10, 20, 50, 100 and 250 µM in the relevant assay buffer were assayed and used to construct a standard curve of concentration against absorbance at 412 nm. This was used to determine concentrations of formaldehyde in the samples from absorbance readings.

## Chapter 3. Isolation, identification and characterization of bacteria possessing RDX degrading activity

### 3.1 Background

The isolation of bacteria with specific degradative activities is commonly performed using selective enrichments, with inocula from contaminated soils. Soil environments are often nutrient limited, and bacteria can develop the ability to scavenge nutrients from polluting compounds, breaking them down in the process. Selective enrichment works by inoculating a source of bacteria into a medium with a defined composition to encourage the growth of specific types of bacteria. Usually the bacteria with the desired degradative ability can grow because they alone can utilize a component of the medium. There are many examples of bacterial isolation using this method in the literature. *Rhodococcus* sp. strain QT-1 was isolated from contaminated soil samples and found to degrade 1,3-dinitrobenzene<sup>81</sup>. Many chlorobenzene degraders were isolated from contaminated groundwater and identified as gram positive strains or *Pseudomonas* species<sup>218</sup>. *Pseudomonas* strain, 4NT, capable of the degradation of 4-nitrotoluene, was isolated from contaminated soil<sup>125</sup>. Further chlorobenzene degraders were isolated from chlorobenzene contaminated wells, whereas uncontaminated wells yielded none with this ability<sup>219</sup>. This study, in particular, illustrates the presence of bacteria possessing the phenotype of interest in areas where the compound is abundant, and the absence of this phenotype in the absence of the compound. The bacteria may possess these activities in order to detoxify the harmful compounds or to obtain nutrients from them in the form of breakdown products. The search for bacteria able to degrade RDX often uses soil contaminated with RDX as a source of micro-organisms (§1.7.2).

In cases where the isolation of the genes which allow these bacteria to degrade xenobiotic compounds are reported, many are found to be carried on plasmids or transposons. Where more than one gene is required for the degradation of a xenobiotic compound using a pathway, these genes are often found in clusters. Examples include the gene cluster for biphenyl degradation in several strains<sup>104, 204, 292</sup>, the *nah* cluster for naphthalene degradation<sup>117, 340</sup>, and the *xyl* cluster for toluene/ xylene degradation<sup>96</sup>. The clustering of genes which are all involved in a degradative pathway will be favoured in that individual pathway components are less useful than the full complement, leading to selective pressure keeping all the components together, and to enable more effective regulation of the genes. However, not all genes involved in a pathway are found together,

as exemplified by the *dntABD* genes for 2,4-dinitrotoluene degradation, which are not clustered, although they are all found on a 180 kb plasmid in *Pseudomonas* sp. strain DNT<sup>298</sup>. Other degradation genes located on plasmids include the genes for alkane, isopropylbenzene, and polyaromatic hydrocarbon (PAH) degradation in strains of *Rhodococcus* and *Pseudomonas*<sup>72, 93, 312</sup>. The presence of these genes on plasmids is thought to aid their mobility within a bacterial population, which also accounts for the presence of such genes on transposons. The *bph* genes in *Alcaligenes eutrophus* are carried on a 59 kb transposon<sup>292</sup>, and the genes for the degradation of chlorocatechol in *A. eutrophus* and alkane in *P. putida* are each found between two insertion sequences<sup>222, 312</sup>. A review of these catabolic transposons can be found in Tan<sup>301</sup>.

RDX is a serious environmental contaminant, and biodegradation has great potential for its remediation. To date, several studies have characterized microbial growth on RDX, and aerobic bacteria appear to form fewer toxic by-products than anaerobic bacteria from RDX degradation, and to perform the degradation more rapidly (§1.7.2). Of the aerobic RDX degrading bacteria reported, all are able to utilize RDX as a sole source of nitrogen<sup>27, 59, 157</sup> but not as a source of carbon. These bacteria include *Stenotrophomonas maltophilia* PB1<sup>27</sup> and *Rhodococcus* sp. strain DN22<sup>59</sup>, both isolated from contaminated soil.

Previously, an RDX degrading bacterium, designated strain 11Y, was isolated by Professor Bruce's group (Professor N. Bruce, personal communication) from explosive contaminated soil through its ability to grow on RDX as a sole source of nitrogen. It is a gram positive strain; partial 16S rDNA gene sequencing and phylogenetic comparison identified it as a species of *Rhodococcus*. National Collection of Industrial and Marine Bacteria (NCIMB) identification found it to be a non-sporulating, non-motile organism, oxidase negative and catalase positive; cell wall and fatty acid analysis showed the presence of mycolic acids which confirmed it to be a strain of *Rhodococcus rhodochrous*, and it has been designated NCIMB 40820. It is desirable to isolate and identify further strains of RDX degrading bacteria, which may give us an insight into the range of bacteria which are possess this activity, and possibly the range of mechanisms by which this occurs.

The isolation and comparison of bacterial strains which can utilize RDX as a sole nitrogen source are described in this chapter.

## 3.2 Materials and methods

### 3.2.1 Isolation and purification of RDX degrading strains

Enrichments were performed in minimal medium with 1 mM RDX provided as the sole nitrogen source and the three carbon sources described (§2.2.3). Contaminated samples were used to inoculate the medium, which was grown for 7 days at 30 °C. Subcultures were performed four times, each time diluting 50 fold into fresh medium, and growing for 7 days at 30 °C. Serial dilutions of the final enrichment cultures were spread onto LA plates. Pure bacterial strains were obtained as individual colonies picked from these plates.

### 3.2.2 Removal of RDX from growth medium

Strains were used to inoculate 5 ml minimal medium containing 1 mM RDX as the sole nitrogen source and three carbon sources. After 5 days at 30 °C, 0.5 ml samples of the media were taken and centrifuged at maximum speed for 2 min. The supernatant was mixed vigorously with 0.5 ml ethyl acetate to extract the RDX and centrifuged for 5 min at maximum speed. The solvent layer was taken and the solvent evaporated at 60 °C. The residue was dissolved in 20 µl acetone and subjected to TLC (§2.4.2). Positive control cultures were inoculated with *Rhodococcus rhodochrous* strain 11Y, known to remove RDX. Two negative controls were performed: a sterile culture and a bacterium known not to degrade RDX, *R. rhodochrous* strain CW25.

### 3.2.3 Zones of clearance on RDX plates

Each bacterial strain was grown in minimal medium containing 1 mM RDX for 3 days. Each culture (1 ml) was harvested and resuspended in 20 µl 40 mM phosphate buffer (pH 7.2) to a high cell density. An aliquot (10 µl) of each isolate was placed on an RDX zone of clearance plate (§2.2.3) which was incubated at 30 °C until zones of clearance became apparent. A positive control using strain 11Y and a negative control using the non RDX degrader strain CW25 were performed.

### 3.2.4 Utilization of RDX as a carbon and nitrogen source

Strains were incubated for 12 days in minimal medium containing no sources of carbon and 5 mM RDX. RDX was assayed by TLC as above, after 5 days and 12 days incubation. Strain CW25 and a sterile culture were included as negative controls for RDX breakdown. No positive controls were available, as no bacteria have been isolated which are able to use RDX as a source of carbon.

### 3.2.5 Identification of bacterial isolates

#### 3.2.5.1 Phenotypic characteristics

All bacterial strains were streaked to single colonies on LA and grown for 3 days at 30 °C. Colonies were assessed phenotypically for colour, morphology, size, whether or not they appeared mucoid, and to what degree.

#### 3.2.5.2 Gram reaction determination <sup>236</sup>

Biomass was taken from LA plates and mixed for 1 min on a glass slide with 3 % w/v KOH. Gram negative strains were identified by a viscous stringy appearance of the mixture, indicating lysis under these conditions. Gram positive strains do not form this viscous gel.

#### 3.2.5.3 16S rDNA identification

A set of oligonucleotide primers specifically designed for annealing to sequences of bacterial 16S rRNA genes were used to amplify nearly full-length 16S rDNA from each of the bacterial isolates. Primers fD1 and rD1, designed around the 5' and 3' ends (respectively) of bacterial 16S rDNA to produce an approx. 1.6 kb fragment <sup>327</sup>, were purchased from Sigma-Genosys Ltd. (Table 3.1). Primer fD1 has an engineered *Sal* I site, and rD1 an engineered *Bam*H I site.

**Table 3.1: Primers used for PCR and sequencing of 16S rDNA fragment.** Engineered restriction sites are shown in bold.

Primer	Sequence	T <sub>m</sub> (°C)
fD1 <sup>327</sup>	5' CCGAATTC <b>CGTCGACA</b> ACAGAGTTTGATCCTGGCTCAG 3'	82.4
rD1 <sup>327</sup>	5' CCCGGGATCCA <b>AGCTTA</b> AGGAGGTGATCCAGCC 3'	82.7
H2f	5' AGCAGCCGCGGTAATAC 3'	54

PCR amplification of DNA was performed using total DNA prepared from each isolate (§2.3.1) as the template. PCR cycles were performed as described (§2.3.6) using an annealing temperature of 55-62 °C and an extension time of 2 min. The approx. 1.6 kb PCR product amplified from each bacterial isolate was gel purified, cloned into pCR-2.1 TOPO<sup>®</sup> vector and transformed into the *E. coli* host TOP10. In order to confirm the size of the insert, plasmids were extracted and digested with *Sal* I and *Bam*H I, which cut within the engineered ends of the fragment. The inserts were sequenced using the standard M13F and M13R primers which prime within the *lacZ* gene of

the pCR-2.1 TOPO<sup>®</sup> vector. The chromatograms obtained were analysed using Sequencher<sup>™</sup> to verify or correct the base assignments that had been made automatically. A further primer, H2f (Table 3.1), was designed to a conserved region within the resulting sequences, and the plasmids sequenced using primer H2f to complete the internal sequence of the 16S rDNA gene. The sequence data were aligned into a single contiguous stretch of DNA (contig) with the use of Sequencher<sup>™</sup>, and the contigs obtained were subjected to BLAST searches. Bacterial identification was based on the most homologous 16S rDNA sequences returned.

### 3.2.6 Antibiotic resistance testing

Plates of LA (20 ml) containing the appropriate concentrations of antibiotic were made. The concentrations of antibiotics used were chosen as the concentrations surrounding the commonly used doses described in Ausubel et al. <sup>8</sup>; those not listed were used at a range of concentrations between 10-100 µg/ml (Table 3.5). Isoniazid was used in concentrations ranging from 50 – 200 µg/ml, around the concentration of 100 µg/ml specified for use when growing cells for electroporation <sup>164</sup>. Bacteria tested included all the RDX degrading isolates, *R. rhodochrous* strain CW25 and *E. coli*. Cells were streaked onto each concentration of antibiotic and growth assessed after 5 days at 30 °C.

### 3.2.7 TNT tolerance testing

LA plates containing TNT of concentrations varying from 0.005 – 0.65 mM were prepared at concentrations: 0.005, 0.01, 0.05, 0.1, 0.5 and 0.65 mM. Bacteria tested included all the RDX degrading isolates, *R. rhodochrous* strain CW25 and *E. coli*. Cells were streaked onto each concentration and growth assessed after 5 days at 30 °C.

### 3.2.8 Extraction of large plasmids

Large plasmids were extracted from rhodococcal strains using the protocol of Hansen et al. <sup>128</sup>. Cells were grown overnight in 40 ml LB, harvested, rinsed in 10 ml of 10 mM sodium phosphate buffer, pH 7.0 and resuspended in 1.35 ml of 25 % w/v sucrose in 50 mM Tris pH 8.0. An aliquot (100 µl) of 10 mg/ml lysozyme in 250 mM Tris pH 8.0 was added and the sample inverted four times before being incubated on ice for 5 min. An aliquot (0.5 ml) of 250 mM EDTA pH 8.0 was added, the sample was inverted eight times, incubated for 15 s at 55 °C, inverted five times and 0.5 ml 3N NaOH was added. The sample was inverted for 3 min, 0.5 ml 2 M Tris pH 7.0

was added and the sample was inverted ten times. A further 0.5 ml 2 M Tris pH 7.0 was added and the sample was again inverted ten times. SDS 20 % w/v in TE (0.65 ml) was added, followed by 1.25 ml 5 M NaCl. The sample was inverted 20 times, incubated at 4 °C overnight, then centrifuged at 12,000 rpm for 30 min at 4 °C. To the supernatant, 1.5 ml 42 % w/v polyethylene glycol (PEG) 6000 in 10 mM sodium phosphate buffer, pH 7.0 was added, mixed in with a plastic pipette and this was incubated at 4 °C overnight. The sample was centrifuged for 5 min at 2,500 rpm using an SS34 rotor in a Sorvall RC5C centrifuge (Sorvall, Kendro, Bishop's Stortford, U.K.) and the pellet resuspended in 150 µl dH<sub>2</sub>O.

### **3.2.9 Resting cell incubations of bacterial isolates**

Cultures (1 litre) of minimal medium containing 0.5 mM RDX as a sole nitrogen source and three carbon sources were inoculated with 5 day starter cultures of RDX grown bacteria. Cultures were grown at 30 °C and assayed for RDX presence twice daily by HPLC. Twenty four hours after complete removal of RDX, cultures were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. Cells were rinsed twice in 40 mM phosphate buffer pH 7.5 and resuspended in 40 mM phosphate buffer pH 7.5 to 0.5 g wet weight/ml. Aliquots (0.9 ml) of 40 mM phosphate buffer pH 7.5 containing 0.25 mM RDX (final concentration) were placed into 7 ml Bijoux containers and equilibrated at 30 °C, as were the resuspended cells. Aliquots (100 µl) of cells were added to the Bijoux containers and incubated, shaking at 110 rpm, for 0, 5, 10, 20, 30 and 60 min at 30 °C. Reactions were stopped by the precipitation of protein through the addition of 1 ml 10 % v/v glacial acetic acid and incubation on ice for 10 min. The resulting supernatant was assayed for RDX concentration by HPLC (§2.4.3) and nitrite concentration using the Griess assay (§2.4.4). Negative controls included sterile RDX sampled after 60 min, and cells incubated in the absence of RDX, sampled after 0, 30 and 60 min.

## 3.3 Results

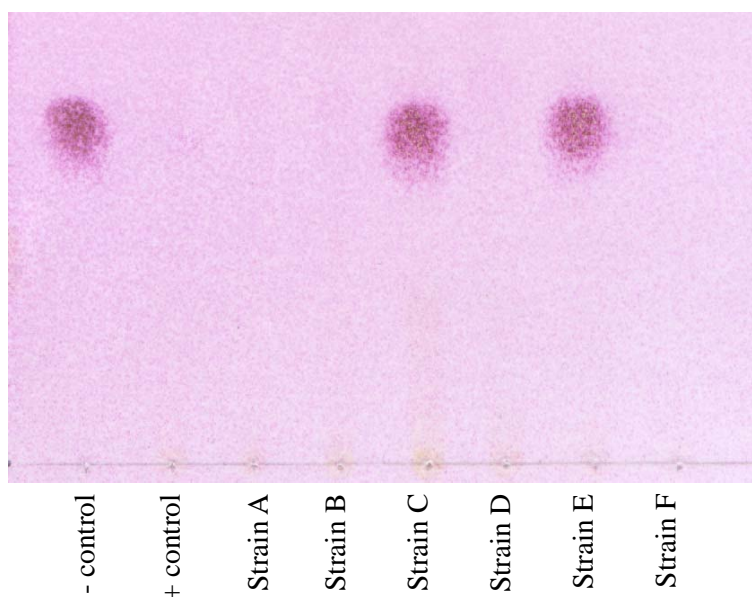
### 3.3.1 Isolation and purification of 19 RDX degrading strains

Material from areas of heavy RDX and HMX contamination were supplied by Dstl and used as the source for isolating RDX degrading bacteria by selective enrichment. Samples were obtained from several sites: soil next to munitions factories where dust from the floor was swept out, soil from areas where explosives were steamed out of shells, and detritus from munition contaminated drains (Table 3.2). The medium used for selective enrichment was similar to that used in the successful isolation of a gram negative RDX degrading bacterium by Binks et al.<sup>27</sup>. This medium contained 1 mM RDX as a sole nitrogen source, and was buffered at pH 7.2 which is within the pH range at which many bacteria isolated from the environment are able to grow.

Following four subcultures in selective medium, the culture was plated onto rich medium and individual colonies picked. Seventy three pure bacterial strains were obtained from the enrichments.

#### 3.3.1.1 Removal of RDX from growth medium

Bacterial growth is typically assessed by optical density at 600 nm, but as 1 mM RDX is above its aqueous solubility limit of 0.27 mM (60 mg/l at 30 °C)<sup>195</sup>, turbidity results from the presence of the undissolved RDX as crystals, as well as the bacterial growth. RDX removal from medium by the bacterial isolates was therefore assessed by TLC to determine which were able to break down the RDX. RDX breakdown would be necessary for release of nitrogen containing compounds for bacterial growth. RDX was visualized through alkaline hydrolysis and detection of resulting nitrite by Griess assay on the TLC plate. Any nitro-containing metabolites produced should also be visualized by this method. The result of a typical TLC analysis of 6 strains is shown in Figure 3.1. Presence of a pink spot indicates that the bacterial isolate was not able to break down RDX. Positive controls containing *Rhodococcus rhodochrous* strain 11Y showed RDX removal, and negative controls, which were either sterile or inoculated with non RDX degrader *R. rhodochrous* strain CW25, showed no removal of RDX.



**Figure 3.1: TLC screening of isolates grown with RDX as a sole nitrogen source.** Presence of a pink spot indicates presence of RDX in the sample assayed. Four of the six isolates shown here could remove RDX. These were later renamed. The positive control consisted of known RDX degrading strain 11Y, and the negative control of uninoculated medium.

A total of 19 bacterial isolates were found to be able to remove 1 mM RDX from medium when it was supplied as a sole source of nitrogen. Complete RDX removal was obtained by these strains over 5 days as analysed by TLC. No other metabolites were observed on the TLC plates. In all cases, the removal of RDX correlated with an increase in turbidity indicative of bacterial growth, although this was not quantified. This was distinguishable by eye from turbidity caused by the presence of RDX, as bacterial growth made the cultures a beige colour as opposed to the white crystals of RDX. It is therefore apparent that the bacterial strains are able to utilize the RDX as a source of nitrogen for growth. Each of the 19 isolates was given a strain designation (Table 3.2). Stocks of the isolates were prepared and stored in glycerol at  $-80\text{ }^{\circ}\text{C}$ .

**Table 3.2: RDX degrading bacterial isolates and the origin of the material used to enrich them.**

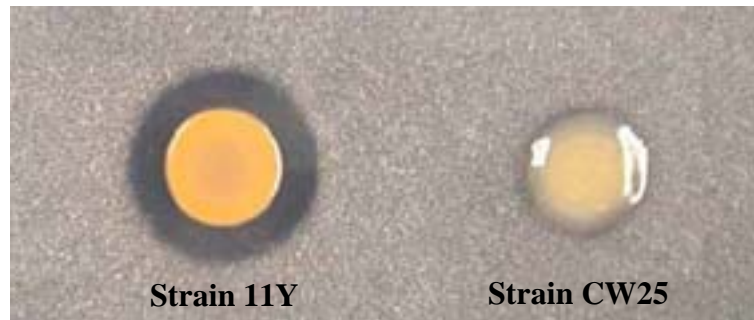
Strain designation	Site of material used for enrichment
Strain HS1	Soil from site of dust sweeping
Strain HS2	Soil from site of dust sweeping
Strain HS3	Soil from site of dust sweeping
Strain HS4	Soil from site of dust sweeping
Strain HS5	Soil from site of dust sweeping
Strain HS6	Soil from site of shell steaming
Strain HS7	Soil from site of shell steaming
Strain HS8	Material from munitions factory drain
Strain HS9	Material from munitions factory drain
Strain HS10	Material from munitions factory drain
Strain HS11	Material from munitions factory drain
Strain HS12	Material from shell steaming area drain
Strain HS13	Material from shell steaming area drain
Strain HS14	Material from shell steaming area drain
Strain HS15	Soil 3 metres from site of shell steaming
Strain HS16	Soil 3 metres from site of shell steaming
Strain HS17	Soil 3 metres from site of shell steaming
Strain HS18	Soil 5 metres from site of shell steaming
Strain HS19	Soil from site of dust sweeping

### 3.3.1.2 Zones of clearance on RDX plates

A screen has been developed to allow visualization of RDX utilization by bacteria on solid media (A. Basran, personal communication). Minimal medium agarose plates containing 5 mM RDX as a sole source of nitrogen have an opaque appearance due to the presence of RDX crystals when it is added above its aqueous solubility limit of 0.27 mM (§2.2.3). RDX degrading bacteria grown on these plates form an area of transparency around the colonies, whereas non RDX degraders do not form a “halo”. This zone of clearance screen was used to confirm the ability of the strains to degrade RDX and to validate the screen.

All the 73 isolates were analysed using this screen. Only the 19 bacteria described in Table 3.2 and *R. rhodochrous* strain 11Y, each of which removed RDX from media as assayed by TLC, formed a zone of clearance around them on RDX zone of clearance plates, indicating RDX utilization (Figure 3.2). A negative control, *R. rhodochrous* strain CW25, did not form a zone of clearance. Therefore the results of the zone of clearance screen correlate entirely with the results of the TLC analysis.

**A**



**B**

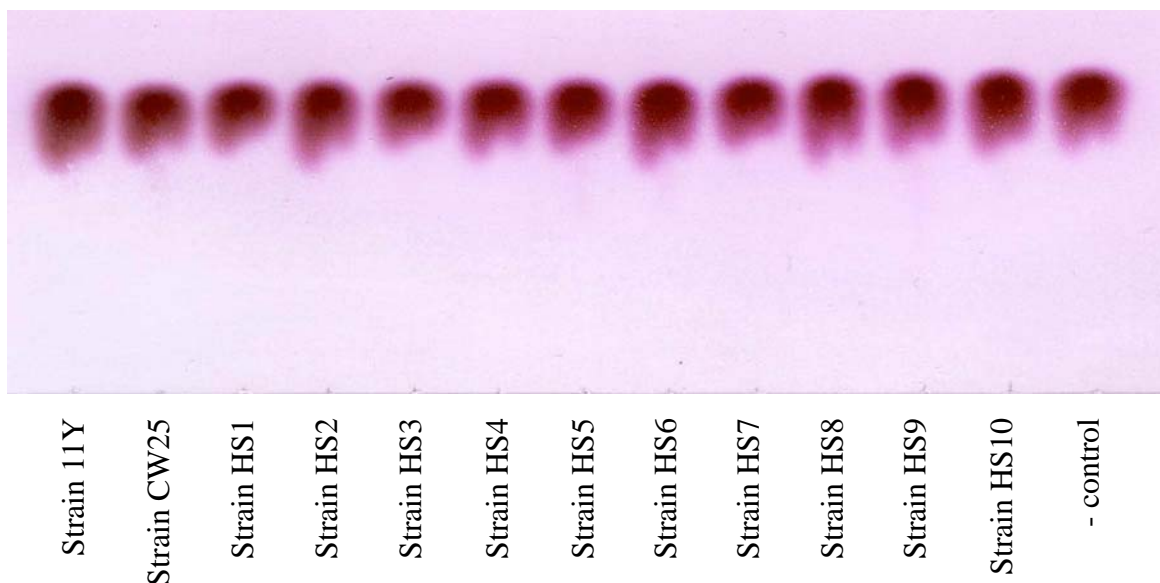


**Figure 3.2: Zone of clearance screening of bacterial isolates.** A. RDX degrading bacterium *R. rhodochrous* strain 11Y produces a zone of clearance whereas non RDX degrading *R. rhodochrous* strain CW25 does not. B. Isolates previously screened by TLC analysis spotted onto RDX zone of clearance plates. All strains formed a zone of clearance, although strain HS5 was mucoid to the extent that the zone is not visible in this image. Strain 11Y was spotted twice.

### 3.3.2 Utilization of RDX as a carbon and nitrogen source

No bacteria have been reported in the literature which utilize RDX as a source of both nitrogen and carbon. To investigate whether bacterial isolates HS1-19 and strain 11Y are able to use RDX as a carbon source, cultures of each were set up in minimal medium containing 5 mM RDX and no other source of carbon. The concentration of RDX was raised to this level, which is far in excess of the solubility limit of 0.27 mM, to allow the bacteria sufficient carbon for growth, as 7-12 times as much as much carbon as nitrogen is required<sup>87</sup>. After incubation for 5 days and 12 days, TLC was performed on the culture media to determine if the RDX had been removed. The RDX would have to be broken down to provide carbon and nitrogen for growth. The resulting TLC plates were interpreted as above (§3.3.1.1).

A typical TLC analysis is shown in Figure 3.3. None of the cultures showed removal of RDX after 5 days. A further incubation and TLC assay showed no obvious RDX disappearance after a further 7 days, indicating that none of the strains are able to utilize RDX as a source of both carbon and nitrogen. No other metabolites were observed using TLC. Assessment of bacterial growth by eye was made impossible by the amount of RDX present in the media.



**Figure 3.3: TLC screening of isolates grown with RDX as a sole carbon and nitrogen source.** None of the strains assayed could remove RDX provided as a source of carbon and nitrogen. The negative control consisted of uninoculated medium.

### 3.3.3 Identification of bacterial isolates

#### 3.3.3.1 Phenotypic characteristics

A preliminary characterization of the isolates was performed, based on colony characteristics after growth on rich medium (LA). Colony differentiation is facilitated by growth on rich agar as opposed to minimal medium plates, as stronger pigmentation is produced<sup>38</sup>. This initial characterization was performed to enable preliminary grouping of the bacteria. The results are shown in Table 3.3.

**Table 3.3: Phenotypic characterization of colonies of bacterial isolates.** \* No single colonies were present to assess colony size

Bacterial isolate	Rich medium				RDX medium	
	Colour	Morphology	Muroid	Colony size after 3 days (diam. mm)	Colour	Muroid
Strain 11Y	Orange	Smooth	-	0.7	Orange	-
Strain HS1	Beige	Smooth	-	0.7	White	++
Strain HS2	Beige	Smooth	+	0.7	White	++
Strain HS3	Beige	Smooth	-	1	White	++
Strain HS4	Beige	Smooth	++	1.5	White	++
Strain HS5	Beige	Smooth	-	1	White	+
Strain HS6	Beige	Smooth	-	0.7	White	-
Strain HS7	Beige	Smooth	-	1	White	-
Strain HS8	Beige	Smooth	-	1.2	White	-
Strain HS9	Beige	Smooth	++	1.5	White	+
Strain HS10	Beige	Smooth	+++	*	White	++
Strain HS11	Beige	Smooth	+	1	White	+
Strain HS12	Beige	Smooth	++	1 – 3	White	+
Strain HS13	Beige	Smooth	-	< 0.5	White	-
Strain HS14	Beige	Smooth	-	1	White	++
Strain HS15	Beige	Smooth	-	0.5	White	-
Strain HS16	Beige	Smooth	-	1	White	-
Strain HS17	Beige	Smooth	-	0.7	White	-
Strain HS18	Beige	Smooth	-	0.7	White	-
Strain HS19	Beige	Smooth	+	1	White	+

All environmental isolates were beige in colour on rich media plates, except 11Y. The colour of the strains was altered by growth on minimal media plates containing RDX, where all were paler, of a white appearance, again with 11Y being the exception by retaining its orange colour. All the strains formed smooth colonies as opposed to rough and the major variations between strains were in colony size and the extent to which they were mucoid. Several of the strains were very mucoid; strain HS10 to the extent that there were no single colonies to use for measurement of the diameter. Colony size varied between 0.5-3 mm in diameter after 3 days growth. When placed on minimal medium, several of the strains became more mucoid than they had been on rich medium.

### 3.3.3.2 Gram reaction determination

A preliminary identification of the bacteria was carried out to determine whether they were gram positive or gram negative. The method used for Gram reaction determination<sup>236</sup> is based on differences in the cell wall composition of gram positive and negative strains. The cell wall of gram negative strains are lysed with the use of 3 % KOH, whereas gram positive strains are not. This is not a staining technique, but appears to give fewer anomalous results than Gram staining<sup>236</sup>. Controls were performed consisting of a known gram positive (*R. rhodochrous* strain CW25) and a known gram negative bacterium (*E. coli*).

The *E. coli* strain produced a viscous string with 3 % w/v KOH treatment, demonstrating lysis. None of the bacterial isolates described here were lysed by this treatment, indicating that all are gram positive.

### 3.3.3.3 16S rDNA identification

The isolates were further identified through the sequencing of their 16S ribosomal RNA genes (rDNA). 16S rDNA sequences are highly conserved and are commonly used to determine phylogenetic relatedness, as they are representative of the variation within the whole genome<sup>111</sup>. In addition to identifying the 19 bacterial isolates described above, the full 16S rRNA gene sequence from *Rhodococcus rhodochrous* strain 11Y (§3.1) was also determined to permit full phylogenetic comparisons between all RDX degrading isolates.

The 16S rRNA gene was amplified through PCR of genomic DNA from each strain using primers fD1 and rD1 (Table 3.1) to give a product of approx. 1.6 kb, containing nearly the entire gene sequence.

The highest homology matches returned from BLAST searches showed that the bacterial isolates fall into three groups. All the isolates from this study were found to be more closely related to each other than to strain 11Y. To demonstrate the degree of relatedness of the bacterial isolates, the sequences of the bacteria with homologous 16S rDNA sequences were retrieved from the databases and aligned with sequences from the bacterial isolates using the PileUp software (GCG). A phylogenetic tree showing the relatedness of all strains was constructed from this alignment using ClustalX<sup>305</sup> and viewed using NJPlot<sup>227</sup> (Figure 3.4).

The strains showing highest homology to each of the bacterial strains are given in Table 3.4. Thirteen of the strains had sequences with greatest homology to *Rhodococcus erythropolis* (accession number U82667)<sup>343</sup>. 16S rDNA sequences from the four strains HS3, HS6, HS10 and HS13 showed highest homology to the 16S rDNA of *Rhodococcus* sp. strain DN22, a previously characterized RDX degrader (X89240)<sup>59</sup>. Two of the strains, HS1 and HS7, had a 16S rRNA gene with highest homology to a nitrile metabolizing *Rhodococcus* sp. (AF420422)<sup>38</sup>. Within these groups, the 16S rDNA sequences of all isolates varied from the other strains by 1 – 8 nucleotides, except those of HS8 and HS18 which were identical. Strain 11Y was found to have a highest homology match with a *Rhodococcus zopfii* strain able to degrade phenol, toluene, biphenyl and chlorinated benzenes (AF191343)<sup>294, 344</sup>. However, previous characterization of the strain by NCIMB using cell wall and fatty acid analysis confirmed strain 11Y to be a strain of *R. rhodochrous*. Both these species fall into the genus subgroup *R. rhodochrous*, within which precise identification of species is not easy<sup>111</sup>. Therefore strain 11Y will be referred to as a strain of *R. rhodochrous*, according to the NCIMB identification.

There is no pattern connecting isolates with particular 16S rDNA homologies to specific contaminated areas. The contaminated samples used for enrichment, with the exception of soil from shell steaming sites, can be seen to contain bacteria with highest homologies to more than one bacterium (comparing Table 3.4 with Table 3.2). All the bacteria are very closely related from 16S rDNA sequence comparison.

Comparing 16S rDNA identification with the colony phenotypes determined above, there is no obvious correlation. It appears that strains with similar 16S rDNA sequences have different colony morphologies.



**Table 3.4: Bacterial isolates and assigned homologous matches based on 16S rDNA phylogenetic analysis.**

Strain designation	Match of highest homology	Accession number
Strain HS1	<i>Rhodococcus</i> sp. 871-AN053	AF420422
Strain HS2	<i>Rhodococcus erythropolis</i>	U82667
Strain HS3	<i>Rhodococcus</i> sp. DN22	X89240
Strain HS4	<i>Rhodococcus erythropolis</i>	U82667
Strain HS5	<i>Rhodococcus erythropolis</i>	U82667
Strain HS6	<i>Rhodococcus</i> sp. DN22	X89240
Strain HS7	<i>Rhodococcus</i> sp. 871-AN053	AF420422
Strain HS8	<i>Rhodococcus erythropolis</i>	U82667
Strain HS9	<i>Rhodococcus erythropolis</i>	U82667
Strain HS10	<i>Rhodococcus</i> sp. DN22	X89240
Strain HS11	<i>Rhodococcus erythropolis</i>	U82667
Strain HS12	<i>Rhodococcus erythropolis</i>	U82667
Strain HS13	<i>Rhodococcus</i> sp. DN22	X89240
Strain HS14	<i>Rhodococcus erythropolis</i>	U82667
Strain HS15	<i>Rhodococcus erythropolis</i>	U82667
Strain HS16	<i>Rhodococcus erythropolis</i>	U82667
Strain HS17	<i>Rhodococcus erythropolis</i>	U82667
Strain HS18	<i>Rhodococcus erythropolis</i>	U82667
Strain HS19	<i>Rhodococcus erythropolis</i>	U82667

### 3.3.4 Antibiotic resistance profiles

Analysis of the antibiotic resistance profiles of each of the bacteria was performed to further differentiate the strains, and to inform the development of transformation strategies for later use. A range of antibiotics was chosen to test, with a range of targets (Table 3.5). The eukaryotic inhibitor, cycloheximide, was tested as transformation protocols can use it as a fungal inhibitor during bacterial growth. Isoniazid was included as some protocols require its addition to media in the preparation of competent nocardioform bacteria to weaken the mycolic acid cell wall <sup>164</sup>.

**Table 3.5: Antibiotics used, commonly used final concentration <sup>8</sup>, concentrations tested and modes of antibacterial action.**

Antibiotic	Final conc. (µg/ml)	Concentrations tested (µg/ml)	Mode of action
Carbenicillin	50	25, 50, 100	Inhibits cell wall peptidoglycan links
Chloramphenicol	20	10, 20, 50	Inhibits peptide synthesis through ribosome 50S subunit
Cycloheximide		10, 50, 100	Inhibits eukaryotic protein synthesis
Gentamycin	15	7.5, 15, 30	Inhibits protein translation
Isoniazid		50, 100, 200	Inhibits cell wall mycolic acid synthesis
Kanamycin	30	15, 30, 60	Inhibits protein translation
Rifampicin	150	75, 150, 300	Inhibits RNA synthesis through RNA polymerase β-subunit
Streptomycin	30	15, 30, 60	Inhibits protein translation
Tetracycline	12	6, 12, 24	Inhibits protein synthesis
Vancomycin		10, 50, 100	Inhibits cell wall peptidoglycan synthesis

All bacteria isolates, with strain 11Y, strain CW25 and *E. coli*, were plated onto all concentrations tested and assessed for growth after 4 days at 30 °C. Table 3.6 shows the minimum inhibitory concentration for each antibiotic, or maximum dose tested, where the strains were tolerant.

Within each group of isolates sharing similar 16S rDNA sequences, all strains have distinct antibiotic resistance profiles, again implying that they are distinct isolates. A comparison of strains HS8 and HS18, which possess identical 16S rDNA sequences, shows that their antibiotic resistance profiles differ significantly, confirming that they are discrete strains.

Sensitivities to the antibiotics carbenicillin, chloramphenicol and kanamycin varied between isolates. Five of the strains were resistant to carbenicillin at 100 µg/ml and the rest of the strains were sensitive at concentrations ranging from 25 – 100 µg/ml. All strains were sensitive to chloramphenicol at the concentrations tested, although the level at which the bacteria were sensitive

varied from 10 – 50 µg/ml. Most of the strains (16) were resistant to 60 µg/ml kanamycin, and the remaining strains were sensitive to either 15 or 30 µg/ml.

**Table 3.6: Antibiotic resistance profiles of bacterial isolates.** Where strain is sensitive (S), minimum inhibitory concentration (MIC) is shown (µg/ml). Where strain is resistant (R), maximum dose tested is shown (µg/ml). Antibiotics shown are carbenicillin (Carb), chloramphenicol (Chl), gentamycin (Gen), kanamycin (Kan), rifampicin (Rif), streptomycin (SS), tetracycline (Tc) and vancomycin (Van). All strains were also resistant to 100 µg/ml cycloheximide and 200 µg/ml isoniazid.

Bacterial isolate	Carb	Chl	Gen	Kan	Rif	SS	Tc	Van
Strain 11Y	S 25	S 10	S 7.5	S 15	S 75	R 60	S 6	S 10
Strain HS1	S 25	S 20	S 7.5	R 60	S 75	R 60	S 12	S 10
Strain HS2	S 25	S 20	S 7.5	R 60	S 75	R 60	S 12	S 10
Strain HS3	S 50	S 20	S 7.5	R 60	S 75	R 60	S 12	S 10
Strain HS4	S 100	S 50	S 7.5	R 60	S 150	R 60	S 6	S 10
Strain HS5	S 50	S 20	S 7.5	R 60	S 75	R 60	S 12	S 10
Strain HS6	S 50	S 50	S 7.5	R 60	S 75	R 60	S 6	S 10
Strain HS7	S 50	S 20	S 7.5	R 60	S 150	R 60	S 12	S 10
Strain HS8	S 100	S 50	S 15	R 60	S 150	R 60	S 12	S 10
Strain HS9	R 100	S 20	S 15	R 60	S 75	R 60	S 12	S 10
Strain HS10	S 100	S 50	S 15	R 60	S 75	R 60	S 12	S 10
Strain HS11	R 100	S 50	S 15	R 60	S 75	R 60	S 6	S 10
Strain HS12	S 100	S 50	S 15	R 60	S 75	R 60	S 12	S 10
Strain HS13	R 100	S 50	S 15	R 60	S 75	R 60	S 12	S 10
Strain HS14	S 100	S 50	S 15	R 60	S 75	R 60	S 6	S 10
Strain HS15	R 100	S 50	S 7.5	R 60	S 75	R 60	S 6	S 10
Strain HS16	S 50	S 20	S 7.5	S 30	S 75	S 30	S 6	S 10
Strain HS17	S 50	S 20	S 7.5	S 15	S 75	S 60	S 6	S 10
Strain HS18	R 100	S 20	S 7.5	S 15	S 75	S 30	S 6	S 10
Strain HS19	S 50	S 50	S 7.5	R 60	S 300	R 60	S 6	S 10
Strain CW25	S 100	S 20	S 15	R 60	R 300	R 60	S 6	S 10
<i>E. coli</i>	R 100	S 10	S 7.5	S 15	R 300	S 15	S 6	R 100

All RDX degrading isolates were sensitive to gentamycin (7.5 – 15 µg/ml), rifampicin (75 – 300 µg/ml) and tetracycline (6 – 12 µg/ml). Strain CW25 and *E. coli* were also sensitive to these with the exception of rifampicin (both strains being resistant to 300 µg/ml). *R. rhodochrous* strain CW25 is a specifically selected rifampicin resistant mutant, and gram negative bacteria such as the control *E. coli* strain are generally less sensitive to rifampicin than gram positive strains, possibly as it can cross the cell wall of gram positive bacteria more easily than those of gram negatives<sup>187</sup>. All but three of the bacterial strains were resistant to streptomycin at 60 µg/ml.

All the rhodococcal strains were sensitive to 10 µg/ml vancomycin, whereas the *E. coli* control was resistant to more than 100 µg/ml. This is indicative of its role in inhibiting peptidoglycan synthesis, which makes up a greater amount of the cell wall of gram positive bacteria than gram negative. In addition, all strains were resistant to the maximum concentrations of cycloheximide (100 µg/ml) and isoniazid (200 µg/ml) tested. Cycloheximide is an inhibitor of eukaryotic protein synthesis, so bacterial resistance would be expected. Isoniazid should weaken the cell walls of nocardioform strains such as *Rhodococcus* by inhibiting mycolic acid production; however, it appears not to affect the viability of these strains at concentrations of 200 µg/ml.

### 3.3.5 Tolerance of bacterial isolates to TNT

The ability of these bacteria to tolerate TNT is of interest as RDX and TNT are often used together in explosive combinations and therefore commonly found co-contaminating land. The ability of the bacteria to tolerate TNT was assessed by their growth on LA plates containing TNT at concentrations between 0.005 and 0.65 mM, which is the solubility limit of TNT in aqueous solution at 30 °C<sup>195</sup>.

The maximum TNT concentration tolerated by the bacteria is shown in Table 3.7. Although six of the isolates could grow in the presence of up to 0.65 mM TNT, growth only occurred in areas where a high cell density had been plated, and not as single colonies. *E. coli* showed no signs of weaker growth at 0.65 mM TNT.

**Table 3.7: TNT tolerance of bacterial isolates.** Maximum tolerated concentration is shown.

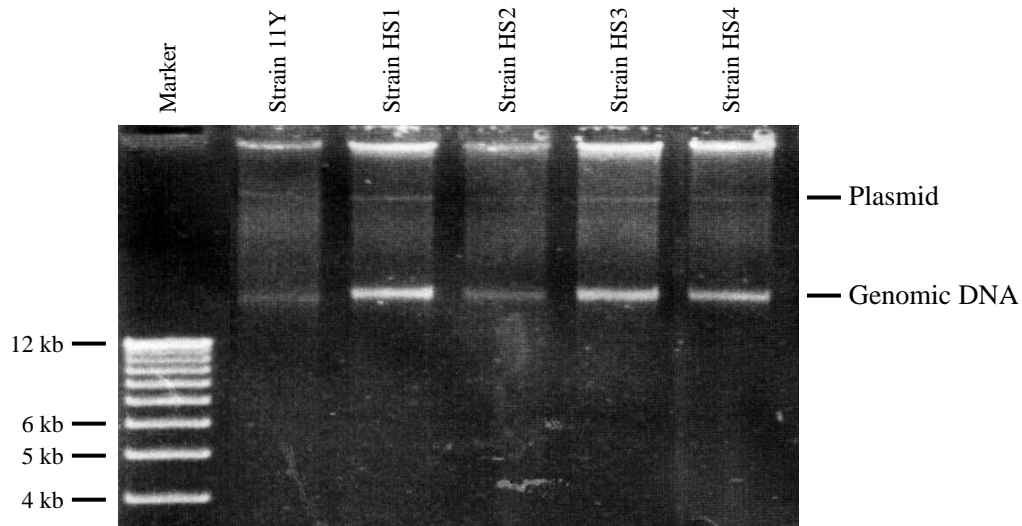
Bacterial isolate	Maximum TNT concentration tolerated (mM)	Bacterial isolate	Maximum TNT concentration tolerated (mM)
Strain 11Y	0.1	Strain HS11	0.65
Strain HS1	0.5	Strain HS12	0.5
Strain HS2	0.5	Strain HS13	0.1
Strain HS3	0.65	Strain HS14	0.5
Strain HS4	0.5	Strain HS15	0.5
Strain HS5	0.65	Strain HS16	0.5
Strain HS6	0.5	Strain HS17	0.1
Strain HS7	0.1	Strain HS18	0.1
Strain HS8	0.65	Strain HS19	0.65
Strain HS9	0.65	Strain CW25	0.1
Strain HS10	0.1	<i>E. coli</i>	0.65

### 3.3.6 Plasmid extractions from isolates

Genes for bacterial properties such as xenobiotic degradation are often found on plasmids (§3.1). The activity which allows the degradation of RDX by *Rhodococcus* sp. strain DN22 is thought to be plasmid-borne<sup>60</sup>. The isolation of a gene carried on a plasmid can be significantly less difficult as the majority of the genome has been eliminated from the search. The presence or absence of plasmids also serves as a way of distinguishing the strains, and may give clues regarding horizontal transfer between species. The RDX degrading bacterial isolates were therefore assessed for plasmid presence.

Initial plasmid extractions on all the bacterial isolates were performed using the boiling lysis method. No plasmids were observed when the extraction was viewed by gel electrophoresis (data not shown).

A protocol for the extraction of large plasmids was performed on all the strains. Gel electrophoresis was performed on the resulting samples and a typical result shown in Figure 3.5. Most of the isolates were found to have large plasmids, and some strains had 2 plasmids (Table 3.8).



**Figure 3.5: Large plasmid preparations from bacterial isolates.** All five of the isolates shown contain one large plasmid, running slower than the genomic DNA as indicated.

**Table 3.8: Bacterial isolates and number of plasmids which they contain.**

Bacterial isolate	Plasmids	Bacterial isolate	Plasmids
Strain 11Y	1	Strain HS10	2
Strain HS1	1	Strain HS11	1
Strain HS2	1	Strain HS12	2
Strain HS3	1	Strain HS13	2
Strain HS4	1	Strain HS14	None
Strain HS5	None	Strain HS15	1
Strain HS6	None	Strain HS16	None
Strain HS7	2	Strain HS17	1
Strain HS8	2	Strain HS18	1
Strain HS9	1	Strain HS19	1

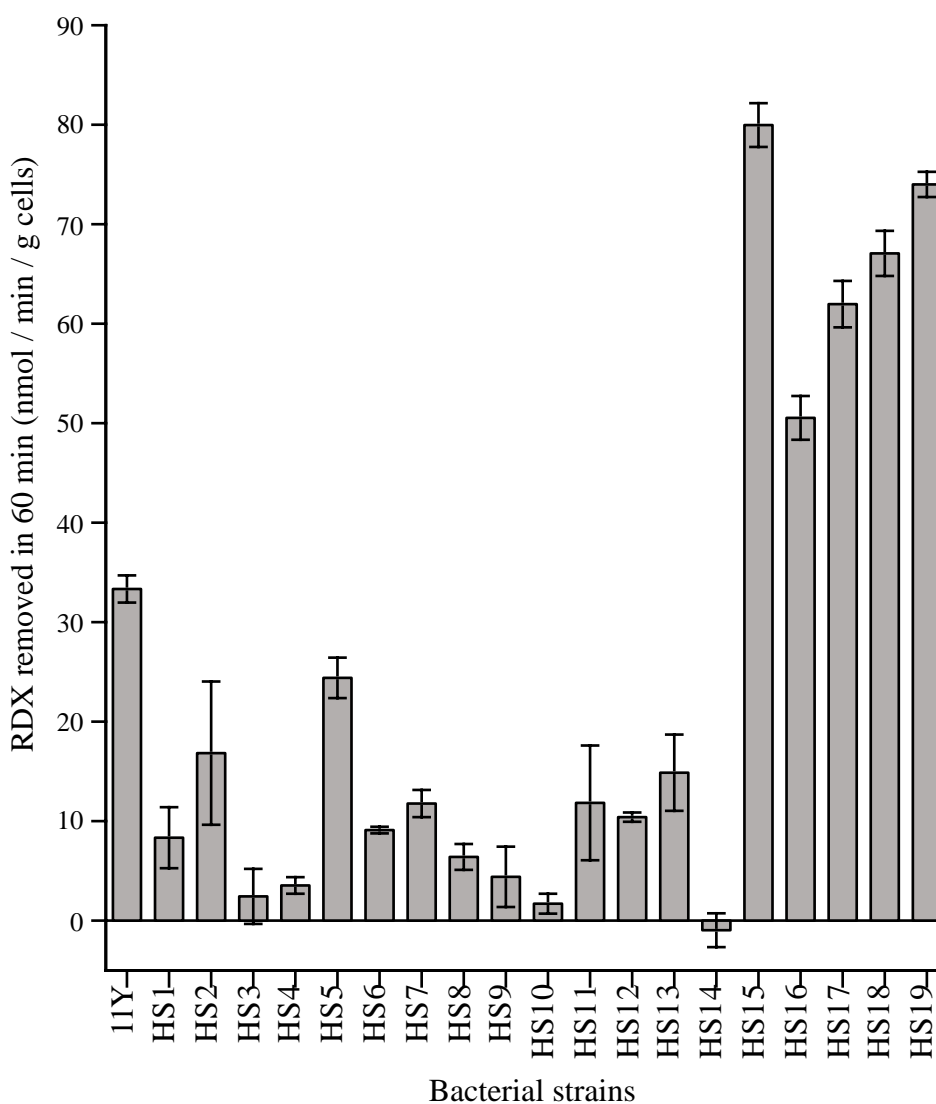
### 3.3.7 Comparison of the RDX degradation rates of bacterial isolates

#### 3.3.7.1 RDX removal during resting cell incubations

To compare the RDX degrading rates of the bacterial isolates, resting cell incubations were performed on all strains. In order for all the strains to be at a similar stage of growth, and for a significant cell density, cells were harvested 24 h after all the RDX had been removed from the growth medium. Harvesting the cells at this stage of growth also eliminated the carry over of any RDX remaining from the culture to the incubation, which could affect RDX concentrations in the

experiment. Resting cell incubations were performed using 0.25 mM RDX in potassium phosphate buffer over 60 min, with aeration, at 30 °C.

In general, the rate of RDX removal by each bacterial isolate was relatively consistent over 60 min. Therefore the amount of RDX removed by each strain over 60 min is represented in Figure 3.6 and Table 3.9.

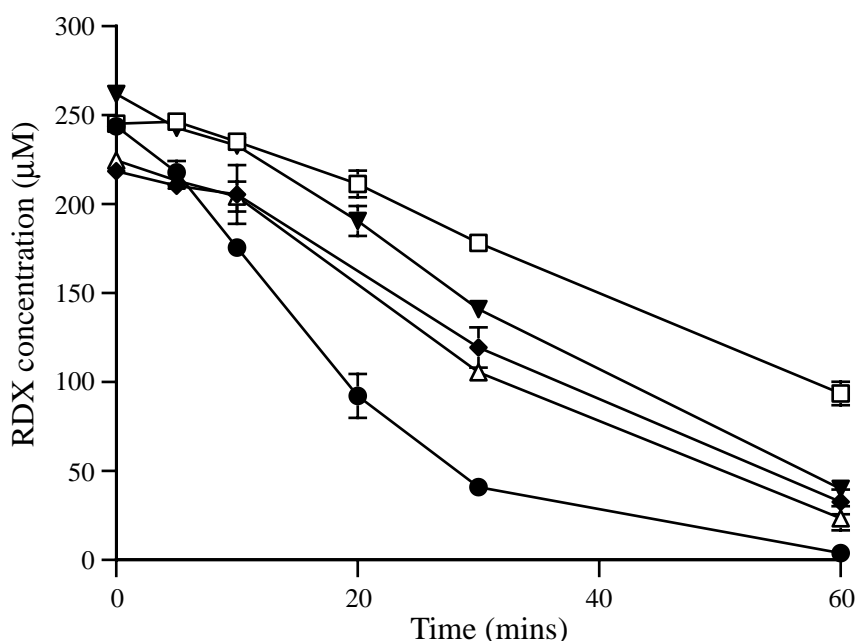


**Figure 3.6: Comparison of RDX degradation rates of bacterial isolates.** Resting cell incubations were used to compare RDX degrading bacterial isolates. Removal of RDX over 60 min shown as nmoles RDX removed per min per g wet weight cells. Figures shown are the average of duplicate or triplicate samples and error bars indicate  $\pm$  one standard deviation.

**Table 3.9: RDX removal by bacterial isolates in resting cell incubations over 60 min.** The number of days taken by each strain to remove the RDX from culture is also shown.

Bacterial isolate	Time taken to remove RDX from culture (days)	RDX removed over 60 min (nmoles/min/g cells)
Strain 11Y	1.5	33.3 ± 1.4
Strain HS1	3.5	8.3 ± 3.1
Strain HS2	3.5	16.8 ± 7.2
Strain HS3	3.5	2.4 ± 2.8
Strain HS4	2.5	3.5 ± 0.8
Strain HS5	2	24.4 ± 2.0
Strain HS6	3	9.1 ± 0.3
Strain HS7	3	11.8 ± 1.4
Strain HS8	4.5	6.4 ± 1.3
Strain HS9	4	4.4 ± 3.0
Strain HS10	4	1.7 ± 1.0
Strain HS11	4.5	11.8 ± 5.8
Strain HS12	4	10.4 ± 0.5
Strain HS13	4.5	14.9 ± 3.8
Strain HS14	4	- 1.0 ± 1.7
Strain HS15	4	80.0 ± 2.2
Strain HS16	3.5	50.5 ± 2.2
Strain HS17	2.5	62.0 ± 2.3
Strain HS18	2.5	67.1 ± 2.3
Strain HS19	1.5	74 ± 1.3

Five of the strains removed over 50 nmoles RDX/min/g cells: HS15, HS16, HS17, HS18 and HS19. The removal of RDX over the 60 min incubation by these five strains is shown in Figure 3.7. Strain 11Y removed over 30 nmoles RDX/min/g cells and the remaining strains removed less than 25 nmoles/min/g cells. The negative value for RDX removal by strain HS14 can be explained, as the shaking incubation over 60 min may have solubilized the RDX more than the sample taken at 0 min, while no RDX was removed by action of the cells under these conditions.

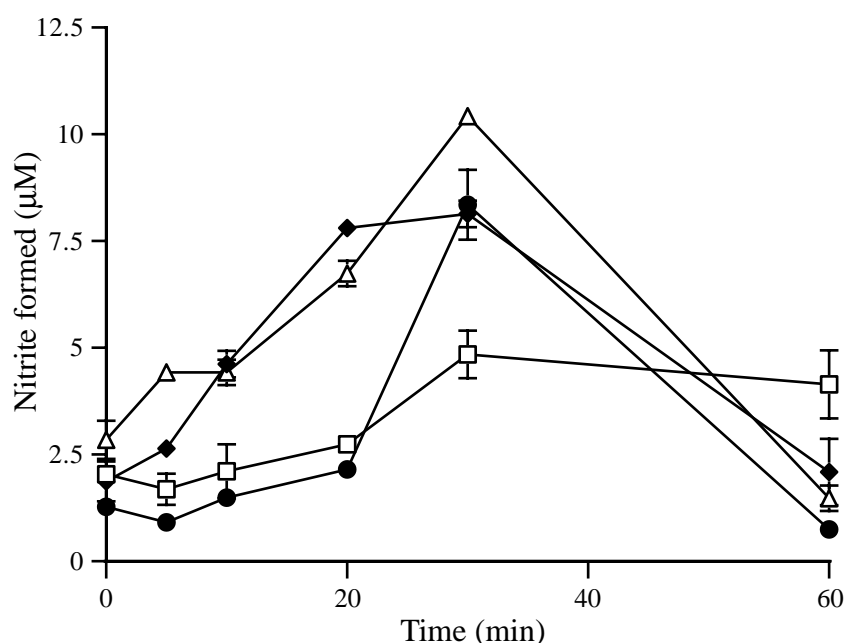


**Figure 3.7: RDX removal from resting cell incubations by five strains.** The RDX concentration over the course of the incubations is shown for the five strains which removed the most RDX. Strains shown are HS15 (●), HS16 (□), HS17 (◆), HS18 (△) and HS19 (▼). Figures shown are the average of duplicate samples and error bars indicate  $\pm$  one standard deviation.

No obvious correlation is seen between the speed of RDX removal from culture during growth, and the amount of RDX removed during the resting cell incubation (Table 3.9). Strains 11Y and HS19 removed RDX from culture the fastest, in 1.5 days, followed by strain HS5, requiring 2 days. This is not reflected in the relative amounts of RDX removed from the incubations.

### 3.3.7.2 Nitrite production during resting cell incubations

In addition to the measurement of RDX removal, nitrite concentrations within the incubation samples were measured, to determine whether all strains produce nitrite from the breakdown of RDX. Nitrite has been seen to be a product of RDX degradation<sup>59</sup> and may be used by the bacteria as a source of nitrogen. Only five of the 20 strains tested produced nitrite at levels above those in the controls containing cells in the absence of RDX: strains 11Y, HS2, HS4, HS5 and HS19. Nitrite levels in the cell only controls varied between 0 – 3  $\mu\text{M}$  (0 – 2 nmoles/min/g cells over 30 min). The concentration of nitrite was generally seen to be greatest after 30 min and subsequently declined (Figure 3.8). The amount of nitrite formed over 30 min by the five strains mentioned is presented in Table 3.10.



**Figure 3.8: Nitrite production from resting cell incubations by four strains.** Nitrite production over the course of a 60 min resting cell incubation. Strains shown are HS2 (●), HS4 (□), HS5 (◆) and HS19 (△). Nitrite concentration peaks at 30 min. Figures shown are the average of duplicate samples and error bars indicate  $\pm$  one standard deviation.

**Table 3.10: Nitrite formed by seven of the bacterial strains over 30 min.** The rate of RDX removal over this time, and the ratio of the two is also shown.

Bacterial isolate	RDX removed over 30 min (nmoles/min/g cells)	Nitrite formed over 30 min (nmoles/min/g cells)	Nitrite formed / RDX removed over 30 min
Strain 11Y	$28.5 \pm 3.7$	$48.7 \pm 3.6$	1.7
Strain HS2	$16.0 \pm 13.1$	$5.5 \pm 0.5$	0.3
Strain HS4	$0.9 \pm 4.0$	$3.2 \pm 0.4$	3.4
Strain HS5	$16.5 \pm 3.6$	$5.4 \pm 0.2$	0.3
Strain HS19	$80.6 \pm 3$	$6.9 \pm 0.1$	0.1

The strains producing nitrite were generally not found to be those which removed the RDX most rapidly. The exception to this is strain 11Y which produced approx. 7 times as much nitrite as the next highest producing strain over 30 min. Comparing the amount of nitrite formed with the amount of RDX removed by these strains, it can be seen that there is no obvious correlation (Table

3.10). This is also evident from strains HS15-HS18, which degraded up to 80 nmoles RDX/min/g cells without the production of any nitrite above control levels. These results indicate that nitrite may not be produced by all bacteria from RDX breakdown and if it is, the rates at which it is removed vary greatly.

### 3.4 Discussion

Nineteen bacterial strains capable of utilizing RDX as a sole source of nitrogen have been isolated from explosive contaminated soil. Previous literature has indicated that the isolation of aerobic RDX degrading bacteria is not easy: in 1981 it was stated that RDX degradation could not occur aerobically<sup>199</sup>, and each study to date has reported the isolation of only one aerobic strain from enrichments. Very few pure aerobic bacterial strains have been described as being able to degrade RDX (§1.7.2.3). The large number of bacteria isolated in this study shows that there are many environmental bacteria which can degrade RDX and utilize it as a sole source of nitrogen, and that their isolation by selective enrichment is possible.

The inability of any of the strains to use RDX as a carbon source is not surprising, as it can be considered to be a one-carbon substrate, in that no breakdown products of RDX are likely to contain carbon-carbon bonds. Only methylotrophic bacteria are able to utilize these one-carbon substrates for growth<sup>188</sup>, and methylotrophy is not a universal property of bacteria. Bacterial utilization of RDX as a carbon source has never been documented, and this activity would not be expected unless an RDX degrading methylotroph were isolated.

Xenobiotic degrading strains isolated from contaminated soil are often found to be species of *Pseudomonas* or *Rhodococcus*, and the selective enrichment conditions used should have been favourable for both. Pseudomonads, rhodococci and closely related species have been found to be capable of the degradation of the herbicide atrazine, phenols, halogenated phenols, chlorinated herbicides and substituted benzenes<sup>16, 91, 124, 325, 339</sup>.

Initial analysis of the strains, using phenotypic characteristics, showed that all strains were beige and had smooth colonies, and that several had a mucoid phenotype. It is not uncommon for colony morphology to change depending on the medium provided<sup>38</sup> which explains the observation that several of the strains became more mucoid on plating onto minimal medium plates containing RDX. Gram reaction determination showed that all isolates are gram positive. Identification was performed using 16S rDNA comparisons; this showed that all strains of the bacteria are strains of rhodococci, which is consistent with the above characteristics<sup>36</sup>.

The genus *Rhodococcus* is found within the order *Actinomycetales*, and comprises twelve described species<sup>111</sup>. Actinomycetes in general are well represented in the soil microbial population and are commonly found in soil at counts of 10<sup>6</sup> per gram, but are not limited to a soil habitat, also being isolated from activated sludge, freshwater and marine habitats<sup>110</sup>. Rhodococci are defined by several characteristics: gram positive, aerobic, non-motile, high G+C content and the ability to

metabolize many substrates; however they are generally defined by their cell wall and envelope composition<sup>36</sup>. More recently, 16S rDNA analysis has become the method of choice for the determination of phylogenetic relatedness, as transfer of 16S genes between species is very rare, and the variation between 16S genes of different species is representative of the variation between the genomes<sup>111</sup>.

Rhodococci have been reported to be able to degrade many xenobiotics including phenols, halogenated phenols, aromatic acids, halogenated alkanes, substituted benzenes, anilines and quinolines<sup>91</sup>. In addition, there are reports of the biotransformations of hydrocarbons, aromatic compounds, nitriles and epoxides<sup>325</sup>. Various rhodococcal species can transform or degrade pentachlorophenol<sup>311</sup>, the herbicides atrazine and s-ethyl dipropylthiocarbamate<sup>16</sup>, and polychlorinated biphenyls<sup>197</sup>. Strains of *R. rhodochrous* have xenobiotic degrading abilities towards styrene and toluene<sup>324</sup>, 2-ethoxyphenol and 4-methoxybenzoate<sup>163</sup>, and 1-chloroalkane<sup>179</sup>. The biotransformations of nitriles by rhodococci is of particular interest, as rhodococcal nitrile hydrolases can catalyse the production of acrylamide from acrylonitrile<sup>46</sup>, which is being exploited industrially through the use of *R. rhodochrous* J1 in acrylamide manufacture<sup>172</sup>. Rhodococci may possess a lignin degrading system, similar to that reported in *Phanaerochaete chrysosporium* (§1.7.2.2), which may be responsible for their ability to degrade such a wide range of compounds<sup>64</sup>,  
110.

The identification of all the isolates as rhodococci, in addition to the reported rhodococcal RDX degrader, strain DN22<sup>59</sup>, indicates that the majority of culturable environmental bacteria possessing the ability to degrade RDX are rhodococci. This could be due to specific features of the strains that enable, for example, uptake of the explosive, or could be due to species specific horizontal gene transfer within the environment. The genomes of rhodococci have been reported to be very flexible, readily undergoing recombination and conjugation, with most strains carrying one or more plasmids, which may increase the likelihood of gene transfer occurring<sup>189</sup>.

The isolation of all gram positive bacteria presents some difficulties with respect to molecular genetical analysis, as their genetics have not been well studied in general and there are few tools available for use with them. Gram positive bacteria also tend to be less easy to manipulate than gram negative bacteria in terms of transformation and protein purification.

The antibiotic resistance profiles showed that all isolates are unique, as none of the strains within the same 16S rDNA classification groups have identical profiles. This information may also be useful in cloning work to aid the choice of vectors for use in these strains in possible knockout

and complementation tests. In particular, as most strains were sensitive to the concentrations of chloramphenicol, gentamycin, rifampicin, tetracycline and vancomycin tested, vectors conferring resistance to these antibiotics will be of use. Few of the RDX degrading bacterial isolates were able to tolerate TNT up to its solubility limit of 0.65 mM. When growth did occur at this concentration it was only when the cells were present at a high density. This may be due to the possible lack of exposure of the isolates to TNT in the environment.

Rhodococcal xenobiotic degradation genes are sometimes found on large plasmids<sup>16, 72, 76, 176, 179</sup>. A plasmid of undetermined size is reported to carry the RDX degrading genes of *Rhodococcus* sp. strain DN22<sup>60</sup>. Of the reported rhodococcal plasmids, many are in the size range 80 – 200 kb<sup>69, 72, 76, 179</sup>, and some of these are linear. A comprehensive list demonstrating the vast range in plasmid size, can be found in Larkin et al.<sup>189</sup>. One strategy for identifying the genes which allow the bacterial isolates to carry out RDX degradation would be to cure the bacteria of the plasmids and, if the bacteria lose their ability to utilize RDX, to isolate the genes responsible from the plasmid, which narrows the search.

Although many of the bacterial isolates were found to carry plasmids, it was not possible to isolate plasmids from all the strains, indicating that some of the strains may carry RDX degrading genes within their genomic DNA. The size of the extracted plasmids was not determined, but their extraction was not achieved using the boiling lysis method, which may have been due to their large size. The estimation of plasmid size by gel electrophoresis would require the extraction and electrophoresis of several large plasmids of known size with which to create a standard curve, although using this method the size determinations are only approximate<sup>128</sup>. The strains may have also contained linear plasmids, which are sometimes found in rhodococci<sup>189</sup> and can carry degradation genes<sup>102</sup>. These would probably have not been isolated by the method used, as it is thought that the extracted plasmids are in the covalently closed circular form, which would not be produced by linear plasmids. As linear plasmids were not analysed, and some of the strains may not have been carrying plasmids, it was decided not to attempt either a plasmid curing or a plasmid gene library approach, although care should be taken in future to isolate total DNA and avoid the loss of large plasmids in any DNA preparations.

Comparisons of the rates at which all bacterial isolates removed 250 µM RDX from resting cell incubations showed a wide range of values, with six of the strains operating at significantly higher rates than the rest. Several showed very little or no RDX removal, which may have been to do with harvesting at less than optimal growth stages. The stage of growth may have significantly

affected the rate at which the strain was able to break down RDX, with particular relevance to strain HS14. However, it was not possible to perform resting cell incubations for each strain throughout the stages of growth, and for that reason the experiment was standardized. There are few reports of resting cell incubations of pure aerobic strains with RDX with which to compare these strains. *Stenotrophomonas maltophilia* strain PB1 would only degrade RDX in the presence of sugars, and removed up to 40 µg/ml (180 µM) over 15 h<sup>27</sup>. *Rhodococcus* sp. strain DN22 removed 150 µM RDX over 3.5 h, 100 µM of that within the first hour<sup>59</sup>. The fastest of the isolates from this study compare favourably with these values, showing removal of up to 250 µM within 1 h.

There was no correlation between the amounts of nitrite produced and RDX removed, which could be due to differing activities of nitrite reductase, or alternate mechanisms of RDX degradation.

It is worth noting that the more mucoid strains failed to form pellets as tight as the less mucoid strains during harvesting and the subsequent rinses. This may have resulted in carry over of buffer during wet weight determination meaning that equivalently less cell paste was added to each sample and a lower rate of RDX removal was observed. However, given the experimental design, this was unavoidable.

It was necessary to select one bacterial strain for further study, as not all the isolated bacteria could be characterized in detail given the time limitations of the project. The characteristics of the chosen isolate should allow further studies to be relatively fast and easy, and allow cloning within that bacterium if a mutation and complementation approach were to be taken. A desirable strain would therefore degrade RDX quickly, possess suitable antibiotic sensitivity profiles for plasmid transformation and be a non-mucoid strain, as these tend to be easier to transform (Prof. E. Dabbs, personal communication).

For these reasons, *Rhodococcus rhodochrous* strain 11Y was chosen for further study. This is not a mucoid strain, has a good rate of RDX removal from both growth medium (1.5 days) and resting cell incubations, degrading a significant amount of RDX over a 60 min incubation (sixth best strain). In addition, strain 11Y was sensitive to the lowest concentrations tested of potentially useful antibiotics. A preliminary survey of *E. coli* - *Rhodococcus* shuttle vectors shows that many rely on conferring resistance to ampicillin (equivalent to carbenicillin), chloramphenicol or kanamycin<sup>70, 73, 77, 79, 85, 135, 146, 270, 317, 349</sup>. Strain 11Y was sensitive to all these at the following concentrations:

carbenicillin (25 µg/ml), chloramphenicol (10 µg/ml) and kanamycin (15 µg/ml). These properties favour strain 11Y as a potential plasmid host, if this should be required.

If metabolite identification from the whole cell breakdown of RDX is to be attempted, strain 11Y may also be a good choice as it produced large amounts of nitrite in resting cell incubation. This, and other metabolites, could be quantified and used to compare to proposed routes of RDX degradation. Strain 11Y was therefore chosen for further investigation.

The results demonstrate the relative ease of isolation of RDX degrading bacteria, and the prominence of rhodococci among these. Future comparison of these strains may provide information on whether the genetic basis of the ability to degrade RDX is common to all the isolates, and how it may have been transferred between strains. However, this is beyond the scope of this project. Further characterization of *Rhodococcus rhodochrous* strain 11Y is required.

## Chapter 4. Characterization of *Rhodococcus rhodochrous* strain 11Y on RDX

### 4.1 Background

Twenty RDX degrading bacteria were compared in the last chapter and of these one strain has been chosen for further characterization: *Rhodococcus rhodochrous* strain 11Y. The ability of strain 11Y to degrade RDX as a sole nitrogen source shall be investigated, and this may indicate its potential for use in bioremediation. The speed of RDX degradation can be assessed by measuring growth on RDX, and the amount of nitrogen used for growth per molecule of RDX provided can be calculated by comparing growth of strain 11Y on RDX with growth on an alternative nitrogen source. Preliminary characterization of the regulation of the RDX degrading ability can be performed by determining whether the activity is induced in the presence of RDX.

It is also useful to investigate the ability of strain 11Y to degrade other nitramine explosives with similar structures to RDX. This may prove useful for future remediation purposes, and might give information on the mechanism of degradation. Few studies have addressed the biodegradation of HMX, and there is no literature regarding microbial action on CL20 (§1.6.3).

Studying the mechanism of RDX biodegradation can provide information on the fate of the RDX: whether it is being mineralized, or degraded to other compounds which may themselves be recalcitrant or toxic. It is therefore necessary to analyse the breakdown products of RDX by strain 11Y. These can then be used to compare to products seen in other studies, and to determine whether any of the currently proposed pathways for RDX biodegradation are applicable to strain 11Y.

## **4.2 Materials and methods**

### **4.2.1 Growth of strain 11Y on RDX as a nitrogen source**

*Rhodococcus rhodochrous* strain 11Y was grown in 1 litre minimal medium with three carbon sources and 0.25 mM RDX as the sole source of nitrogen at 30 °C, inoculated with a 24 h seed culture which had been grown in the same medium, under the same conditions. Samples of 2 ml were removed from the cultures at regular intervals throughout the growth cycle. Bacterial growth was measured by determination of culture optical density at 600 nm, with appropriate dilution of the culture when necessary. Degradation of RDX was assayed by monitoring the decrease in concentration by HPLC. Bacterial cells were removed by centrifugation and the supernatant (100 µl) analysed by HPLC. Nitrite and ammonium concentrations were assayed using ion chromatography. Controls consisted of a sterile culture, and a culture of the non RDX degrader *R. rhodochrous* strain CW25.

### **4.2.2 Determination of nitrogens from RDX used by strain 11Y**

Strain 11Y was grown on minimal medium containing three carbon sources and varying concentrations of NH<sub>4</sub>Cl and RDX. Concentrations of NH<sub>4</sub>Cl used were 0, 0.25, 0.5, 0.75, 1, 1.25 and 1.5 mM. Concentrations of RDX used were 0, 0.042, 0.083, 0.125, 0.167, 0.208 and 0.25 mM which correspond to 0, 0.25, 0.5, 0.75, 1, 1.25 and 1.5 mM nitrogen supplied respectively. Growth was measured regularly over three weeks using OD<sub>600</sub>, and the maximum OD reached by each culture recorded and plotted.

### **4.2.3 Inducibility of RDX degrading ability**

Strain 11Y was grown from a 4 day starter culture in minimal medium containing three carbon sources and either 3 mM NH<sub>4</sub>Cl or 1 mM RDX as a sole source of nitrogen at 30 °C for 2 days. HPLC was used to confirm that all RDX had been removed from the RDX grown culture. Cells were harvested and rinsed twice in 40 mM phosphate buffer pH 7.2. Cells were resuspended in phosphate buffer to 0.5 g wet weight/ml, and resting cell incubations were performed, with the samples assayed for RDX and nitrite concentration by HPLC and Griess assay (§3.2.9).

### **4.2.4 Strain 11Y tolerance of HMX and CL20**

Strain 11Y was streaked onto LA plates containing CL20 or HMX at concentrations of 0, 0.5, 1, 5 and 10 mM. Growth was assessed after 5 days and 8 days.

#### **4.2.5 Determination of growth on HMX and CL20 as nitrogen sources**

Strain 11Y was used to inoculate minimum medium containing varying concentrations of  $\text{NH}_4\text{Cl}$ , HMX, CL20 and no nitrogen source. HMX and CL20 were diluted from stocks routinely made in DMF. Concentrations used were:  $\text{NH}_4\text{Cl}$  – 0.5, 1, 1.5 mM, CL20 – 0.5, 1, 5 mM, HMX – 1, 5 mM.  $\text{OD}_{600}$  values were measured regularly over a period of 3 weeks, and the maximum values recorded. A control using DMF as a sole nitrogen source was performed.

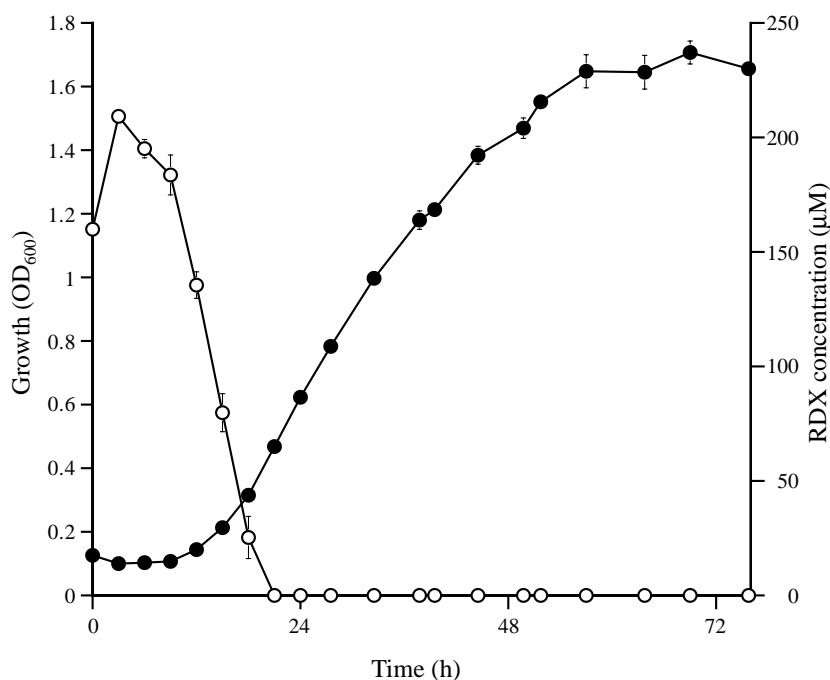
#### **4.2.6 Resting cell incubations of strain 11Y**

Cells were grown to late-log phase (48 h), harvested and rinsed in 40 mM potassium phosphate buffer, pH 7.2. Cells were used at a final concentration of 0.05 g wet weight/ml and incubated in 25 ml 40 mM potassium phosphate buffer pH 7.2 with 0.25 mM RDX at 30 °C with inversion every 5 min. Samples were taken at intervals, centrifuged immediately at 4 °C for 1 min and the supernatant was assayed immediately on the anion Dionex column for nitrate, nitrite and formate. Further samples taken at the same time had an equal volume of 10 % v/v glacial acetic acid added followed by a 10 min incubation on ice to precipitate any protein present and the resulting supernatant was analysed for RDX by HPLC, for ammonium by cation Dionex, and for formaldehyde (§2.4.6). Controls contained either cells incubated in the absence of RDX, or RDX incubated in the absence of cells.

## 4.3 Results

### 4.3.1 Growth of strain 11Y on RDX as a nitrogen source

*Rhodococcus rhodochrous* strain 11Y was characterized with respect to its growth on RDX as a sole source of nitrogen. In addition to measurements of growth and RDX concentration, the growth medium was assayed for the presence of nitrite and ammonium. As the RDX is used as a source of nitrogen, it was thought that nitrogen containing metabolites such as nitrite and ammonium might appear in the medium before being utilized by the bacteria. Such metabolites have been seen during microbial RDX breakdown in previous studies<sup>30, 59, 95</sup> and strain 11Y has previously been shown to be able to utilize both as sources of nitrogen for growth (E. Travis, personal communication). Strain 11Y was grown in minimal medium containing RDX as a sole nitrogen source at the concentration of 0.25 mM, which was chosen to be below the aqueous solubility limit of 0.27 mM in order for the growth to be able to be determined by OD<sub>600</sub> measurements.



**Figure 4.1: Growth of strain 11Y with RDX as a sole nitrogen source.** Optical density at 600 nm (●) and RDX concentration (○). Figures shown are the average of triplicate cultures and error bars indicate  $\pm$  one standard deviation.

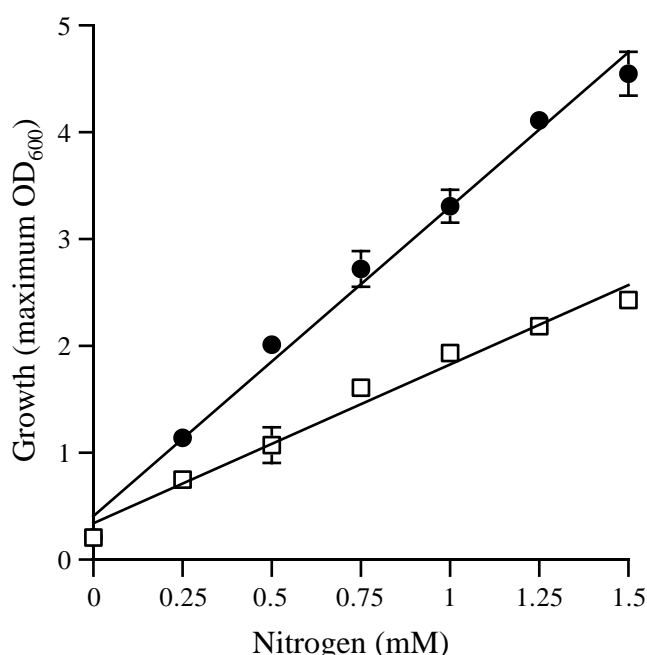
The growth curve confirmed that strain 11Y can utilize RDX as a nitrogen source for growth (Figure 4.1). RDX disappearance was complete within 21 h, and the OD<sub>600</sub> continued to rise until a

final value of 1.8 was obtained after approx. 60 h. Negative controls using a non RDX degrading strain showed no increase in  $OD_{600}$  and no loss of RDX over the course of the experiment. No appearance of ammonium or nitrite was seen in any of the cultures.

#### 4.3.2 Number of nitrogen atoms from RDX used by strain 11Y

In order to give an indication of how many atoms of nitrogen from RDX are used by strain 11Y, growth of strain 11Y in media containing varying concentrations of either RDX or ammonium (as  $NH_4Cl$ ) as the sole source of nitrogen was compared.

The maximum growth reached by strain 11Y in all cultures was measured (Figure 4.2). The values plotted are growth per mM of nitrogen supplied, as there are 6 nitrogens in each molecule of RDX. Therefore, a concentration of 1.5 mM  $NH_4Cl$  was equivalent to 0.25 mM RDX (1.5/6). Best-fit lines were added to both sets of data (using Microsoft Excel). The R-squared value, which gives an indication of how well the trend-line fits the data, was calculated for both lines. R-squared values can range between 0 – 1, with 1 indicating that all the points fall on the line. The  $NH_4Cl$  data gave a line with  $r^2 = 0.991$  and the RDX data gave a line with  $r^2 = 0.981$ ; these values show that the data can be represented by a straight best-fit line.

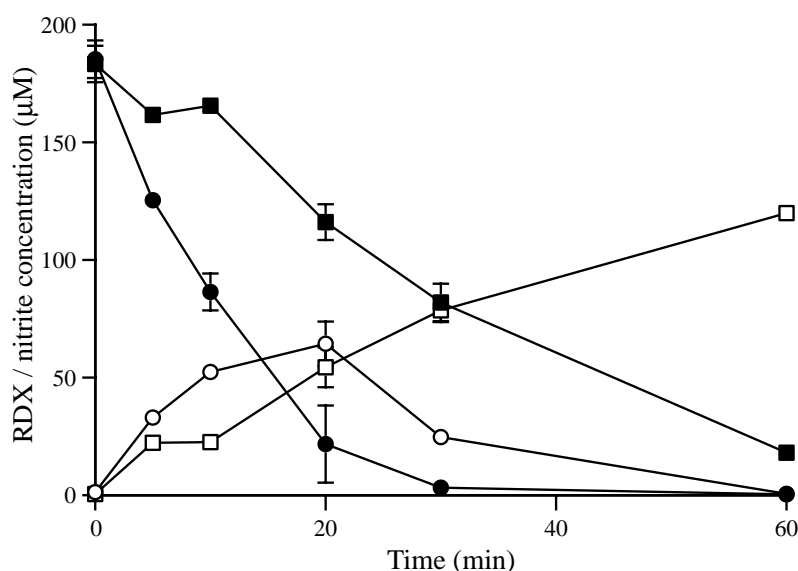


**Figure 4.2: Maximum growth of strain 11Y on RDX and ammonium.** Maximum  $OD_{600}$  on increasing concentrations of RDX (□) and ammonium (●). The RDX best-fit line has a gradient of 1.486 and  $r^2 = 0.981$ . The ammonium best-fit line has a gradient of 2.894 and  $r^2 = 0.991$ . Figures shown are the average of triplicate cultures and error bars indicate  $\pm$  one standard deviation.

Gradients of the trend-lines were calculated to compare the slopes of the lines. The gradient of the  $\text{NH}_4\text{Cl}$  best-fit line was found to be 2.894, and 1.486 for the RDX data. The ratio of these gradients ( $\text{NH}_4\text{Cl}$  : RDX) is 2.01: 1, which indicates that strain 11Y can obtain twice as much nitrogen from  $\text{NH}_4\text{Cl}$  than it can from RDX, for each mM of nitrogen present. These results strongly suggest that strain 11Y uses only 3 of the 6 nitrogen atoms from RDX for growth.

### 4.3.3 Inducibility of strain 11Y RDX degrading activity

In order to determine whether expression of the RDX degrading gene(s) is (are) induced, resting cell incubations were performed using strain 11Y which had been grown on ammonium (as  $\text{NH}_4\text{Cl}$ ) or RDX. RDX grown cells should have high levels of any inducible enzymes, compared to the  $\text{NH}_4\text{Cl}$  grown cells. Strain 11Y was grown on 1 mM RDX or 3 mM  $\text{NH}_4\text{Cl}$  as sole sources of nitrogen, which should contain equivalent amounts of nitrogen for growth and provide equal biomass (§4.3.2). Cells were harvested from both cultures after all the RDX from the RDX grown culture had been removed, as assayed by HPLC. This ensured no carry over of RDX, and indicated that the cells were in mid-log phase (§4.3.1). Wet weights of cells were determined: 2.3 g from the RDX culture, and 1.7 g from the  $\text{NH}_4\text{Cl}$  culture. Resting cell incubations with RDX using cells from both cultures were performed, and the concentrations of RDX and nitrite assayed.



**Figure 4.3: Resting cell incubation of strain 11Y previously grown on RDX or  $\text{NH}_4\text{Cl}$ .** RDX grown strain 11Y RDX concentration (●) and nitrite concentration (○).  $\text{NH}_4\text{Cl}$  grown strain 11Y RDX concentration (■) and nitrite concentration (□). Figures shown are the average of duplicate cultures and error bars indicate  $\pm$  one standard deviation.

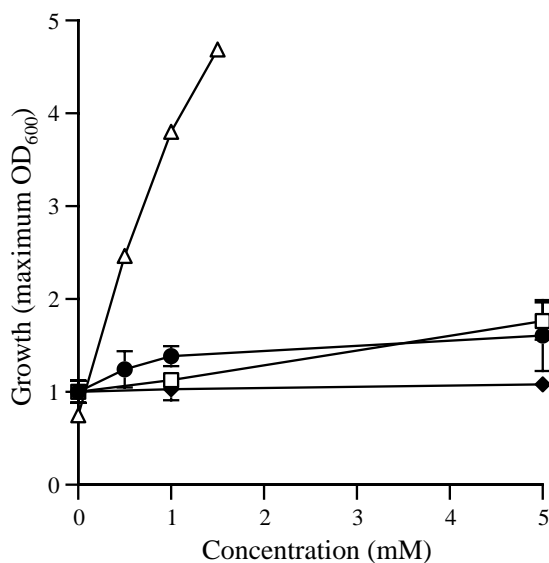
RDX degradation occurred to a significant extent in both samples (Figure 4.3), indicating that RDX degrading enzyme(s) are produced in the absence of RDX. However, the removal of RDX was increased approx. three-fold in RDX grown cells: time taken to remove approx. 100  $\mu\text{M}$ : 10 min (RDX grown), 30 min ( $\text{NH}_4\text{Cl}$  grown); time taken to remove approx. 165  $\mu\text{M}$ : 20 min (RDX), 60 min ( $\text{NH}_4\text{Cl}$ ).

Nitrite was seen to accumulate, and the pattern of its production differs between the two samples. Nitrite increases in a similar way for both samples over the first 30 min and then decreases in the RDX grown samples, but continues to increase in the  $\text{NH}_4\text{Cl}$  grown samples (Figure 4.3).

#### 4.3.4 Growth of strain 11Y on other nitramine explosives

The growth of strain 11Y on two further nitramine explosives, HMX and CL20, was investigated. The tolerance of strain 11Y to both compounds was initially tested. Each explosive was added to LA plates up to concentrations of 10 mM and the plates were then streaked with strain 11Y. The bacterium grew at the same rate, and to the same degree, at all concentrations of both nitramines compared to LA in the absence of either compound, indicating that neither compound appears to affect the growth of strain 11Y (data not shown).

The ability of strain 11Y to utilize these two explosives was assayed using minimal medium containing the compounds as sole nitrogen sources at concentrations between 0 – 5 mM. The maximum  $\text{OD}_{600}$  values reached over a period of 3 weeks are plotted against the total nitrogen concentration in Figure 4.4. Growth of strain 11Y was not observed on the nitramines CL20 and HMX. A slight increase in the optical density at 600 nm is noticeable as the concentrations of the nitramines increase, which is attributable to their insolubility causing light scattering at 600 nm. The positive control of  $\text{NH}_4\text{Cl}$  showed growth and the negative control of DMF showed that no significant growth was occurring on the solvent in which the explosives were dissolved. Zone of clearing plates were also made containing 5 mM each of HMX and CL20, and no zones of clearance were observed when strain 11Y was either streaked or spotted at a high cell density (§3.2.3) onto them. These results indicate that strain 11Y cannot release any nitrogen from either HMX or CL20 for growth, despite being able to grow on the very similar compound RDX.



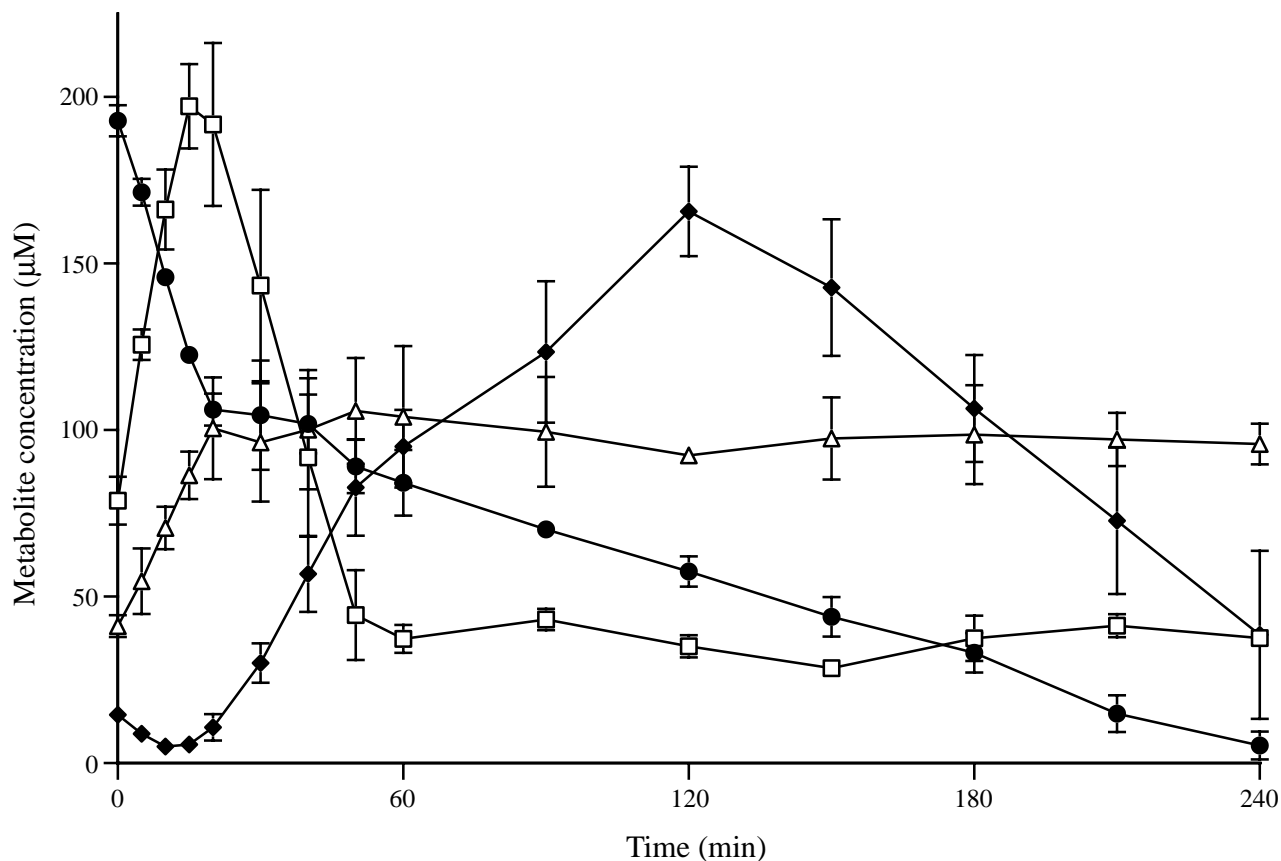
**Figure 4.4: Growth of strain 11Y on nitramine explosives CL20 and HMX.** The maximum OD<sub>600</sub> on increasing concentrations of CL20 (●), HMX (□), DMF (◆) and NH<sub>4</sub>Cl (Δ). Figures shown are the average of triplicate cultures and error bars indicate  $\pm$  one standard deviation.

#### 4.3.5 Products of resting cell incubations of strain 11Y with RDX

In order to give an indication of the mechanism of RDX breakdown by strain 11Y, and to see if it fits the pathways proposed in the literature (§1.7.2), some of the common products of RDX degradation were analysed. The nitrogen containing metabolites nitrite and ammonium were not seen during the growth of strain 11Y on RDX (§4.3.1), which may indicate either that they are not products of RDX degradation by strain 11Y, or that they are rapidly metabolized by the cells. Nitrite was identified during resting cell incubations (§3.3.7.2 and §4.3.3). To clarify this, resting cell incubations were performed. Metabolites should accumulate using this system, as the cells should not be taking products up for growth. Metabolites assayed for were nitrite, nitrate, ammonium, formate and formaldehyde.

The RDX and metabolite concentrations over the course of the resting cell incubation are shown in Figure 4.5. Metabolites which appeared during the experiment were nitrite, formate and formaldehyde. RDX disappeared over 4 h, although the rate of RDX disappearance slowed considerably after 20 min. Nitrite appeared transiently in approx. equimolar concentrations with the original concentration of RDX within 15 min, and then decreased. Production of formaldehyde was slower, but it remained in the supernatant, and formate was produced over a longer period of time and subsequently declined. The concentration of ammonium produced was not above that seen in controls (0-8  $\mu$ M), and no nitrate was produced. Controls consisted of incubations of RDX without

cells, or cells without RDX. These showed low levels of formate (maximum 22  $\mu\text{M}$  formed from cells in the absence of RDX) and formaldehyde (maximum 33  $\mu\text{M}$  formed from cells in absence of RDX). No nitrite was observed and no RDX was degraded in the absence of strain 11Y. No other products were observed using the HPLC and ion chromatography procedures described.



**Figure 4.5: Resting cell incubation products of strain 11Y on RDX.** RDX concentration (●) nitrite concentration (□), formate concentration (◆), and formaldehyde concentration (△). Figures shown are the average of triplicate cultures and error bars indicate  $\pm$  one standard deviation.

## 4.4 Discussion

*Rhodococcus rhodochrous* strain 11Y has been found to grow on, and remove 250  $\mu\text{M}$  RDX from culture within 21 h. Comparing this rate of RDX disappearance to those using other strains and consortia is made difficult by the different conditions and concentrations of RDX used. Most reported anaerobic consortia and pure strains take from a week to over a month to produce significant decreases in RDX concentration<sup>30, 32, 98, 103, 199, 274, 275, 308, 345</sup>. The aerobic fungus *Phanerochaete chrysosporium* degraded approx. 96 % of the 0.125  $\mu\text{M}$  RDX in its growth medium over 30 days<sup>89</sup>, taking 60 days when the RDX concentration was increased to 280  $\mu\text{M}$ <sup>276</sup>. Aerobic strains appear to be able to degrade RDX more quickly: 180  $\mu\text{M}$  within 32 h using a coryneform bacterium<sup>338</sup>, 230  $\mu\text{M}$  within 160 h using *Stenotrophomonas maltophilia* strain PB1<sup>27</sup>, and 200  $\mu\text{M}$  within 40 h using the unidentified strain A<sup>157</sup>. Comparing strain 11Y to a similar bacterial strain, the rate at which it removes RDX is similar to that of *Rhodococcus* sp. strain DN22 which eliminated 160  $\mu\text{M}$  RDX within 20 h<sup>59</sup>. A lag period of approx. 12 h was observed with strain 11Y, even though the bacteria used to inoculate the media had been grown on RDX. It is possible that the bacteria in the starter culture had reached stationary phase, which would explain the lag. The growth of the bacteria for many hours after the disappearance of RDX is probably due to the continued utilization of nitrogen containing products of RDX degradation. The attempt to identify whether nitrite or ammonium accumulated in the growth medium and were possibly responsible for this continued growth showed that neither was present throughout the course of the experiment. This would imply either that other nitrogen containing products must be present, or that any nitrogen source was not released by the bacteria into the medium, being metabolized slowly intracellularly to allow growth to continue for a further 36 h.

Comparing growth on ammonium with growth on RDX indicated that strain 11Y uses 3 moles of nitrogen from each mole of RDX. The same ratio has been observed with other aerobic bacterial systems<sup>27, 59</sup>. With the data obtained, it is impossible to tell which form(s) the nitrogen takes before being utilized by the bacteria.  $\text{N}_2\text{O}$  has been proposed as a significant product of RDX breakdown<sup>276</sup>, and may account for some or all of the nitrogens not taken up. One of the products of RDX degradation which is likely to be used as a nitrogen source by the bacteria is nitrite, which was identified in the growth culture of *Rhodococcus* sp. strain DN22<sup>59</sup> but not that of strain 11Y, although it has been identified during resting cell incubations of RDX with strain 11Y. The absence of nitrite from the growth medium may be due to a more efficient nitrite reductase in strain 11Y than strain DN22, leading to a more rapid turnover of nitrite.

Many bacterial systems which enable bacteria to degrade xenobiotics are induced by the compound which they can degrade. These include the styrene oxidizing enzyme from *Rhodococcus rhodochrous*<sup>324</sup>, the triazine hydrolase from *R. corallinus*<sup>209</sup>, the naphthalene dioxygenase of *Rhodococcus* sp.<sup>4</sup>, the pyrene degradation genes in *Mycobacterium*<sup>165</sup> and ETBE degrading enzymes in *R. ruber*<sup>53</sup>. In the cases when induction in these strains was measured by activity, it was undetectable in the absence of the inducer, increasing greatly in its presence, and when measured by SDS-PAGE there was a dramatic increase in intensity of a specific band after induction. RDX degradation has previously been observed to be inhibited in the presence of ammonium, using strains of corynebacteria<sup>338</sup> and rhodococci<sup>59</sup>. In this latter study, resting cell incubations performed with cells grown on ammonium showed very little RDX removal compared to cells grown on RDX, although cells grown on nitrite or nitrate showed an intermediate level of RDX removal<sup>59</sup>. Binks et al.<sup>27</sup> observed diauxic growth on minimal medium containing both RDX and an additional nitrogen source, using a mixed culture containing *S. maltophilia* strain PB1. The activity towards RDX in strain 11Y was seen to be present in cells grown on ammonium, and it increased approx. three-fold in cells grown on RDX. This indicates that biodegradation of RDX using strain 11Y will occur even in the presence of alternative nitrogen sources, which may make strain 11Y a useful strain for investigation. Nitrite was produced during these resting cell incubations, although decreased after 30 min using cells grown on RDX, and continued to accumulate using cells grown on ammonium. The differences in nitrite uptake may indicate the upregulation of an enzyme required to utilize the nitrite, such as an assimilatory nitrite reductase, whose involvement has also been invoked in a previous study<sup>59</sup>, and which has been found to be inducible by nitrite or nitrate in many bacterial species<sup>194</sup>.

There are several other explosives containing the same nitramine group as RDX. *R. rhodochrous* strain 11Y was not able to grow on the two nitramine compounds tested. Both HMX and CL20 were supplied to strain 11Y as sole sources of nitrogen, and no growth was observed. Whether this is to do with an inability of the larger molecules to interact with an enzyme active site, or an inability to get into the cell in the first place could only be determined using purified enzyme. There is only limited information regarding the biodegradation of these compounds, with no reports of biological action on CL20 and only a few reports of HMX degradation by aerobic and anaerobic consortia<sup>130, 139</sup>.

Metabolites identified during cell growth or in resting cell incubations can give clues to the mechanism of RDX degradation. Anaerobic biodegradation of RDX has been reported to occur

through sequential reduction of the nitro groups to nitroso, followed by possible, unstable, hydroxylamino intermediates and ring cleavage (Figure 1.6). This mechanism was postulated by McCormick et al.<sup>199</sup> using anaerobic sludge, and some or all of the nitroso intermediates have been seen in other anaerobic studies<sup>98, 137, 167, 345</sup>. End products of this route appear to include formaldehyde or methanol and potentially toxic hydrazine derivatives, and it has been suggested that a type I nitroreductase is responsible for this activity<sup>168</sup>. However, aerobic bacterial degradation would seem not to follow this route as no nitroso intermediates have been observed during the aerobic degradation of RDX using bacteria.

The metabolites identified during the degradation of RDX by strain 11Y are nitrite, formaldehyde and formate. The production of small organic molecules, such as formaldehyde and formate indicate that the RDX has been degraded rather than transformed. All the above metabolites have been identified in the breakdown of RDX by thermal decomposition, alkaline hydrolysis and photolysis (§1.7.1), and of the mechanisms proposed for the degradation of RDX by these routes, two involve the initial cleavage of the N-N bond (Figures 1.4 and 1.5). This mechanism would fit in with the observation that the first metabolite produced during the resting cell incubations with strain 11Y was nitrite. Denitration of RDX as a first, important, step in its breakdown has been suggested in a scheme put forward by Fournier et al.<sup>95</sup> working on *Rhodococcus* sp. strain DN22 (§1.7.2.3). This reaction would destabilize the RDX to the extent that a spontaneous ring cleavage would occur<sup>138</sup>. Using strain DN22, products observed include nitrite, nitrous oxide, ammonium, formaldehyde, carbon dioxide and a dead end product (Figure 1.8), which do not correlate entirely with those seen using strain 11Y. The differences may be due to different competing reactions within the bacteria after the initial denitration.

No formate is observed during RDX degradation by strain DN22, but it is produced by strain 11Y. This may be because strain DN22 possesses a more efficient formate dehydrogenase than strain 11Y; formate dehydrogenase enzymes are widely found in bacteria, catalysing the oxidation of formate to carbon dioxide<sup>234</sup>. Previous work on RDX degradation using anaerobic sludge has identified formate as a product, and proposed a role for acetogens in its formation from formaldehyde<sup>138</sup>. This reaction is catalysed by formaldehyde dehydrogenase enzymes, which are ubiquitous, found in plants, animals and bacteria<sup>12, 122</sup>, and an inducible formaldehyde dehydrogenase has been characterized in *Rhodococcus erythropolis*<sup>86</sup> which is closely related to strain 11Y. Therefore it seems likely that this reaction could be occurring within strain 11Y. However, the formate may be produced independently of the formaldehyde, as the formaldehyde

concentration does not decrease during the resting cell incubation, while the formate concentration increases. Both formate and formaldehyde are produced from the alkaline hydrolysis, photolysis and thermal decomposition of RDX<sup>62, 138, 148, 229</sup>, indicating that formate can result directly from RDX breakdown.

Ammonium was absent from the resting cell incubations with strain 11Y, although it was formed from RDX breakdown by strain DN22. Strain 11Y may not produce ammonium from the breakdown of RDX, but if it does its assimilation, through the action of the enzymes glutamine and glutamate synthetase<sup>205</sup>, may be faster in strain 11Y than strain DN22. Nitrous oxide was not assayed for, and no other intermediates or dead end metabolites were observed using the HPLC parameters described.

The decrease in the rate of RDX disappearance after 20 min of the incubation could be due to the metabolites affecting the cells deleteriously. In particular, a large amount of nitrite was produced at that time and it may have toxic effects on the bacteria<sup>247</sup>. Further characterization of this pathway is necessary to ensure that full mineralization is occurring, meaning that there are no dead end products, and that no toxic by-products accumulate. Given that toxic nitroso compounds are formed during anaerobic biodegradation, and a dead end product is found using *Rhodococcus* strain DN22, use of strain 11Y may be confirmed to be the safest method of RDX bioremediation characterized to date.

## Chapter 5. Cloning, sequencing and analysis of the gene cluster responsible for RDX degrading ability

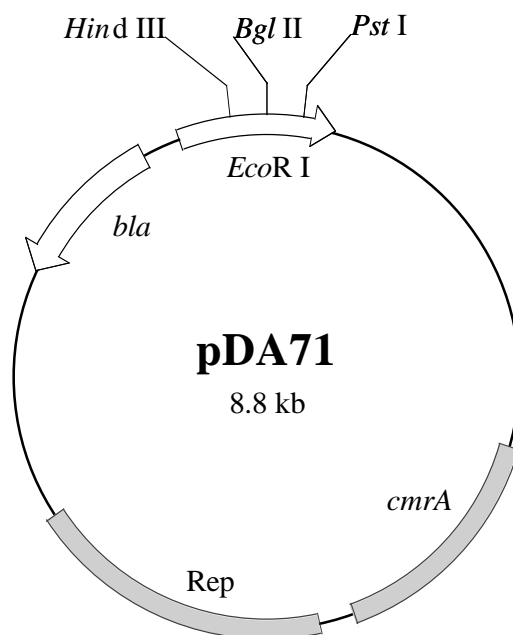
### 5.1 Background

The characterization of *Rhodococcus rhodochrous* strain 11Y with respect to its growth on RDX has been described, and some of the metabolites it produces from the degradation of RDX have been elucidated. This chapter describes the cloning of the gene(s) responsible for the RDX degradation using a molecular genetic approach. This will involve the creation of a DNA library from strain 11Y and the transfer of the library to a non RDX degrading host in order to obtain clones which have gained the ability to degrade RDX .

The molecular biology and genetics of rhodococci are not well characterized and few tools for genetic manipulation have been developed for use within this genus. *Escherichia coli* is the preferred host for genetic manipulation as it has been more thoroughly characterized and there are more tools available for use with it, so several *E. coli* – *Rhodococcus* shuttle vectors have been developed, which are able to be maintained in both strains. The plasmids can be manipulated in the more amenable *E. coli* host, and transferred to a rhodococcal host where genes of rhodococcal origin are more likely to be expressed. *E. coli* – *Rhodococcus* shuttle vectors are created from cryptic rhodococcal plasmids (and their derivatives) fused to *E. coli* cloning plasmids such that origins of replication for maintenance in both organisms are present<sup>70, 73, 77, 79, 85, 135, 146, 270, 317, 349</sup>. These vectors have been used to clone rhodococcal genes involved in dibenzothiophene desulphurization<sup>230</sup>, herbicide EPTC degradation<sup>271</sup>, isopropylbenzene degradation<sup>164</sup>, rifampicin resistance<sup>7</sup>, azo dye degradation<sup>143</sup> and cocaine degradation<sup>40</sup>. Such shuttle vectors have also been used to introduce fragments into rhodococcal hosts for heterologous expression of proteins from related species, or to study upstream sequences<sup>174, 175, 193, 198, 210, 272</sup>.

The *E. coli* – *Rhodococcus* shuttle vector pDA71 (or its predecessor pDA37<sup>70</sup>) was used in the cloning of four of the rhodococcal genes listed above, and was chosen for library construction in this study. Plasmid pDA71 (Figure 5.1) was constructed from pEcoR251<sup>35</sup> and rhodococcal plasmid pDA21<sup>70, 71, 238</sup>. The plasmid carries a gene for ampicillin resistance (*bla*) and a positive selection system. If the *EcoR* I suicide gene is not disrupted by the insertion of a fragment of DNA in one of its unique cloning sites, *Bgl* II, *Hind* III or *Pst* I, *EcoR* I endonuclease is produced which cleaves the host cell DNA<sup>54</sup>. Thus only plasmids carrying an insert allow their host to survive when

grown on ampicillin or carbenicillin. This positive selection system can be used to select for insert-carrying plasmids in *E. coli*, and this library can later be transferred to a rhodococcal host.



**Figure 5.1: Plasmid map of pDA71.** pDA71 is an *E. coli* - *Rhodococcus* shuttle vector containing cloning sites within the *EcoR* I endonuclease gene. This suicide gene is inactivated when an insert is present. The *bla* gene, which confers ampicillin resistance to *E. coli* hosts, the rhodococcal replicon, Rep, and the *cmrA* gene, which confers chloramphenicol resistance to rhodococcal hosts, are shown. The *E. coli* elements in pDA71 are from pBR322, and the construction of the plasmid is discussed to Dabbs, 1998<sup>71</sup>. Only unique restriction sites which disrupt the *EcoR* I gene are shown.

Many methods for the transformation of rhodococci have been developed, involving either electroporation or the use of protoplasts. In a survey of papers describing the transformation of gram positive bacteria, none of the protocols appears to be efficient for all strains, and many papers describe the optimization of conditions for specific strains. Electroporation is the most commonly used method for the transformation of gram positive bacteria<sup>79, 83, 135, 144, 146, 160, 164, 184, 197, 206, 216, 266, 270, 314</sup> and protoplast methods have also been described, involving the removal of the cell wall prior to transformation to aid DNA uptake<sup>69, 317</sup>. *E. coli* to *Rhodococcus* conjugation has also been reported, but requires specific donor strains<sup>316</sup>.

This chapter discusses the cloning and characterization of RDX degrading gene(s) from *Rhodococcus rhodochrous* strain 11Y.

## 5.2 Materials and methods

### 5.2.1 Library construction

#### 5.2.1.1 Preparation and partial digestion of DNA

Total DNA prepared from strain 11Y (§2.3.1) was used in trial digestions with *Sau3A* I using 10 µg DNA in a total volume of 10 µl, at an enzyme concentration of 0.1 unit per µl, at 37 °C for 20, 40, 60 and 80 min. The reaction was stopped by the addition of EDTA pH 7.5 to a final concentration of 20 mM<sup>159</sup>. The size ranges of the resulting digestions were determined by gel electrophoresis. Partial digestion was optimized at 30 min, and a 10x scale up performed. Gel electrophoresis confirmed that the scale up had the same range of fragment sizes as the trial digestion.

#### 5.2.1.2 DNA fragment size fractionation on sucrose gradients

Fragments of *Sau3A* I digested DNA were size fractionated using an adaptation of the method described in<sup>8</sup>. Solutions containing 10 %, 15 %, 20 %, 25 %, 30 %, 35 % and 40 % w/v sucrose were made in a buffer of 100 mM sodium chloride, 20 mM Tris pH 8.0 and 10 mM EDTA pH 8.0. Aliquots (550 µl) of each were layered in a gradient from 40 % to 10 % in a Beckman 11 x 60 mm centrifuge tube and left to equilibrate overnight at 4 °C. DNA was layered on top and spun for 3 h at 60,000 rpm at 20 °C in a Beckman SW60Ti rotor using a Beckman XL-90 ultracentrifuge (Beckman, High Wycombe, Buckinghamshire, UK.). The bottom of the centrifuge tube was pierced with a 0.8 mm diameter syringe needle, fractions were collected from decreasing sucrose concentrations and samples from each fraction were visualized on an agarose gel. DNA was precipitated from fractions containing the desired size ranges by incubating on ice for 10 min with T500 dextran at a final concentration of 200 µg/ml, sodium acetate pH 6.0 to 0.3 M and 2 volumes of ethanol and resuspended in 200 µl dH<sub>2</sub>O. A further precipitation was performed, and the DNA resuspended in 50 µl dH<sub>2</sub>O.

### 5.2.2 Microtitre plate assays of *E. coli*

Microtitre plates were used with 150 µl LB containing 100 µg/ml carbenicillin in each well. Five library transformants were used to inoculate each well, the plate was covered and incubated at 37 °C overnight. Samples from this plate were transferred to method A and B plates using microtitre plate replicating tools. Method A plates contained 150 µl LB, 100 µg/ml carbenicillin and 0.5 mM RDX in each well, method B plates contained the above without the RDX. Both were covered and

incubated at 30 °C overnight. Lysozyme (20 µl of a 50 mg/ml stock) was added to each well of method B plates, which were incubated at 37 °C for 2 h. Aliquots (50 µl) of 440 mM Tris pH 8.0 and 3 mM RDX were added to each well and the plate was incubated at 30 °C for 3-4 h. Plates from both methods were assayed for the presence of nitrite. Sulphanilamide solution (50 µl of 1 % w/v sulphanilamide, 6.7 % v/v concentrated HCl) was added to each well and incubated for 5-10 min, 20 µl N-(1-naphthyl)ethylenediamine (NED) solution (0.5 % w/v NED) was added and the reaction was allowed to proceed for 10 min. The presence of any pink coloration was recorded. A positive control of the known RDX degrader, strain 11Y, was used, as was a sterile negative control. An uninoculated well with added lysozyme was used to determine any interaction between lysozyme and the components of the Griess assay, and no pink colouration was seen.

### 5.2.3 Transfer of library into rhodococcal host

#### 5.2.3.1 Electroporation method 1 <sup>164</sup>

Strain CW25 was grown in 100 ml LB containing 1.8 % w/v sucrose, 1.5 % w/v glycine and 0.01 % w/v isonicotinic acid hydrazide (isoniazid) at 30 °C to an OD<sub>600</sub> of 3, cooled on ice, harvested by centrifugation at 8000 rpm at 4 °C for 15 min, washed twice in ice cold sterile water and resuspended in 1 ml 30 % w/v polyethylene glycol (PEG) 6000. Aliquots of 100 µl were mixed with up to 1 µg DNA, transferred to a chilled 0.2 cm electroporation cuvette and incubated on ice for 10 min. Electroporation was performed at 2.4 kV, 400 Ω, 2.5 µF using a Biorad MicroPulser (Biorad, Hemel Hempstead, Hertfordshire, U.K.). LB (1 ml) was added immediately after electroporation and the cells incubated at 30 °C for 3 h before being plated onto selective medium and incubated further at 30 °C.

#### 5.2.3.2 Electroporation method 2 <sup>135</sup>

Strain CW25 was grown in 10 ml LB containing 1.8 % w/v sucrose and 1.5 % w/v glycine at 30 °C to mid-exponential phase (15 h), harvested by centrifugation at 6500 rpm at 4 °C for 10 min, washed 3 times in ice cold, sterile water, resuspended in 1 ml ice cold water and kept on ice. Aliquots (10 µl) were mixed with 1 µg DNA in a chilled 0.2 ml electroporation cuvette and kept on ice. Electroporation was performed at 2.4 kV as above. After electroporation the cells were incubated on ice for 10 min followed by a 10 min heat shock at 37 °C. The cells were recovered in 1 ml LB at 30 °C for 2 h before being plated onto selective medium and incubated further at 30 °C.

### 5.2.3.3 Electroporation method 3<sup>160</sup>

Strain CW25 was grown in 50 ml LB containing 1.8 % w/v sucrose and 1.5 % w/v glycine at 30 °C to an OD<sub>600</sub> of 0.5, harvested, washed twice in ice cold water and resuspended in 2.5 ml ice cold water. Aliquots of 400 µl of cells were mixed with up to 1 µg DNA and incubated at 40 °C for 5 min. The electroporation mixture was placed into 0.2 mm cuvettes and electroporation was performed at 2.4 kV as above. LB (600 µl) was added to the cells after electroporation and recovery was performed at 30 °C for 4 h before plating onto selective medium.

### 5.2.3.4 PEG-mediated protoplast method<sup>69</sup>

Strain CW25 was grown in LBSG medium (1 % w/v tryptone, 0.5 % w/v yeast extract, 0.5 % w/v sodium chloride, 10.3 % w/v sucrose and 3 % w/v glycine) to an OD<sub>600</sub> of 2-3 and stored for up to 1 month at 4 °C. A 50 µl volume of cells was used for each transformation. Cells were rinsed twice in autoclaved B buffer (0.3 M sucrose, 0.01 M MgCl<sub>2</sub> and 0.025 M N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) pH 7.2) and resuspended in the original volume of B buffer containing 5 mg/ml lysozyme. After 90 min incubation at 37 °C, cells were rinsed gently in freshly made P buffer (B buffer with 2 % v/v 1 M CaCl<sub>2</sub> and 1 % v/v 0.03 M KH<sub>2</sub>PO<sub>4</sub>), gently resuspended in the original volume of P buffer and 2 µl plasmid DNA was added (approx. 0.7 µg). After 5 min, an equal volume of PPEG (P buffer with 50 % w/v PEG 6000) was gently mixed in and left for 5 min. The cells were spread onto cold regeneration plates (as LBSG, with glycine omitted and 1.5 % w/v agar and 0.4 % w/v MgCl<sub>2</sub> added. After autoclaving, and immediately before pouring the plates 2 % v/v 1 M CaCl<sub>2</sub>, 1 % v/v 0.03 M KH<sub>2</sub>PO<sub>4</sub> and 1.7 % v/v 0.25 M TES pH 7.2 were added). After a 12 h recovery at 30 °C, chloramphenicol was introduced underneath the agar, using a sterile spatula, to a final concentration in the agar of 40 µg/ml. Cells were incubated at 30 °C for approx. 7 days, until distinct colonies appeared.

### 5.2.4 Growth of strain CW25(pHSX1) on RDX

Strains CW25 and CW25(pHSX1) were grown on minimal medium containing three carbon sources and 0.25 mM RDX. Chloramphenicol (40 µg/ml) was added to the growth medium of strain CW25(pHSX1). RDX concentrations were measured by taking samples at regular intervals, assaying by HPLC and comparing to standard curves of RDX concentrations run with every set of samples. Nitrite and ammonium were assayed using ion chromatography. Negative controls were

sterile, inoculated with strain CW25 or with strain CW25 transformed with pDA71 carrying an insert which did not confer RDX degrading ability.

### 5.2.5 Primers used for sequencing and subcloning pHSX1

**Table 5.1: Primers used for sequencing and subcloning 7.5 kb insert**

Primer	Sequence	T <sub>m</sub> (°C)
H1f	5' GGGAAAAGAGGAGATCAAG 3'	52.7
H1r	5' CTAAGAAAAGGACGTAAGG 3'	50.6
H4f	5' GCTGTACGTCGTCAACGC 3'	56.6
H6r	5' GGTCCTCGCGACGGGCC 3'	62
H7r	5' GGTACCTGATCGCCTCG 3'	56
H8f	5' CCGCGAGGTCGACGC 3'	54
H9f	5' CCTGCGCGCGGATCG 3'	54
H11f	5' CGTGGAGCGCACCACC 3'	56
H12f	5' GTAGAAGACGCGCCGG 3'	54
H13f	5' GCCGAACACCACCGAG 3'	54
H14f	5' CGAGGCGATCAGGTACC 3'	56
H15f	5' GGTAGGACTTGCGG 3'	46
H16f	5' CGTGTCGTCGTTGC 3'	46
H17f	5' CGGGCTCGGCAACC 3'	50
H22f	5' CGACGCTCATGATGG 3'	48
H24f	5' GGACAGGACGATCGGC 3'	54
H31f	5' CCGCCGCGATCATGC 3'	52

### 5.2.6 Subcloning insert to determine RDX degrading region

PCR amplification of pHSX1 using primers H26r and H30f (Table 5.2), an annealing temperature of 55 °C and 4 min extension, yielded a product of the correct size (3.5 kb) as visualized by gel electrophoresis. The fragment was excised, purified by gel extraction, cloned into pCR<sup>®</sup>2.1-TOPO and transformed into an *E. coli* host. Plasmid DNA was extracted and digested with *Hind* III, as was pDA71, the required bands excised, extracted and ligated as described (§2.3.5). The ligation mixture was transformed into an *E. coli* host. The insert sizes were confirmed by digestion with

*Hind* III followed by gel electrophoresis, and the plasmids transformed into strain CW25 (§5.2.3.4). This plasmid construct was named pHSX1a.

The method was performed as described above to subclone one ORF into pDA71, using primers H26r and H37f, with a PCR annealing temperature of 55 °C and an extension time of 3 min to obtain a fragment of 2.4 kb. This construct was named pHSX1b.

**Table 5.2: Primers used to construct insert fragments in pDA71.** Engineered restriction sites are shown in bold.

Primer	Sequence	T <sub>m</sub> (°C)
H26r	5' GGATA <b>AAGCTT</b> CAGGACAGGACGATCGG 3'	74.6
H30f	5' CTGA <b>AAGCTT</b> ACCGAACCCGAAC 3'	60
H37f	5' GCGTGA <b>AAGCTT</b> CGCACCTGGCGTCG 3'	82.3

## 5.2.7 RNA manipulation

### 5.2.7.1 RNA extraction

RNA was extracted according to the second method described in Nagy et al.<sup>261</sup>. Cells were grown overnight in 5 ml LB with 0.6 mg/ml carbenicillin added 2-3 h before harvesting. The RNeasy mini kit (Qiagen) was used to extract the RNA according to the manufacturer's protocol. All solutions were made using 0.1 % v/v diethylpyrocarbonate (DEPC) in dH<sub>2</sub>O, which had been shaken vigorously and left to stand overnight at 37 °C before being autoclaved<sup>261</sup>.

Contaminating DNA was removed using RQ1 RNase-free DNase (Promega) according to the supplied manual, after which the enzyme was removed through a phenol: chloroform extraction. An equal volume of phenol: chloroform: isoamyl alcohol 25:24:1 was added to the RNA sample, mixed and centrifuged at maximum speed for 20 min. The RNA in the aqueous layer was precipitated by the addition of sodium acetate pH 6.0 to 0.3 M and 2 volumes of ethanol, and resuspended in 50 µl 0.1 % v/v DEPC in dH<sub>2</sub>O. RNA concentration was measured using absorbance at 260 nm, taking 1 absorbance unit to equal 40 µg/ml RNA<sup>261</sup>.

### 5.2.7.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was performed with the Access RT-PCR system (Promega) according to the manufacturer's protocol. The system contains AMV reverse transcriptase (AMV RT) for first strand DNA synthesis and *Tfl* DNA polymerase for second strand cDNA synthesis and DNA amplification.

The reaction was performed in a final volume of 25  $\mu$ l, using 1  $\mu$ g RNA with 25 pmol each primer (Table 5.3), 0.2 mM each dNTP, 1 mM MgSO<sub>4</sub> and a final concentration of 0.1 unit of each enzyme per  $\mu$ l. RT-PCR cycles consisted of the following: 48 °C for 45 min for reverse transcription, 94 °C for 2 min to inactivate the AMV RT, followed by 40 cycles of 94 °C for 30 s (denaturation of template), 60-68 °C for 1 min (primer annealing gradient) and 68 °C for 2 min (extension using *Tfl* DNA polymerase). A final cycle of 68 °C for 10 min was used for extension. Controls were performed at the lowest annealing temperature in the absence of AMV RT to confirm that the products were a result of amplification from RNA and not any contaminating DNA. The reactions were visualized by gel electrophoresis, fragments of the desired sizes were extracted, gel purified, cloned into pCR<sup>®</sup>2.1-TOPO and transformed into an *E. coli* host. Plasmids were extracted and the inserts sequenced using M13F and M13R primers, complementary to the *lacZ* gene surrounding the insert site.

**Table 5.3: Primers used for RT-PCR.**

Primer	Sequence	T <sub>m</sub> (°C)	Reaction
H14f	5' CGAGGCGATCAGGTACC 3'	56	A, B
H41f	5' CGACGTCGCAGATCTGGCCG3'	75.6	A
H42f	5' CCATCGATCGCGAGCTCGTGC 3'	76.3	B
H21f	5, CCACGGCAGAGTCC 3'	48	C
H22f	5' CGACGCTCATGATGG 3'	48	C
H6r	5' GGTCCTCGCGACGGGCC 3'	62	D
H40f	5' GCCAGCATCGCGTCGACCTCG 3'	78.2	D
H22f	5' CGACGCTCATGATGG 3'	48	E, F
H45f	5' GCCGCGTTCGACGTCGTGGTCC 3'	80.3	E
H46f	5' CGTGCGACACCGTCATCACCG 3'	76.4	F

### 5.2.8 Resting cell incubations with metyrapone

Resting cell incubations were performed using strain 11Y as previously described (§3.2.9), with 0, 0.1, 0.5, 1 and 10 mM 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone) added. The samples were assayed for RDX by HPLC and nitrite using the Griess assay, at times 0, 10, 30 and 60.

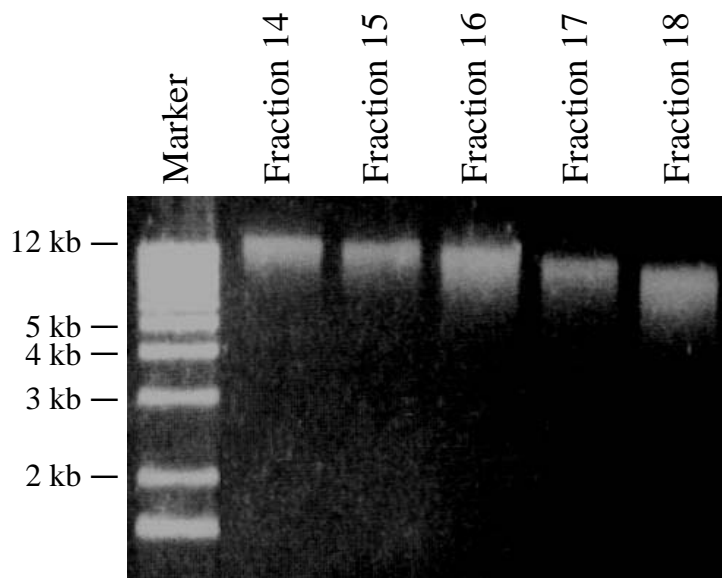
## 5.3 Results

### 5.3.1 Construction of strain 11Y library in *E. coli*

The approach taken to clone the gene(s) which allow(s) *Rhodococcus rhodochrous* strain 11Y to degrade RDX involved the creation of a library from strain 11Y DNA, and the transfer of this library to a non RDX degrading host strain. If one of the library clones, each of which carry a fragment of DNA from strain 11Y, develops the ability to degrade RDX, it must be due to the presence of a fragment containing the gene(s) which allow strain 11Y to degrade RDX.

To obtain a representative library, overlapping fragments are required. For this, a restriction enzyme which cuts very frequently must be chosen, such as one which has a recognition site of only four base pairs. An enzyme commonly used for library construction is *Sau3A* I, which has the recognition sequence GATC and produces cohesive ends that are compatible with those created by *BamH* I and *Bgl* II. Therefore DNA cut with *Sau3A* I can be ligated to pDA71 cut with *Bgl* II (§5.1). A partial digestion of DNA with *Sau3A* I results in a wide range of different fragments to result, one or several of which will hopefully carry the gene(s) of interest. The degree of partial digestion which will give the sizes of fragments required must be determined through trial digestions. Total DNA was used in order to provide fragments of any large plasmids present, as well as fragments of genomic DNA.

The desired range of partially digested DNA fragment sizes chosen was 5-10 kb so that any fragments would contain intact genes, regulatory regions and possibly clustered genes (§3.1). In order to obtain the 5-10 kb fragments, strain 11Y DNA was partially digested, and a size fractionation was performed on a sucrose gradient. Thirty one fractions were obtained, and those which contained fragments between 5-10 kb were identified through gel electrophoresis (Figure 5.2), precipitated, resuspended in water and pooled. Several ligations of the fragments to *Bgl* II digested pDA71, and transformations of the ligation mixtures into *E. coli* were performed, and a total of approx. 23,000 transformants obtained. Stocks of the library in the *E. coli* host were made and stored in glycerol at -80 °C (§2.2.3).

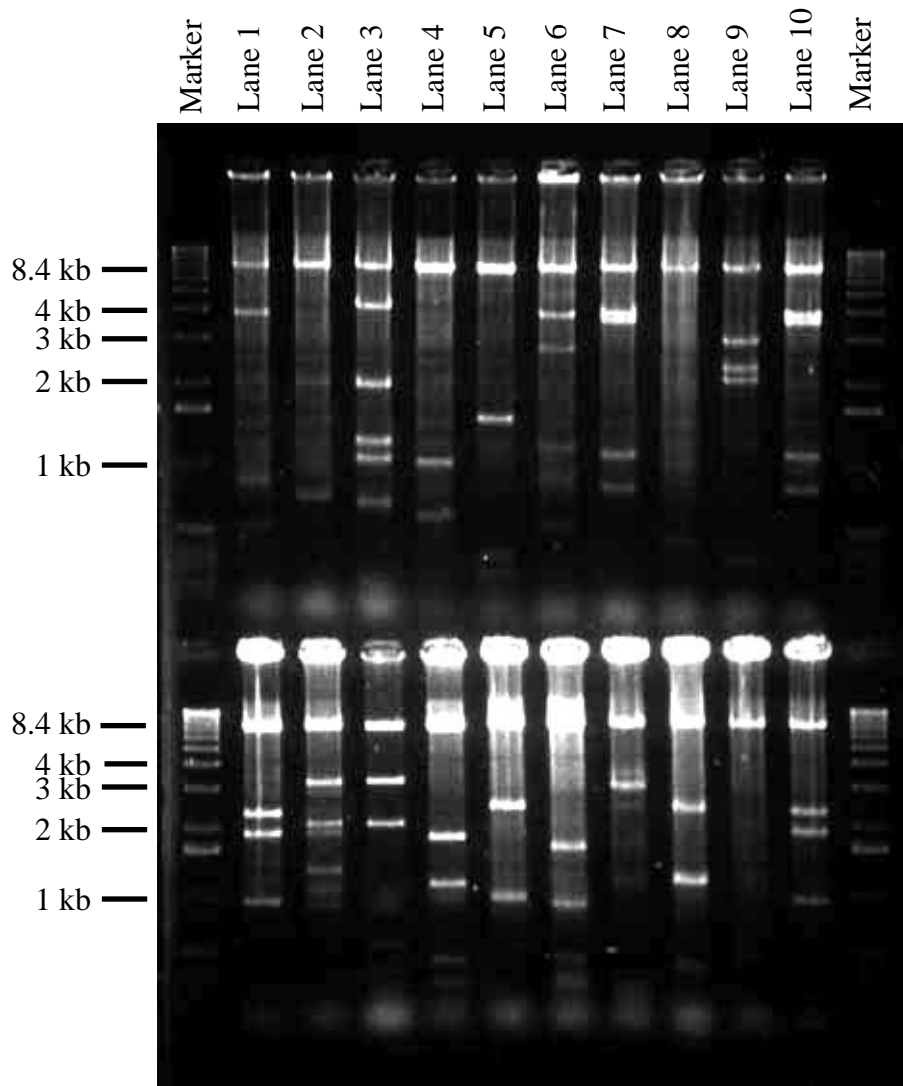


**Figure 5.2: Gel electrophoresis of chosen fractions from the sucrose gradient.** Fractions 14-18 of the sucrose gradient contain fragments between 5 and 10 kb.

#### 5.3.1.1 Analysis of insert sizes

A representative library is required to provide coverage of the whole genome. To determine the number of clones required it was necessary to first calculate the average pDA71 insert size in the library. To release the insert, in order to calculate its size, restriction sites either side of it were required. The unique *Bgl* II site which had been used to create the library was eliminated in the process, as a *Sau*3A I cohesive end was ligated to it, removing the full *Bgl* II consensus sequence. Therefore, the unique *Hind* III and *Pst* I sites flanking the insertion site were selected (Figure 5.1). There are 415 bp of vector DNA between these sites, either side of the original *Bgl* II site. Twenty library clone colonies were picked, grown overnight and plasmids extracted by the boiling lysis method (§2.3.1). Digestions with *Hind* III and *Pst* I were visualized by electrophoresis.

Digestions of library plasmids with *Hind* III and *Pst* I were used to determine insert size and are shown in Figure 5.3. The insert size of each plasmid was calculated from the sum of the sizes of all the bands, excluding the 8.4 kb vector band. The average insert size, correcting for the extra 0.4 kb of vector DNA, was 7.4 kb, and all the colonies gave different restriction patterns indicating that all carried different inserts.



**Figure 5.3: Restriction analysis of twenty genomic library clones.** Restriction analysis of 20 transformants from the genomic library of strain 11Y in *E. coli*, digested using Hind III and Pst I. The bands at 8.4 kb represent linearized pDA71.

### 5.3.1.2 Determination of required number of clones

A calculation, based on the Poisson distribution, was used to estimate the number of clones required for a 99 % probability of getting a gene of interest.

$$N = \ln(1 - P) / \ln[1 - (I/G)]$$

(where  $N$  is the number of clones that must be screened to isolate a particular sequence with probability  $P$ ,  $I$  is the size of the average cloned fragment in base pairs and  $G$  is the size of the target genome in base pairs <sup>8</sup>)

The size of strain 11Y genome was unknown but an estimated value of 6 Mb was used. This was approximated from known genome sizes of related bacteria<sup>25, 57, 58, 65, 232, 242</sup>.

Using the average insert size of 7.4 kb calculated above (§5.3.1.1), 3732 clones should provide a 99 % probability of isolating any particular gene. With 23,000 transformants produced from strain 11Y DNA, this library was considered to be highly representative.

### 5.3.2 Screening of library in *E. coli*

As the library was in an *E. coli* host, and cloning techniques in *E. coli* are better developed than those in rhodococci, the isolation of an RDX degrading clone from the library in *E. coli* was attempted. If the RDX degrading protein(s) were found to be active in an *E. coli* host, it would remove the need for subsequent transformation of the library into a rhodococcal host.

The presence of an RDX degrading clone could not be assessed using a growth screen. The only identified nitrogen containing metabolite from the breakdown of RDX by the mechanism of strain 11Y is nitrite (§4.3.5), which *E. coli* is not able to use as a nitrogen source when grown aerobically<sup>56</sup>. Therefore, alternative screens were devised to allow the detection of activity against RDX, without requiring the bacteria to be able to use it as a source of nitrogen.

To ensure that the *E. coli* TOP10 cells were able to grow in the presence of RDX, cells were plated onto LA containing 5 mM RDX. Growth was assessed after 2 days and was found to be unaffected when compared to growth on LA containing no RDX. Therefore, under the conditions required for this screen, RDX does not appear to affect the growth of *E. coli*.

#### 5.3.2.1 Zone of clearance on adapted RDX plate

To determine whether the activity against RDX is being expressed in any of the *E. coli* clones, an adaptation of the zone of clearance plate screen was used. LA containing 100 µg/ml carbenicillin made up the bottom layer, to encourage growth of the cells and achieve a sufficient biomass of each clone to potentially allow for expression of RDX degrading genes. The top layer consisted of phosphate buffer and agarose containing 5 mM RDX. Glycerol stocks of the library in *E. coli* were spread onto these plates at a dilution of 10<sup>-7</sup> to give approx. 300 colonies/plate, and the plates grown at 30 °C for up to 9 days.

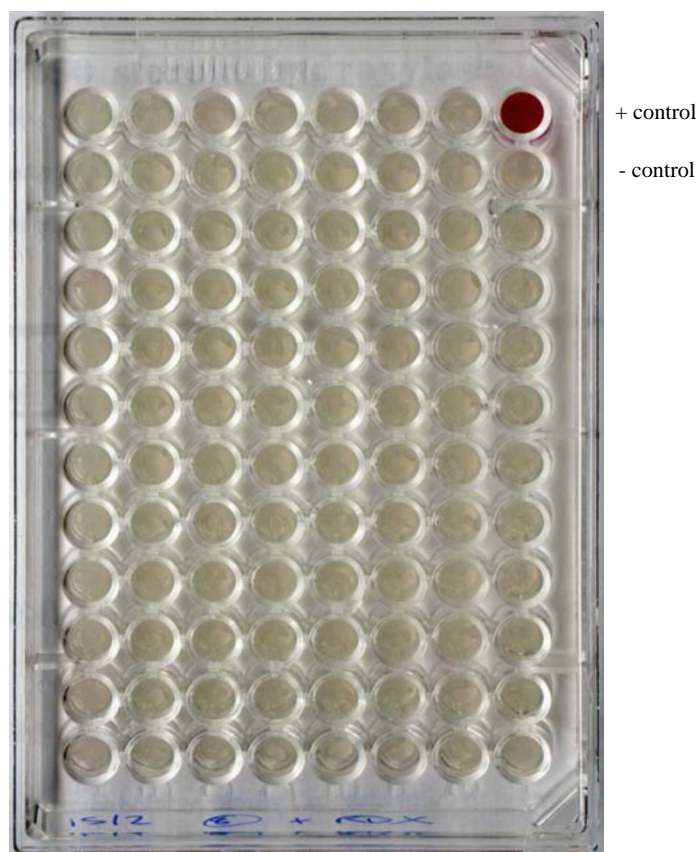
A total of approx. 11,000 library clones were screened on 35 plates. Each colony grew to a diameter of approx. 3 mm indicating that the cells could obtain nutrients from the LA layer for growth, but none produced a zone of clearance (data not shown).

### 5.3.2.2 Microtitre plate assays using whole cells and cell extract

A more sensitive screen was devised, assaying for the production of nitrite from RDX breakdown using the Griess assay. The library in *E. coli* was screened in 96 well microtitre plates for the ability of both whole cells (method A) and crude cell extract (method B) to produce nitrite from RDX. Crude cell extract was used to allow the RDX to interact with any expressed degradation enzymes in the absence of a cell wall, which could possibly inhibit RDX uptake.

Method A involved the growth of *E. coli* library clones in LA with RDX overnight and assayed for nitrite production. Method B involved the growth of *E. coli* library clones in LA in the absence of RDX. After overnight growth, lysozyme was added to each well to lyse the culture and produce a crude cell extract. RDX was then added and the nitrite presence assayed after 3-4 h incubation.

A representative library of 3,800 clones was screened using both methods. A typical microtitre plate result is shown in Figure 5.4. In some cases a faint pink coloration (a positive result of the Griess assay, indicating nitrite presence) was observed, but this was not reproducible when repeated. The faint pink wells were always at the edges of the plate and commonly at the corners. The positive control using strain 11Y showed an intense pink colouration.



**Figure 5.4: Microtitre plate screening of library in *E. coli*.** Clones from the *E. coli* library of strain 11Y were grown in microtitre plate wells with RDX and subjected to Griess assay analysis for nitrite presence, indicative of RDX breakdown. A positive control of strain 11Y developed a pink colouration when assayed and a negative, sterile control showed no change in colouration.

### 5.3.3 Transfer of library into rhodococcal host

Screening the library in *E. coli* for the RDX degrading activity was not successful. It was therefore necessary to transfer the library into a rhodococcal host, within which the RDX degrading genes would be more likely to express due to promoter recognition within similar strains. The library screen involved the transfer of RDX degrading activity to a strain which could not previously degrade RDX. *Rhodococcus rhodochrous* strain CW25 (described in Quan and Dabbs<sup>238</sup>) is closely related to strain 11Y, is routinely used for DNA transformations, is unable to degrade RDX, as determined by a previous screen, and can use nitrite, produced from the degradation of RDX by strain 11Y (§4.3.5), as a nitrogen source for growth. Therefore, strain CW25 was chosen to be the host of the strain 11Y library. When grown on minimal medium, optimal growth of strain CW25 is achieved when the medium is supplemented with glutamate and thiamin. However, as it can use glutamate as a source of nitrogen, which would interfere with growth screens using RDX as the sole

nitrogen source, and it can grow in the absence of either supplement, neither were subsequently used.

#### 5.3.3.1 Electroporation to transform cells

A literature survey of rhodococcal transformation techniques was performed, to determine the conditions most likely to provide a large number of transformants. The methods vary with respect to stage at which the cells are harvested, treatment of the cells prior to electroporation and treatment of the cells after electroporation. The overriding theme from the survey is that no method appears to work for all rhodococcal hosts and that in many cases the conditions need to be optimized for the specific host strain. With so many parameters to vary, there is a lot of choice in which electroporation protocol to use.

The method described in Kessler et al.<sup>164</sup> involves growing strain CW25 cells to saturation in LB containing sucrose and glycine, which inhibits peptidoglycan synthesis, supplemented with isoniazid to inhibit mycolic acid synthesis. Both of these cause the rhodococcal cell wall to weaken and increase the likelihood of foreign DNA uptake by the cell. The cells are resuspended in PEG which increases the effective DNA concentration and thus transformation efficiency. A maximum efficiency of 373 CFU (colony forming units) per  $\mu\text{g}$  DNA was achieved, which was much lower than the figure of  $10^5$  which should have been possible. Altogether 166 colonies were obtained by this method, on 6 plates.

To get a higher transformation efficiency, the method described in Hashimoto et al.<sup>135</sup> was used, adapted by growing the cells in the medium described above, rather than MYP as specified. Cells were harvested at mid-exponential phase, after 15 h. No PEG is used in this method; instead the cells are resuspended in ice cold, sterile water. Approximately the same electroporation conditions are used, but the recovery period involves 10 min incubation on ice, followed by 10 min at 37 °C, and a further 2 h in rich medium at 30 °C. Only 2 transformants were obtained using this method using 1  $\mu\text{g}$  DNA, instead of a predicted  $10^7$  CFU per  $\mu\text{g}$  DNA.

Finally, an electroporation method described in Kalscheuer et al.<sup>160</sup> was used. LB containing sucrose and glycine was again used to grow the cells, which were harvested at an  $\text{OD}_{600}$  of 0.5 and incubated with DNA at 40 °C for 5 min (heat shocked) before electroporation. A maximum transformation efficiency of 349 CFU per  $\mu\text{g}$  DNA was attained, which is still much lower than expected. This method was also not reproducible using strain CW25, with some attempts resulting in no transformants.

None of the electroporation protocols attempted provided sufficient numbers of clones to enable screening of the library. Another transformation method was required.

#### 5.3.3.2 PEG-mediated protoplast method to transform cells

A protoplast method of transformation was attempted, performed with the help of Professor Eric Dabbs at the University of Witwatersrand, South Africa. Strain CW25 was grown in LBSG to weaken the cell wall. The cell wall of the host was removed with the use of lysozyme, creating protoplasts; this, with the use of PEG to increase the effective concentration of DNA, aids DNA uptake. Several thousand transformants were obtained per plate, giving a transformation efficiency of approx. 5000 CFU per  $\mu\text{g}$  DNA. Due to the transfer of the library from *E. coli* to a rhodococcal host, 5-6 times as many transformants were required as were previously calculated (§5.3.1.2) for a good chance of representing all clones (E. R. Dabbs, personal communication). A total of approx. 50,000 transformants were finally obtained, giving a representative library. The clones were rinsed off the regeneration plates using LB, pooled and vortexed to mix. The pooled library was halved and divided into aliquots, each aliquot of which should have contained representatives of each clone. The aliquots were stored with glycerol as stocks at  $-80\text{ }^{\circ}\text{C}$ .

### 5.3.4 Screening of library in rhodococcal host

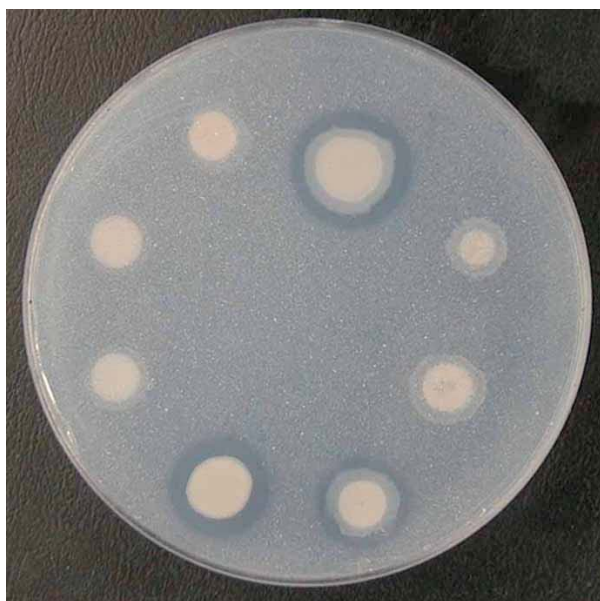
#### 5.3.4.1 Selective enrichment in RDX medium

Selective enrichment was used to screen the rhodococcal library for RDX degrading clones. Minimal medium containing three carbon sources and 1 mM RDX as the sole source of nitrogen was inoculated with the library, to encourage the growth of transformants expressing RDX degradation gene(s) (§3.1). Four aliquots of library clones in strain CW25 were used to inoculate four flasks of selective medium, which were grown at  $30\text{ }^{\circ}\text{C}$  for 2 weeks. The flasks were subcultured and grown for a further 2 weeks.

TLC analysis of the media of subcultured flasks (§3.2.2) showed that no RDX remained in two of the flasks (data not shown). The flasks that showed loss of RDX contained medium with higher turbidity than the flasks which showed no loss of RDX, indicating that growth was occurring in these flasks. Samples from these two flasks were spread onto LA plates containing chloramphenicol, and placed at  $30\text{ }^{\circ}\text{C}$ . The clones on these plates formed a lawn which was streaked to single colonies.

#### 5.3.4.2 Zone of clearance on RDX plates

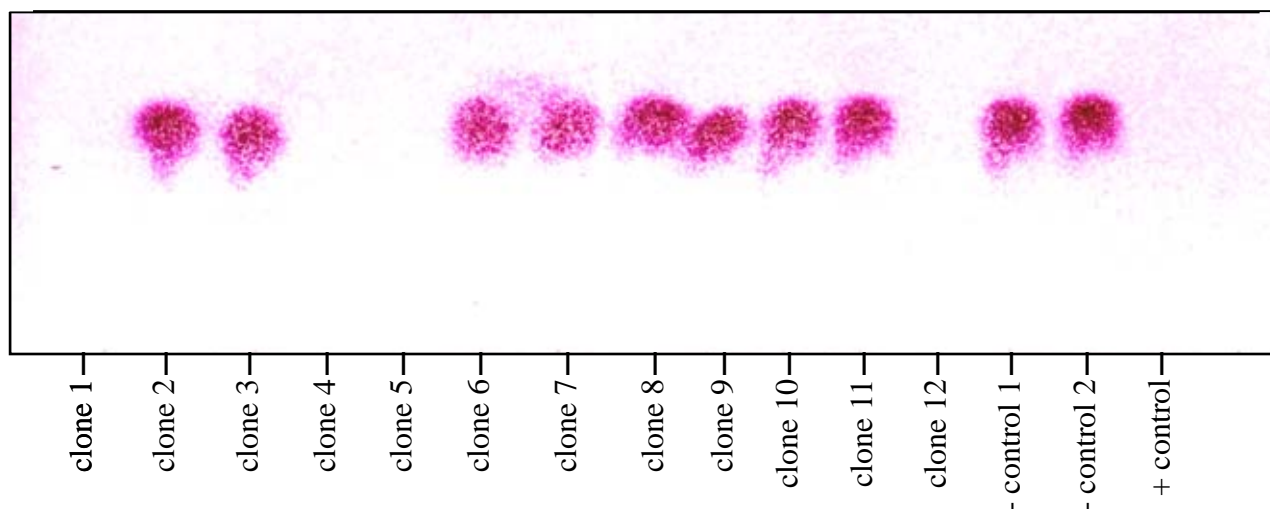
To determine which, if any, of the single colonies from the enrichments had gained the ability to degrade RDX, the zone of clearance screen was used. To ensure sufficient biomass for the screen, individual clones were grown up in LB containing chloramphenicol, harvested and spotted onto RDX zone of clearance plates at high cell density. Twelve clones were screened and four clones were found to produce a zone of clearance (Figure 5.5).



**Figure 5.5: Zone of clearance screening of library clones.** Eight transformants were spotted onto RDX zone of clearing plates. Three of the eight gave a zone of clearing indicative of RDX degradation.

#### 5.3.4.3 Loss of RDX from growth medium assayed by TLC

To confirm that the four clones which could produce zones of clearance could remove RDX from growth medium, TLC was used to screen them for RDX utilization. Each of the 12 individual colonies were used to inoculate minimal medium containing three carbon sources and 1 mM RDX. After 7 days, samples were taken and analysed by TLC (§3.2.2). The four clones which gave zones of clearance (1, 4, 5 and 12) also removed all the RDX from culture, whereas none of the other clones possessed this ability (Figure 5.6). A negative control containing untransformed strain CW25 showed no removal of RDX. No metabolite accumulation was observed in any of the cultures as assayed by TLC. It was therefore concluded that the four clones had gained the ability to degrade RDX, and they were chosen for further study.

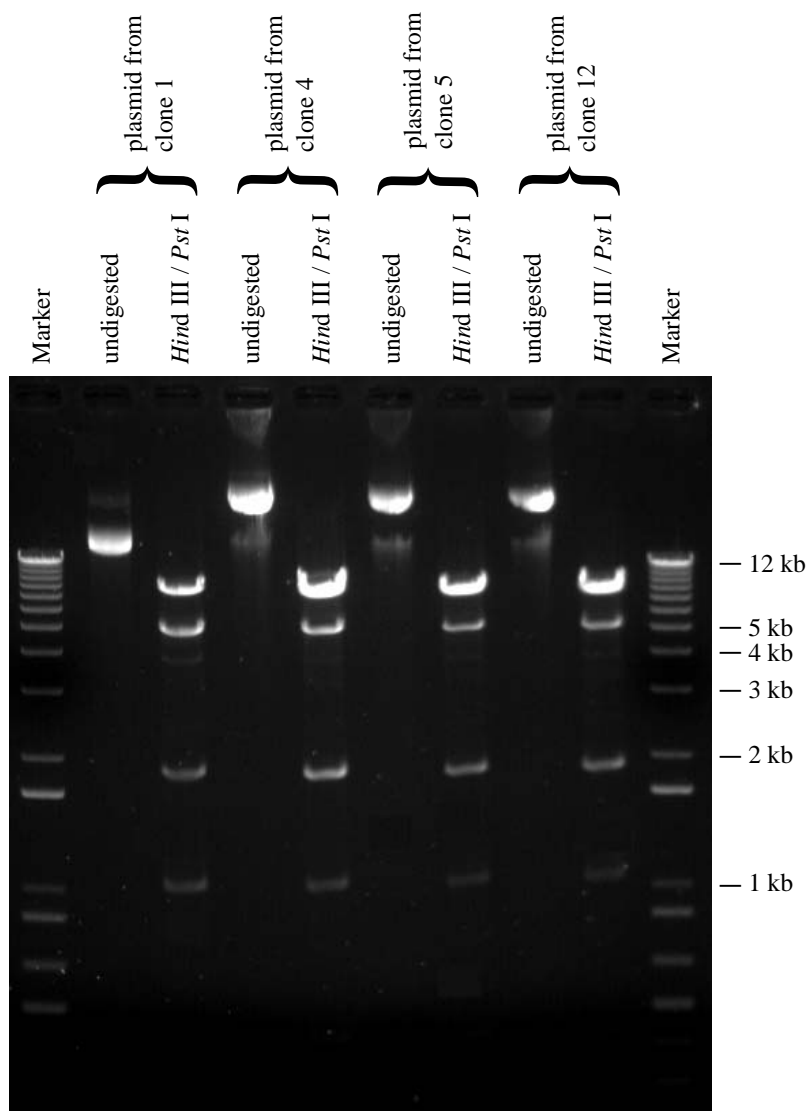


**Figure 5.6: TLC screening of library clones.** Cultures of 12 transformants from the genomic library in *Rhodococcus* host strain CW25 were assayed for ability to remove RDX from culture. Controls included a sterile control (- control 1), strain CW25 (- control 2) and strain 11Y as a positive control.

#### 5.3.4.4 Preliminary analysis of four clones

To analyse the plasmids of these clones it was necessary to extract them and propagate them for future use in an *E. coli* host. The extraction of plasmids was attempted using the alkaline lysis protocol with additional lysozyme in solution I (§2.3.1). No plasmid was extracted as visualized using gel electrophoresis. A method was therefore developed to obtain these plasmids from rhodococci. The method involved the extraction of total DNA from the rhodococci, containing both genomic and plasmid DNA, followed by standard transformation of this into an *E. coli* host. In this way only circularized plasmid DNA would transform the cells, and only cells carrying the plasmid would be able to grow on selective plates. The plasmids could then be easily extracted from the *E. coli* host.

Plasmids were extracted from all four clones and subjected to preliminary restriction analysis using *Hind* III and *Pst* I, to estimate the size of the insert (§5.3.1.1). All four gave the same restriction banding pattern (Figure 5.7), suggesting that they all contained the same insert. The bands produced, as visualized by gel electrophoresis, were of the approximate sizes: 5 kb, 1.8 kb, 1.1 kb and the vector band at 8.4 kb. This gives an insert size of approx. 7.5 kb, correcting for the extra 0.4 kb of vector DNA produced from the digestion (§5.3.1.1).



**Figure 5.7: Preliminary restriction analysis of plasmids extracted from four RDX degrading clones.** Restriction analysis of the plasmids from RDX-degrading clones showed that all plasmids produced the same banding pattern. Undigested plasmid DNA and DNA restricted with *Hind* III and *Pst* I was analysed by gel electrophoresis.

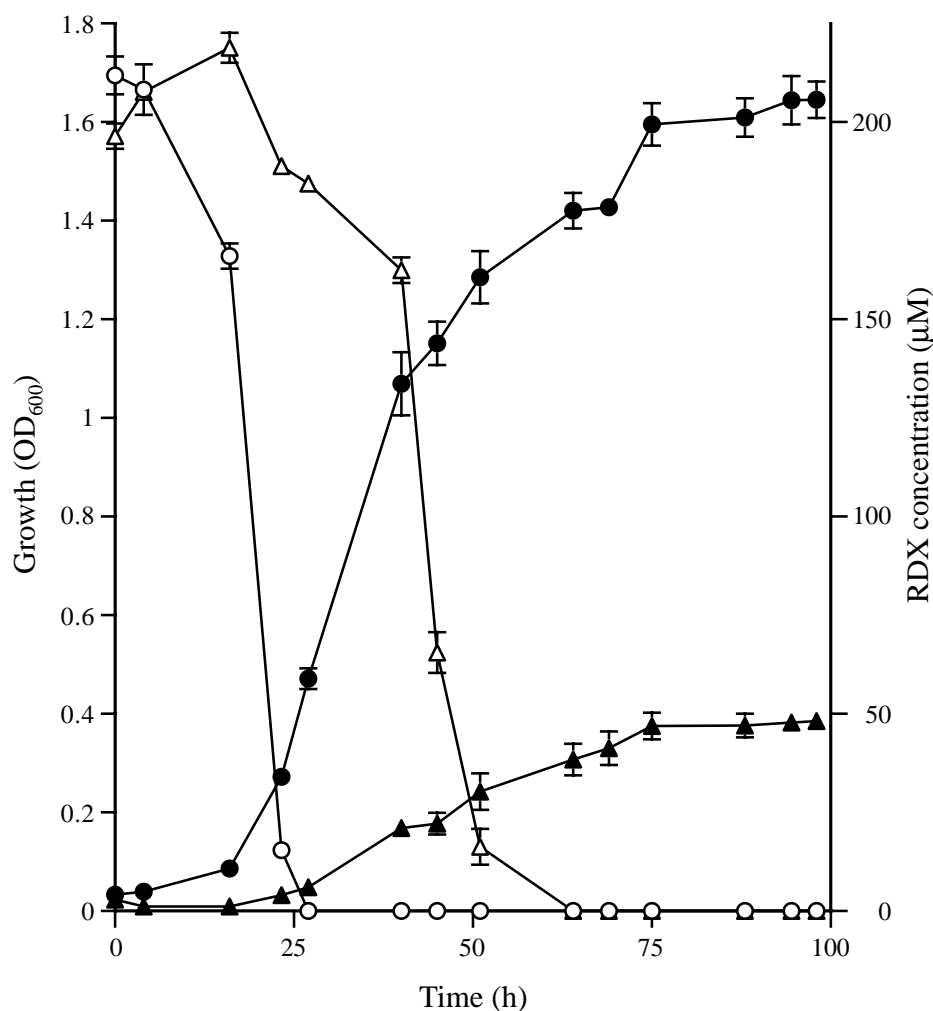
To confirm that this plasmid is responsible for the degradation of RDX in these clones, all plasmids were re-transformed into strain CW25, and the resulting transformants assayed on RDX zone of clearance plates. All four plasmids produced transformants which could produce zones of clearance. The plasmid from clone 1 was selected and named pHSX1.

### 5.3.5 Growth of strain CW25(pHSX1) on RDX

The ability of *Rhodococcus rhodochrous* strain CW25 carrying pHSX1 (CW25(pHSX1)) to degrade RDX was compared to that of strain 11Y. Both strains were grown from starter cultures in

minimal medium with 0.25 mM RDX, growth was assessed by  $OD_{600}$ , and RDX concentration by HPLC.

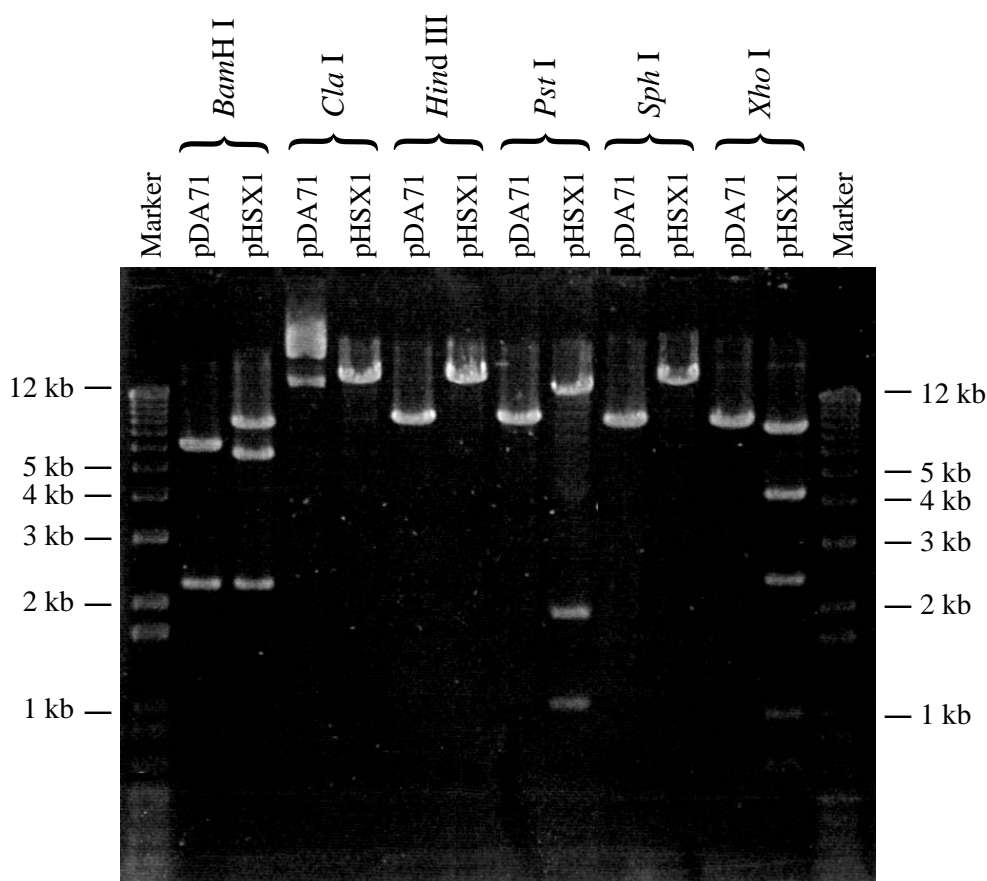
Growth curves showed that CW25(pHSX1) can grow on RDX as a sole nitrogen source and degrade it over time (Figure 5.8). This confirmed that pHSX1 confers the ability to degrade RDX. RDX removal by strain CW25(pHSX1) was complete within 64 h, compared to 27 h taken by strain 11Y. Strain CW25(pHSX1) has a longer lag period than strain 11Y (approx. 24 h as opposed to approx. 12 h), and a lower final  $OD_{600}$  (0.4 as opposed to 1.6). Neither nitrite or ammonium were observed to accumulate during growth of either bacterium, and no RDX removal was detected in a sterile control, an untransformed strain CW25 control, or a control of strain CW25 carrying pDA71 with a random insert.



**Figure 5.8: Growth of strain 11Y and strain CW25(pHSX1).** Optical density at 600 nm of strain 11Y (●) and strain CW25(pHSX1) (▲), RDX concentration when supplied to strain 11Y (○), and strain CW25(pHSX1) (△). Figures shown are the average of triplicate cultures and error bars indicate  $\pm$  one standard deviation.

### 5.3.6 Restriction analysis of insert conferring RDX degrading ability

A more thorough restriction analysis of pHSX1 was performed. A selection of enzymes was used, of which several gave too many or too few bands for useful information to be gained, when the digests were visualized by gel electrophoresis. The following were used in single and double digestions: *Bam*H I, *Cla* I, *Hind* III, *Pst* I, *Sph* I and *Xho* I (single digestions shown in Figure 5.9). Digestions patterns of pHSX1 were compared with those of pDA71, for which there is no full sequence data or restriction map available. The deduced restriction map of the insert is shown in Figure 5.10A. The *Xho* I site at 3.8 kb was later resolved to two sites, 40 bp apart, using sequence data.



**Figure 5.9: Restriction analysis of pHSX1.** Analysis of pDA71 and pHSX1 using restriction enzymes: *Bam*H I, *Cla* I, *Hind* III, *Pst* I, *Sph* I and *Xho* I.

### 5.3.7 Sequence analysis of insert conferring RDX degrading ability

#### 5.3.7.1 Sequence information using restriction digest clones

The restriction map deduced above provided information for the subcloning of sections of pHSX1, in order to obtain internal sequence of the insert. The fragments chosen to provide useful sequence information were: 1.9 kb *Pst* I fragment covering 0-1.8 kb of the insert, 2.4 kb *Xho* I fragment covering 3.8-6.2 kb and 3.8 kb *Bam*H I/ *Cla* I fragment covering 2.4-7.2 kb of the insert (Figure 5.10A).

Cloning vectors were selected to contain appropriate restriction sites for the subcloning of the fragments. Plasmid pGEM<sup>®</sup>-5Zf+ was used to clone the *Pst* I fragment due to the unique *Pst* I site in its multiple cloning site. Plasmid pGEM<sup>®</sup>-7Zf+ was used to clone the *Xho* I and *Bam*H I/ *Cla* I clones as it has all three restriction sites within its multiple cloning site. Appropriate digestions, gel extractions, ligations and transformations were performed and the resulting plasmids used as a template for sequencing using primers M13F and M13R. This method provided useful information on the internal sequence of pHSX1 which was used for the subsequent design of sequencing primers.

#### 5.3.7.2 Completion of insert sequence

The DNA sequence of the *Eco*R I endonuclease gene in pDA71<sup>114, 217</sup> (accession number J01675) was obtained from the GenBank Sequence Database (National Centre for Biological Information, NCBI). The sequence was used to design primers flanking the *Bgl* II cloning site to enable sequencing of the plasmid insert. The software package Sequencher<sup>™</sup> was used to aid the design of primers H1f (30 bp upstream of the *Bgl* II site) and H1r (56 bp downstream of the restriction site), shown in Table 5.1. These primers, and primers designed subsequently, were checked to ensure that they would not form stem-loops, and that they would not fold back on themselves. In addition, primers were designed to be G/C rich at the 3' end wherever possible, to ensure tight binding to the template at the site of extension.

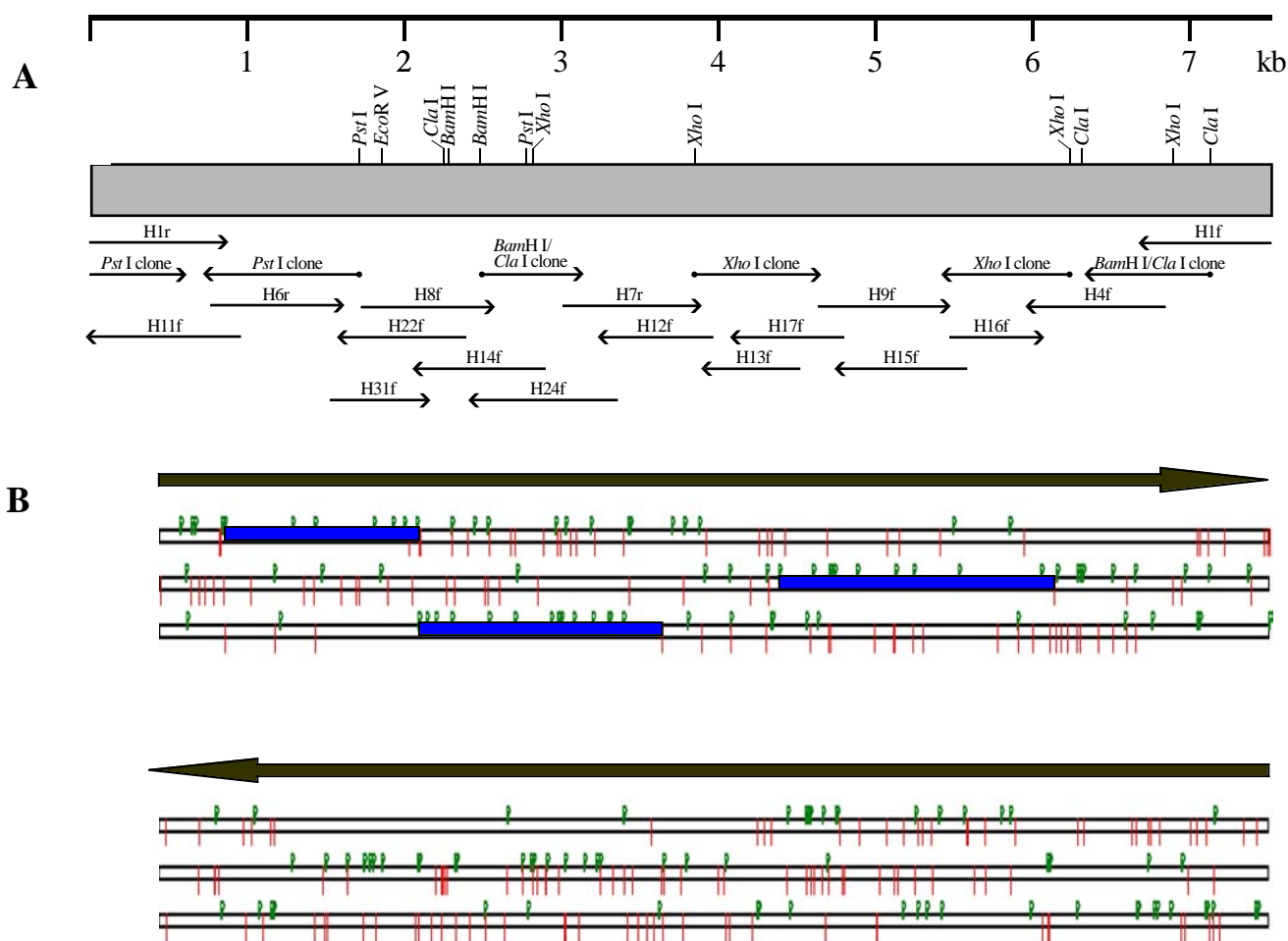
Plasmid pHSX1 DNA was prepared from an *E. coli* host and used as a template for automated sequencing using primers H1f and H1r. The chromatograms obtained from each sequencing reaction were analysed using Sequencher<sup>™</sup> to verify or correct the base assignments that had been made automatically. This sequence information, and the sequence information from the restriction clones (§5.3.7.1), was used to design primers to sequence the next section of the insert. Further primers were designed from the sequences derived until the full insert was sequenced. The

oligonucleotide sequences of all primers designed are shown in Table 5.1. Sequencher™ was used to align all sequence data into a single contiguous stretch of DNA (contig). The full DNA sequence was determined in one direction, and all but the 3' end was sequenced in both directions. This allowed for the comparison of chromatograms and final verification of the sequence data. The fragments used to assemble the final contig are illustrated in Figure 5.10A.

### 5.3.7.3 Primary analysis of insert sequence

The contig was analysed for all possible open reading frames, in each of the six possible frames. This data is presented in Figure 5.10B. Amino acid translations of all open reading frames (ORFs) were subjected to BLAST searches. Only three were identified as having homology to any known proteins. Viewing the contig left to right (Figure 5.10B), the first open reading frame encodes a polypeptide of 425 amino acids, the second consists of 552 amino acids, and the third of 626 amino acids.

The amino acid sequence of the first ORF has highest similarity to bovine mitochondrial adrenodoxin reductase (27 % identities and 42 % similarities; BLAST E value <sup>6</sup> of  $1 \times 10^{-75}$ ; accession number P08165)<sup>220, 254</sup> of the proteins of known function. Several putative mycobacterial reductases also have high amino acid similarity. The highest similarity score of the deduced amino acid sequence of the second ORF (26 % identity and 42 % similarities; E value of  $4 \times 10^{-35}$ ) was found with a P450-like protein bioI (CYP107H1) from *Bacillus subtilis* involved in biotin biosynthesis (accession number U51868)<sup>37</sup>, and similar scores were found with putative cytochrome P450s from the *Mycobacterium tuberculosis* H37Rv genome<sup>57</sup>. This ORF also has a region at the N-terminus which has homology to a flavodoxin domain. The amino acid sequence of the third ORF has highest similarity (44 % identities and 60 % similarities; E value of 0) to acetyl coenzyme A synthetase from *Tetrahymena pyriformis* (accession number AB026298)<sup>323</sup>.



**Figure 5.10: Restriction enzyme cleavage map and sequencing information for pHSX1.** A. The box represents the 7567 bp of the pHSX1 insert with deduced restriction sites from restriction mapping. Horizontal arrows indicate the direction and extent of sequencing from the primers indicated, with the circles representing the sequencing from clones made using the indicated restriction sites. B. Start and stop codons in all six reading frames from the sequence data, analysed by Sequencher™. Start codons are indicated by **P** symbols and stop codons by red bars. Only three of the open reading frames have any degree of homology to other proteins, and these are shown in blue.

### 5.3.8 Subcloning insert to determine RDX degrading region

#### 5.3.8.1 Subcloning two ORFs into pDA71

In order to determine which of the ORFs of pHSX1 are responsible for the degradation of RDX, areas of the insert were subcloned into pDA71 (Figure 5.11). It was thought that the more likely candidates for the RDX degrading genes were the first two ORFs corresponding to cytochrome P450 and reductase, as P450s have previously been implicated as being involved in the biodegradation of RDX<sup>60, 302</sup>.

To subclone the fragment containing the P450 and reductase-like genes into pDA71, it was necessary to engineer restriction sites corresponding to one of the unique sites within the *EcoR* I

endonuclease gene into the sequence. Sequence data of the relevant section was analysed for restriction sites and it was found that this region contains recognition sites for both *Pst* I and *Bgl* II. Neither of these enzymes could be used, as digestion with either would fragment the insert region. Therefore *Hind* III sites were engineered at either end of the insert for use with the *Hind* III cloning site within pDA71. As the promoter regions of these genes are unknown, the full region upstream of the genes within pHSX1 was maintained in the subclone.

PCR primers were designed to amplify the required sections of the insert, from the beginning of the existing insert in pHSX1, 426 bp upstream of the first ORF, to the transcription termination site of the second. *Hind* III restriction sites were engineered within the primers such that PCR products created using these primers could be restricted using *Hind* III, creating overhangs compatible with *Hind* III-digested pDA71. The primers were designated H26r and H30f (Table 5.2). The PCR product resulting from this reaction was cloned into the *Hind* III site of pDA71 as described to create pHSX1a. This construct was transformed into strain CW25 using the PEG-mediated protoplast method.

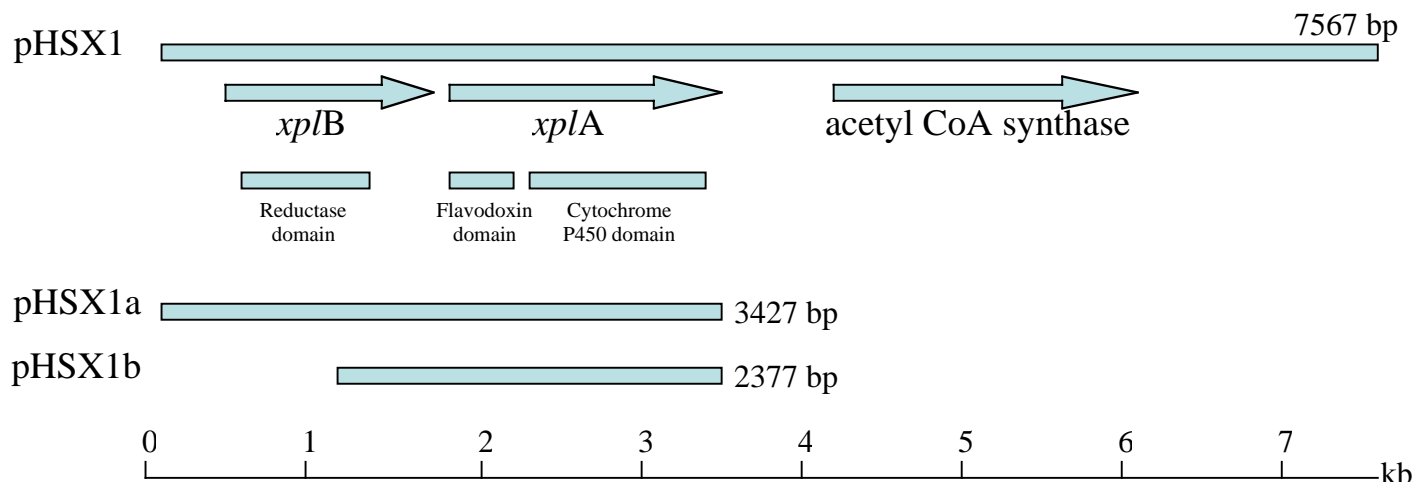
Transformants of strain CW25 containing pHSX1a were screened on zone of clearing plates for RDX degrading activity. All transformants had the ability to form zones of clearance, indicating that it is the cytochrome P450 and reductase-like genes which confer this ability, and that the acetyl CoA synthase gene is not involved.

#### 5.3.8.2 Subcloning one ORF into pDA71

A further subcloning experiment was performed to see if the reductase-like gene is required for the activity, or if the P450-like gene is the unique component. As before, as much of the upstream sequence was maintained as possible. Therefore a primer was designed to be complementary to a region 697 bp upstream of the P450-like gene ATG start codon. Although the 3' end of the reductase-like gene is present, only the P450-like gene should be expressed from this fragment. This primer had a *Hind* III site engineered into it as before, and was designated H37f (Table 5.2). A PCR product was obtained using primers H37f and H26r, and cloning was performed as above.

The plasmid resulting from the cloning of this PCR product into pDA71 was designated pHSX1b, and gave a zone of clearance when present in host strain CW25. This indicates that it is the expression of the P450-like gene which is responsible for the ability of strain 11Y to degrade RDX, and that strain CW25 is able to provide any other components required for activity.

The inserts of plasmids pHSX1a and pHSX1b are illustrated, along with the three ORFs described, in Figure 5.11. The first ORF, with homology to a reductase, was named *xplB*, and the second, with homology to a cytochrome P450, named *xplA*, for *explosive* degrading.



**Figure 5.11: Map of the 7567 bp region responsible for conferring the ability to degrade RDX.** The open reading frames correspond to proteins with homology to adrenodoxin reductase (*XplB*), cytochrome P450 (*XplA*), and acetyl CoA synthase. Areas identified as protein domains are indicated. The subcloned DNA fragments inserted into pDA71 to create pHSX1a and pHSX1b are shown. Both conferred activity against RDX to strain CW25.

1 CTGACGCGCA CCGAACCCGA ACTGGCACAC GAGACTCTGC ACTGTGCTGC CCTGGGGATC  
 61 GACGTCCCCG CGGGAAAGTG CATCGGCTTC ACCGACATCG ACGAGATCCG CACGGAAGAG  
 121 GGCCCGGTCT TCGTCTTCCG GATGTCCGGC CGGCTGTACG GCCCCGACGA CAGCGATGTC  
 181 AACGAATGGA CGATCCACGG CGAACCCGAT CTGGTGATGT CCAACGGCAC CCCGCCGACG  
 241 ATGGCCACCA CCTGCACCCA ATTGGTGAAC CGTATCCCCG ACGTGCTCGA CGCCGACCCG  
 301 GGATTCGTGA CCGTCGTGTA TCTGCCCAGG CTGCGCTACC GGCACGGTCG GCTGCACGAC  
 361 CACCTGAGCA GGTGGTCATC GGATCGTTAC ATCGTGCGCG AAGAACTCTA ACAAT**AGAGT**  
 421 ATTTGG**ATGG** ACATCATGAG TGAAGTGGAC GTGGCACCGC GCGTGGCGGT GGTGCGCGCG  
 1 M D I M S E V D V A P R V A V V G A  
 481 GGACCGTCGG GTTGTTTCAC CGCACAGCAA CTCCGCAAGC AGTGGCCGGA GGTGGAGGTC  
 19 G P S G C F T A Q Q L R K Q W P E V E V  
 541 ACCGTCTTCG ACCGGCTACC CACCCCCTTC GGGCTCCTTC GCTACGGCGT GGCTCCCGAC  
 39 T V F D R L P T P F G L L R Y G V A P D  
 601 CATCAGGGCA CGAAGAACGT GATCCGGCAA CTCTCGCGGG TATTCGACGA TCGGACTCGC  
 59 H Q G T K N V I R Q L S R V F D D R T R  
 661 TTCGTGCGCA ACATCGAGCT CGGCCGGAAC CTGTGATCG AGGACCTGCG AGCCGCGTTC  
 79 F V G N I E L G R N L S I E D L R A A F  
 721 GACGTGCTGG TCCTCGCGAC GGGCCTCAGC GGAGACCGCC GTCTCGGCAT CCCGGGCGAT  
 99 D V V V L A T G L S G D R R L G I P G D  
 781 GACCTGCCCC GCATCGTGGG GTCCGGGGCGC TTCACCCGAT GCGTCAACGA CCATCCCGCC  
 119 D L P G I V G S G R F T R C V N D H P A  
 841 GCCGGCGACC TGCCGACGGT GGGCCGGAGG GTGGTCCTGG TGGGCGGCGG CAACGTGCGC  
 139 A G D L P T V G R R V V L V G G G N V A  
 901 ATGGACATCA TCCGGCTGCT GTCGAAGCAG CCCGACGAAT TCACCGGTTT CGATCTGCAC  
 159 M D I I R L L S K Q P D E F T G S D L H  
 961 CCGGACACCC TCGGGAGACT GAGATCGGAG GGCCACGGC GGATCGACGT GGTGGTGCGC  
 179 P D T L G R L R S E G P R R I D V V V R  
 1021 TCCACGCCCA CCGACGCCAA GTTCGACCCG GTGATGATGC GTGAACTGGC GCACCTGGCG  
 199 S T P T D A K F D P V M M R E L A H L A  
 1081 TCGACGGAGT TCCGCCTCGC CGATGCGGGG GTGCTCGCCA CGGCAGAGTC CTCCGACCCG  
 219 S T E F R L A D A G V L A T A E S S D P  
 1141 CGGAGTGCGG CCCTGGCCCA CGTCGTCGAA CGCGAGAGCC CGGCGGCACC GGCCACGACG  
 239 R S A A L A H V V E R E S P A A P A T T  
 1201 GTGGTGTTCC ACTTCGGATC GACCCCGGTC GAGGTGATCG GCACCGACCG CGCGGAGGCC  
 259 V V F H F G S T P V E V I G T D R A E A  
 1261 GTCAAGGTGC GCACCGGCGT GCACACCACG ACTCTCGCGT GCGACACCGT CATCACCGCA  
 279 V K V R T G V H T T T L A C D T V I T A  
 1321 ATCGGTTTCG AGTCGGCCGT AAACGACGAC CTCGACCTGA CGCTGTACCG CGACGCCGAT  
 299 I G F E S A V N D D L D L T L Y R D A D

1381 CCGGGAGAGG ATTTTCTTGC TCCGGGCCTG TACCGCACCG GGTGGCTGCA CAGTTCCACC  
 319 P G E D F L A P G L Y R T G W L H S S T

1441 GGAGCGCTTC CCGAGATGCG GGCCCGTGCC CGGGCGTTGG CGGCCCGAAT CCGCCGCGAT  
 339 G A L P E M R A R A R A L A A R I R R D

1501 CATGCCGGTC ACCATCCGGC CCGTCCGGGT CTGGTGGCAA TCCCTTACGA AGTGATCGAC  
 359 H A G H H P A R P G L V A I P Y E V I D

1561 CGGACAGTCG ATTTTCGACGG CTGGATGCGG ATCGACGAGG CGGAGGTCGC CTCGGCCTCG  
 379 R T V D F D G W M R I D E A E V A S A S

1621 CCGGGCCGCA TCCGGCAGAA GGTCCGCGAG GTCGACGCGA TGCTGGCGTT GGCCCGCACC  
 399 P G R I R Q K V R E V D A M L A L A R T

1681 GTGCCGGCCG AATCGGTCTG **CTG**A GACGGC AGGCCTGCAG TGAACCCGAG AATCACCCGA  
 419 V P A E S V C \*

1741 ACACCCGAGA TGGAGTGT**GG** **AGAATCATGA** CCGACGTAAC TGTCTGTTC GGAACCGAGA  
 1 M T D V T V L F G T E T

1801 CCGGCAACGC CGAGATGGTC GCCGACGACA TCGCTTCTGC CCTGGGGGAA TTCGATATCG  
 13 G N A E M V A D D I A S A L G E F D I E

1861 AGGCCACCGT GGTGGGGATG GAGGACTTCG ACGTCGCAGA TCTGGCCGCC TCCGGCACGG  
 33 A T V V G M E D F D V A D L A A S G T V

1921 TCGTGCTCGT CACCTCCACC TACGGAGAGG GTGAACTGCC GGCGACGACC CAGCCCTTCT  
 53 V L V T S T Y G E G E L P A T T Q P F F

1981 TCGATGCGAT GAAGGCGGCC GAGCCTGACC TCACGGGTCT GCGGTTCGGG GCCTTCGGCC  
 73 D A M K A A E P D L T G L R F G A F G L

2041 TCGGCGACAG CACCTACGAC ACCTACAACA ACGCGATCGA CATCCTCGTC GGTGCGGTGA  
 93 G D S T Y D T Y N N A I D I L V G A V T

2101 CAGACGCAGG AGCGACACAG GTCGGCGCAA CGGGCCGGCA CGATGCCGCG TCCTTCCAGC  
 113 D A G A T Q V G A T G R H D A A S F Q P

2161 CGGCGGACGG ACCCGTGGCC GAGTGGGCCA AACAGTTCGC CGAAGCCCTC TCTGATCGAA  
 133 A D G P V A E W A K Q F A E A L S D R T

2221 CTCGACGAGG AGGACATGAG ATGACCGCTG CGTCCATCGA TCGCGAGCTC GTGCCGTGGT  
 153 R R G G H E M T A A S I D R E L V P W S

2281 CGGATCCCGA ATTCAGGAAC AACCCCTATC CCTGGTATAG GCGACTGCAA CAGGACCATC  
 173 D P E F R N N P Y P W Y R R L Q Q D H P

2341 CGGTGCACAA GCTGGAGGAC GGCACCTACC TGGTGTCCCG GTACGCCGAC GTGAGTCATT  
 193 V H K L E D G T Y L V S R Y A D V S H F

2401 TCGCGAAACT GCCCATCATG AGCGTCGAAC CCGGATGGGC CGACGCCGGG CCCTGGGCGG  
 213 A K L P I M S V E P G W A D A G P W A V

2461 TCGCGAGCGA CACCGCGCTG GGTTCGGATC CCCCAGATCA CACCGTGTTC CGCCGGCAGA  
 233 A S D T A L G S D P P H H T V L R R Q T

2521 CCAACAAGTG GTTCACGCCG AAACCTGGTGG ACGGCTGGGT CAGGACCACC CGTGAGCTGG  
 253 N K W F T P K L V D G W V R T T R E L V

2581 TCGGCGACCT GCTCGACGGT GTCGAGGCCG GTCAGGTGAT CGAGGCCAGG CGCGACCTCG  
 273 G D L L D G V E A G Q V I E A R R D L A

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2641 CCGTCGTCCC CACCCACGTC ACGATGGCAC GCGTCCTACA ACTACCCGAA GACGATGCCG
293   V V P   T H V   T M A R   V L Q   L P E   D D A D

2701 ACGCCGTGAT GGAGGCGATG TTCGAGGCGA TGCTGATGCA AAGCGCGGAG CCCGCGGACG
313   A V M   E A M   F E A M   L M Q   S A E   P A D G

2761 GCGATGTCGA CCGGGCTGCA GTCGCTTTTCG GCTATCTGAG TGCGCGCGTC GCCGAGATGC
333   D V D   R A A   V A F G   Y L S   A R V   A E M L

2821 TCGAGGACAA GCGGGTGAAC CCGGGCGACG GCCTGGCCGA TTCGTTGCTC GACGCCGCC
353   E D K   R V N   P G D G   L A D   S L L   D A A R

2881 GCGCCGGGGA GATCACCGAG AGCGAGGCTA TCGCCACGAT TCTGGTGTTT TATGCCGTGG
373   A G E   I T E   S E A I   A T I   L V F   Y A V G

2941 GGCACATGGC CATCGGGTAC CTGATCGCCT CGGGTATCGA ACTGTTTCGCT CGTCGGCCCCG
393   H M A   I G Y   L I A S   G I E   L F A   R R P E

3001 AGGTTTTTAC CGCATTCCGC AACGACGAGT CAGCACGTGC GCGGATCATC AACGAGATGG
413   V F T   A F R   N D E S   A R A   A I I   N E M V

3061 TCCGGATGGA CCCGCCACAA CTGTCTTTTCC TGCGGTTTCC TACCGAGGAC GTGGAGATCG
433   R M D   P P Q   L S F L   R F P   T E D   V E I G

3121 GCGGTGTGCT CATCGAGGCC GGATCGCCCA TCCGCTTCAT GATCGGTGCG GCCAATCGCG
453   G V L   I E A   G S P I   R F M   I G A   A N R D

3181 ACCCCGAGGT CTTTCGATGAC CCGGATGTCT TCGACCACAC CCGCCCCGCT GCGGCCAGCC
473   P E V   F D D   P D V F   D H T   R P P   A A S R

3241 GCAACCTGTC GTTCGGTCTG GGGCCGCATT CCTGTGCGGG ACAGATCATC TCGCGTGCCG
493   N L S   F G L   G P H S   C A G   Q I I   S R A E

3301 AGGCAACGAC TGTCTTCGCC GTGCTGGCCG AACGGTACGA GCGGATCGAG TTGGCCGAGG
513   A T T   V F A   V L A E   R Y E   R I E   L A E E

3361 AACCCACCGT GGCACACAAC GATTTCGCCC GGCGCTACCG AAAACTGCCG ATCGTCCTGT
533   P T V   A H N   D F A R   R Y R   K L P   I V L S

3421 CCTGA 3425
      *

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**Figure 5.12: Nucleotide sequence of *xpl* gene cluster and deduced amino acid sequence.** Nucleotides are numbered with 1 being the first nucleotide of the insert DNA. Start (ATG) and stop (TGA) codons of *xplB* and *xplA* respectively are shown in bold. Putative ribosome binding sites are underlined in bold. The sequence was submitted to Genbank and assigned the accession number AF449421.

### 5.3.9 Sequence analysis of amino acid translation of ORFs

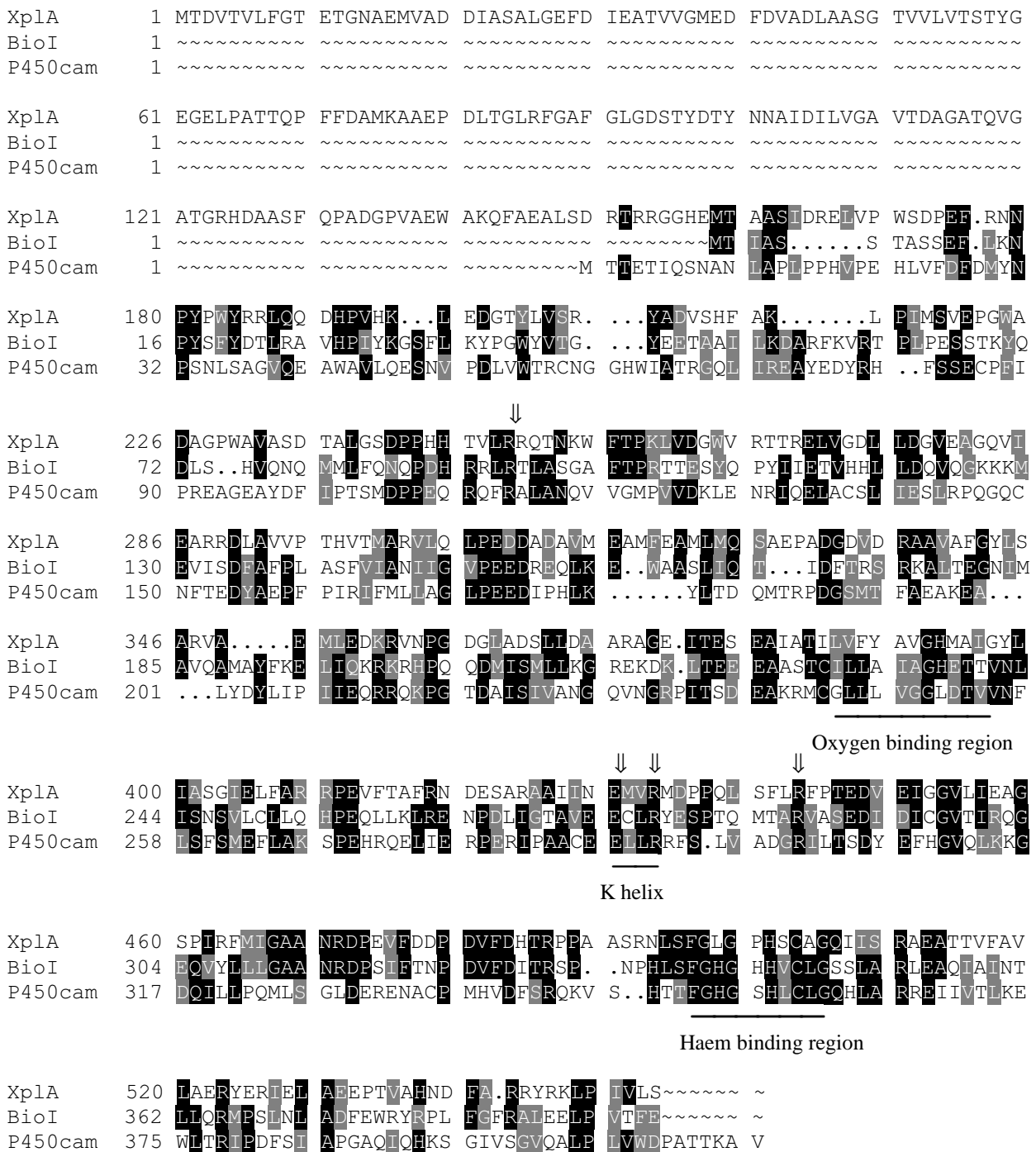
The deduced amino acid sequences of *xplA* and *xplB* were subjected to further analysis. Nucleotide sequence and deduced amino acid sequence of this region are shown in Figure 5.12. The putative Shine-Dalgarno sequences are shown in bold <sup>278</sup>.

#### 5.3.9.1 P450-like region of *XplA*

Comparing *XplA* with known P450s, there are several areas of high conservation. These are indicated in an alignment of the amino acid sequences of *XplA* with *BioI* of *B. subtilis*, with which

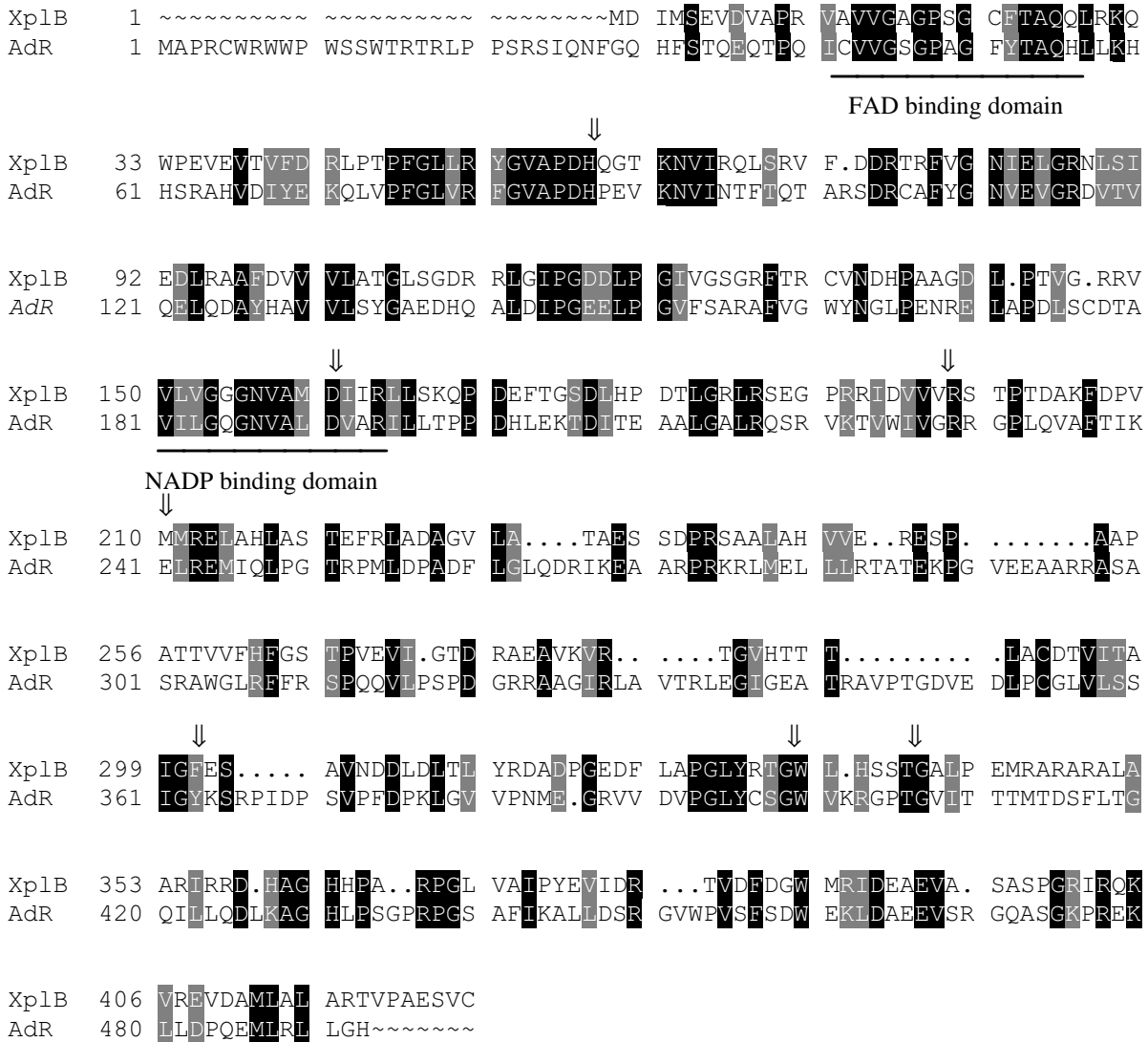
it has most homology, and P450<sub>cam</sub> of *P. putida*, which is often used as a P450 paradigm, in Figure 5.13. Generally the C-termini of P450s are more highly conserved than the N-termini.

The haem binding region has the signature FXXGXXXCXG (<http://drnelson.utmem.edu/PIR.P450.description.html>), which is more specifically described on the PROSITE database (<http://ca.expasy.org/prosite/><sup>149</sup>) as [FW]-[SGNH]-x-[GD]-x-[RKHPT]-x-C-[LIVMFAP]-[GAD] (accession number PS00086). The haem binding region of XplA (residues 496-505) fulfils the requirements of both signatures. The cysteine residue within the signature is the haem binding ligand<sup>263</sup> and is absolutely conserved. Arg-249, Glu-430, Arg-433 and Arg-443 (numbers refer to XplA) are also conserved<sup>133, 223</sup>. The oxygen binding region consensus A-[AG]-x-[ED]-T (<http://drnelson.utmem.edu/PIR.P450.description.html>), present in many cytochrome P450s, is not complete in XplA. This region lines up with the residues V-G-H-M-A in XplA (residues 397-401).



**Figure 5.13: Alignment of protein encoded by *xplA* with known P450s.** Alignment of the deduced amino acid sequence of *xplA* with *Bacillus subtilis* P450-like BioI (CYP107H1; accession number U51868)<sup>37</sup> and P450<sub>cam</sub> from *Pseudomonas putida* (CYP101; accession number P00183)<sup>126, 310</sup>. Identical residues are shaded black and similar residues shaded grey. Domains are underlined and residues described in the text indicated by arrows.



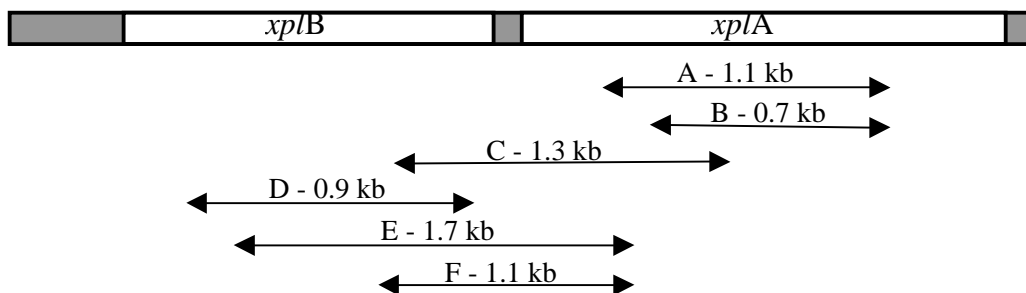


**Figure 5.15: Alignment of protein encoded by *xplB* with a known adrenodoxin reductase.** Alignment of the amino acid sequence of Xp1B with adrenodoxin reductase (AdR) of *Bos taurus* (accession number P08165)<sup>220, 254</sup>. Identical residues are shaded black and similar residues shaded grey. Domains<sup>129</sup> are underlined and residues described in the text indicated by arrows.

### 5.3.10 RT-PCR analysis of *xplA* and *xplB* transcription

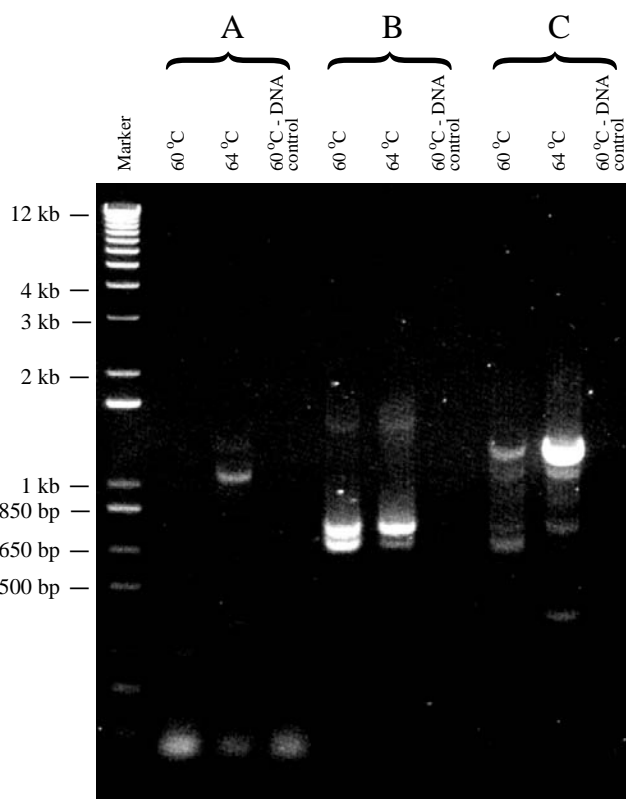
#### 5.3.10.1 Confirmation of transcription of *xplA* and *xplB*

To confirm that both *xplA* and *xplB* are transcribed in strain 11Y, RNA was harvested from cells and subjected to RT-PCR. Primers (Table 5.3) were either chosen from those used for sequencing, or designed from the sequence of pHSX1 to produce fragments of interest (Figure 5.16).



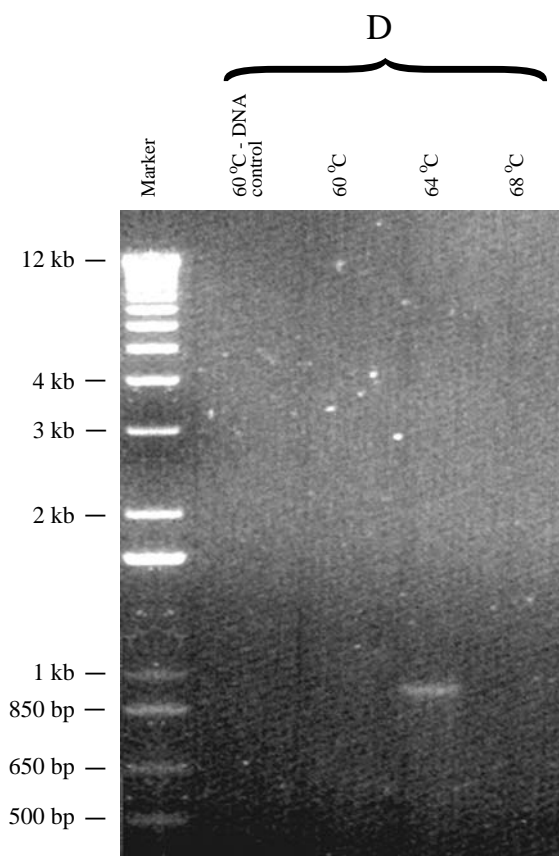
**Figure 5.16: Schematic of the fragments chosen for amplification by RT-PCR.**

To determine transcription of *xplA*, three RT-PCR reactions were performed, each using a gradient of annealing temperatures from 60–64 °C. Reaction A was performed with the primers H14f/ H41f, reaction B with H42f/ H14f, and reaction C with H21f/ H22f. All gave products of the appropriate sizes (1.1, 0.7, 1.3 kb respectively) as visualized by gel electrophoresis (Figure 5.17). The fragments were cloned, sequenced and confirmed to be the correct products, as the sequence was identical when compared to the original sequence.



**Figure 5.17: RT-PCR analysis of *xplA* transcript.** Two annealing temperatures were used in the DNA amplification cycles (60 °C and 64 °C) to maximize the likelihood of getting the appropriate amplification. The negative control contained no AMV RT. The most prominent bands at 64 °C annealing temperature were of the predicted size (A – 1.1 kb, B – 0.7 kb, C – 1.3 kb).

The RT-PCR procedure was also performed to determine the transcription of *xplB* using primers H6r/ H40f (reaction D). A band of the predicted size of 0.9 kb was produced (Figure 5.18). As before, the fragment was cloned and sequenced and its identity confirmed.



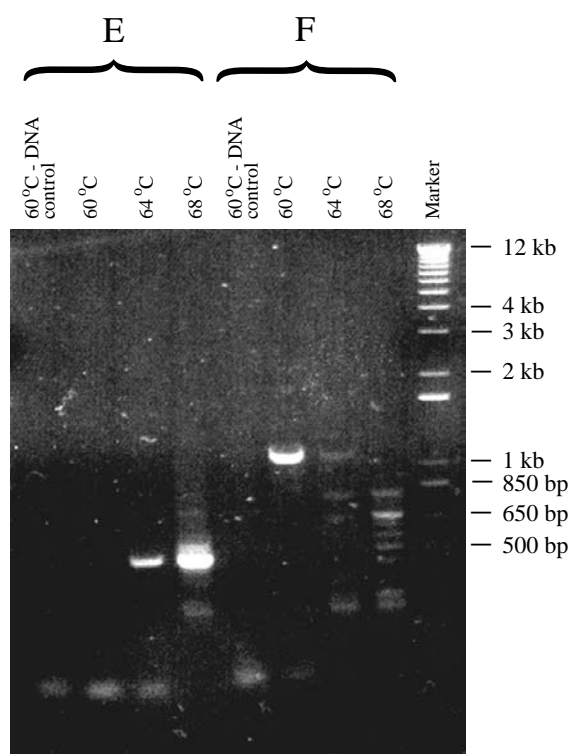
**Figure 5.18: RT-PCR analysis of *xplB* transcript.** Annealing temperatures of 60 °C, 64 °C and 68 °C were used in the DNA amplification cycles and a control without the AMV RT was used. A transcript of the predicted size (0.9 kb) was observed using 64 °C annealing temperature.

### 5.3.10.2 Determination of length of *xplA* transcript

The result of reaction C (§5.3.10.1) indicates that the transcription start site of *xplA* is at least 649 bp upstream of the ATG start codon, as that is the site at which H21f anneals. RT-PCR was performed to determine the approximate transcription start site of *xplA*. Further forward primers were designed to sequences upstream of *xplA*. Reaction E was performed with primers H22f/ H45f and reaction F with H22f/ H46f (Table 5.3).

Reaction F produced a band at the appropriate size of 1.1 kb, when visualized by gel electrophoresis, and its identity was confirmed by sequencing as above. However, reaction E did not produce a fragment of the expected size (1.7 kb) at any of the annealing temperatures (Figure

5.19). This implies that the initiation of the transcription of *xplA* occurs between 649 bp (from reaction C) and 1033 bp (from reaction E) upstream of the start codon.

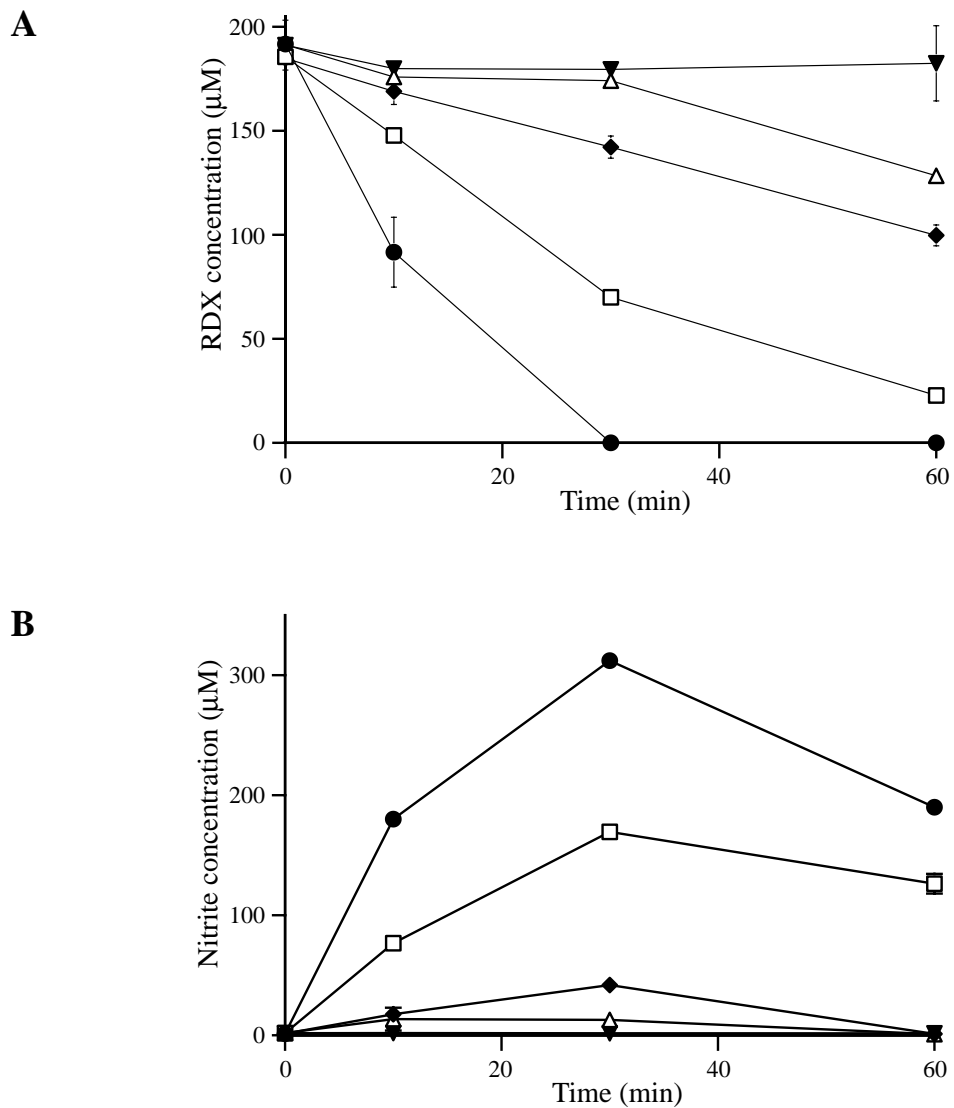


**Figure 5.19: RT-PCR analysis of upstream region of *xplA* transcript.** Annealing temperatures of 60 °C, 64 °C and 68 °C were used. Controls were performed without the AMV RT. Reaction E showed no transcripts of the predicted size (1.7 kb) at any annealing temperature, whereas F showed a transcript of the predicted size (1.1 kb) at 60 °C annealing temperature.

### 5.3.11 Evidence for P450 function using an inhibitor

To provide further evidence of the involvement of a P450 in the degradation of RDX by strain 11Y, resting cell incubations were performed in the presence of metyrapone, a specific inhibitor of P450s<sup>311</sup>. Metyrapone was added to the incubations at several concentrations, and RDX and nitrite concentrations were monitored.

The addition of metyrapone had an inhibitory effect on the degradation of RDX (Figure 5.20). Significant reductions in the rate of RDX removal were seen using 0.1, 0.5 and 1 mM metyrapone, and no RDX removal was observed in the presence of 10 mM metyrapone. A similar pattern was seen in the nitrite production, with no nitrite being produced in the presence of the greatest concentration of the inhibitor.



**Figure 5.20: Metyrapone inhibition of strain 11Y resting cell incubation with RDX.** A. RDX concentration in the presence of metyrapone at: 0 mM (●), 0.1 mM (□), 0.5 mM (◆), 1 mM (△) and 10 mM (▼). B. Nitrite concentration in the presence of metyrapone at: 0 mM (●), 0.1 mM (□), 0.5 mM (◆), 1 mM (△) and 10 mM (▼).

## 5.4 Discussion

A DNA library was used to clone the RDX degrading genes from *Rhodococcus rhodochrous* strain 11Y. A representative library of strain 11Y DNA was made first in *E. coli* and was subsequently transformed into a non RDX degrading rhodococcal strain. Screening the library in *E. coli* was attempted, but was not successful. No zones of clearance were produced when the library was screened using an adapted zone of clearance screen, so a more sensitive assay was developed to allow the detection of nitrite resulting from the breakdown of RDX. Both whole cells and lysed cells were screened in this manner and although there were some apparent positives, these turned out to be non reproducible. This phenomenon of unreproducible positives using the Griess assay in microtitre plates has been reported before, and the presence of nitric oxides in ambient air is thought to be responsible<sup>288</sup>, although evaporation from edge and corner wells may also play a role.

The failure of these methods to identify an RDX degrading clone indicates that the necessary components were not actively produced in *E. coli*, as representative libraries were screened in each case. This may be due to a lack of transcription of the gene(s) from their native promoter(s), or the production of inactive protein. There are only a few cases in which promoters of rhodococcal genes have been seen to function in *E. coli*. The promoters of the genes for *Rhodococcus* sp. N-774 amidase<sup>134</sup>, *R. rhodochrous* J1 nitrile hydratase<sup>169</sup>, *R. rhodochrous* CTM catechol 2,3-dioxygenase gene<sup>52</sup>, *R. opacus* MR11 NAD-reducing hydrogenase<sup>118</sup> and *R. rhodochrous* NCIMB 13259 catechol 1,2-dioxygenase<sup>297</sup> are active in *E. coli*. This is likely to be because these rhodococcal promoters conform to the *E. coli* promoter consensus. Sequences homologous to *E. coli* promoter -10 and -35 elements have been found upstream of the gene *hppC* in *R. globerulus* PWD1, which may be responsible for its expression in *E. coli*<sup>13</sup>. In addition there may be components present in rhodococci but not in *E. coli* which allow the correct folding of the protein, or extra cofactors or pathway components required for activity which do not occur in *E. coli* cells.

A clone from the rhodococcal library carrying a 7.5 kb insert was isolated by selective enrichment and found to be able to remove RDX from culture. When comparing the growth of this clone, CW25(pHSX1), with the growth of strain 11Y on RDX, CW25(pHSX1) grew more slowly than, and reached a lower final OD<sub>600</sub> than, strain 11Y. The presence of chloramphenicol in the growth medium of CW25(pHSX1) may have been responsible for the longer lag phase of this strain, and strain CW25 does not grow as well as strain 11Y on nitrite (E. Travis, personal communication), which is the only identified nitrogen source released from the breakdown of RDX by strain 11Y

(§4.3.5). The insert in pHSX1 contains 426 bp upstream of *xplB*, and it is possible that further upstream sequences are required for optimal gene expression.

The 7.5 kb fragment was found to contain three ORFs whose deduced amino acid sequences have homology to an adrenodoxin reductase, a cytochrome P450 and an acetyl CoA synthase. Subcloning sections of the 7.5 kb fragment from pHSX1 demonstrated that the gene for the acetyl CoA synthase was not involved in RDX degradation. A further subcloning experiment showed that it is the gene for the cytochrome P450-like protein (*xplA*) which is the unique component for RDX degradation possessed by strain 11Y and not strain CW25. Strain CW25 appears to be able to provide any other components required.

P450s are haem containing monooxygenase proteins, widely distributed in animals, plants and bacteria and are able to catalyse a wide range of reactions which are commonly involved in the detoxification of substances, particularly xenobiotics<sup>262</sup>. The name P450 describes the maximum of the peak (450 nm) in the absorption spectrum of the reduced carbon monoxide complex. They are not true cytochromes; a more accurate term would be haem-thiolate protein<sup>215</sup>, describing the haem binding to the sulphur molecule on the cysteine residue. P450<sub>cam</sub> is the best characterized of the bacterial P450s, and catalyses the hydroxylation of camphor in *Pseudomonas putida*.

The deduced amino acid sequence of XplA was analysed and found to contain all the absolutely conserved residues of P450s (Figure 5.13). In particular the residues Arg-249 and Arg-443 are thought to be involved in hydrogen bonding the haem<sup>223</sup>, and Glu-430 and Arg-433 are found within the K helix (EXXR sequence) which appears to be involved in maintaining the position of a “meander” region of the protein, helping to either stabilize the haem pocket, or interact with redox partners<sup>133</sup>.

The threonine residue in the oxygen binding region consensus, absent in XplA, corresponds to the Thr-252 of P450<sub>cam</sub>, and has been reported to play a crucial role in the monooxygenation reaction, as reduced activity and uncoupling of the electron transfer are seen when it is substituted by another amino acid<sup>153, 239</sup>. However, it appears not to be highly conserved in P450s, as alignments at <http://www.icgeb.trieste.it/~p450srv/> indicate that approx. 10 % of sequences have alternative amino acids at this position, including Ala, Glu, Ser, Asn, Pro and Ile. The substitution of Ala for Thr found at this position in XplA is seen in several other P450s including P450<sub>eryF</sub> from *Saccharopolyspora erythraea*, involved in erythromycin biosynthesis. Interestingly, the reverse mutation in P450<sub>eryF</sub>, Ala245Thr, gave a massively reduced activity and uncoupling of the reducing equivalent utilization<sup>68</sup>. The H-bonding formed by the Thr in P450<sub>cam</sub> is thought to be supplied by a

water molecule in the absence of Thr<sup>67</sup>. This substitution may be involved in the accommodation of large substrates by reducing steric hindrance between them and the amino acid side chain, and may impair the ability of the protein to cleave the O-O bond of the oxygen<sup>120</sup>.

Eukaryotic P450s, such as those used to detoxify substances in the liver, require a reductase component for electron transfer and fall into a group called Type II, whereas bacterial and mitochondrial P450s are Type I, requiring ferredoxin and ferredoxin reductase to complete the multicomponent enzyme system<sup>74</sup>. The reactions which P450s can catalyse in rhodococci and related species include biotransformations of xenobiotics, such as the dechlorination of pentachlorophenol by *Rhodococcus chlorophenolicus*<sup>311</sup>, and the degradation of ethoxyphenol and methoxybenzoate by P450<sub>RR1</sub> and P450<sub>RR2</sub> of *R. rhodochrous*<sup>163</sup>, the herbicide EPTC by *thcB* from *Rhodococcus* sp.<sup>210</sup>, ethyl-*tert*-butyl ether by *ethB* from *R. ruber*<sup>53</sup> and piperidine and pyrrolidine by *pipA* from *Mycobacterium smegmatis*<sup>235</sup>. Rhodococcal P450s involved in xenobiotic degradation are sometimes found in operons with genes coding for ferredoxin reductases and ferredoxins<sup>53, 210</sup>. In some cases regulatory genes and other ORFs, to date of unknown function, are found in the operon<sup>37, 53, 66, 210, 235</sup>.

The use of a P450 in the biodegradation of RDX has been speculated upon in *P. chrysosporium*<sup>276</sup> and inhibition studies have also implied the involvement of a P450 in work on rhodococcal RDX degraders<sup>60, 302</sup>. Inhibitor studies using the specific P450 inhibitor metyrapone showed that the removal of RDX from resting cell incubations by *Rhodococcus rhodochrous* strain 11Y is greatly reduced in the presence of metyrapone. This provides further evidence of the use of a P450 in the degradation of RDX by strain 11Y and adds weight to the cloning evidence.

The *xplA* gene has homology to a P450 with a flavodoxin domain at the N-terminus. Bacterial P450s are usually about 400 amino acids, and this N-terminal extension adds a further approx. 140 residues, which is in the size range of the short chain bacterial flavodoxins. Met-158 could be postulated as being a start codon for the original P450 as it falls between the two domains. In some situation, flavodoxins are able to perform the same physiological roles as ferredoxins, despite there being no sequence homology between the two<sup>309</sup>. Both are involved in electron transport, but flavodoxins use a flavin (FMN) moiety as the redox centre instead of an iron-sulphur centre. Therefore, the flavodoxin domain of XplA could possibly dispense with the necessity for a separate ferredoxin. The domain contains most of the residues which form the flavodoxin signature for the binding of the FMN phosphate group, with the exception of Thr-15 (Figure 5.14). This threonine residue has been implicated in electron transfer, or in the maintenance of the conformation

of the protein<sup>315</sup>. The flavin is proposed to be sandwiched between residues Trp-60 and Tyr-98 (numbers refer to *D. vulgaris*)<sup>309</sup>. The equivalent of Tyr-98 is present in XplA, but Trp-60 is replaced by Tyr. However, both have aromatic sidechains such that it is possible that Tyr could function in the place of Trp.

A flavodoxin-like gene has recently been found in a cluster with a novel P450 and a reductase in *Citrobacter brakii*<sup>140</sup>, although the function of the flavodoxin as a redox partner of the P450 has not been demonstrated. In XplA, the flavodoxin appears to be part of a compound protein with the putative P450, and fused genes containing components of the P450 system are not uncommon. The most well known example is that of P450<sub>BM-3</sub> from *Bacillus megaterium*, in which the reductase is fused to the P450 in one self-sufficient protein<sup>212, 213</sup>. Crespi et al.<sup>66</sup> reported an operon containing a P450 and a ferredoxin-like domain at the N-terminus of a protein otherwise dissimilar to any protein involved in P450 reactions. A two-component bacterial P450 system was reported by Serizawa and Matsuoka<sup>268</sup>, with the reductase component containing both FAD and FMN groups, and no ferredoxin being required for activity. Very recently a new class of fused P450 has been cloned from a *Rhodococcus* sp., comprising an N-terminal P450 domain with a C-terminal reductase domain, and possibly containing a ferredoxin type 2Fe2S domain, although the function of these domains has not been proved<sup>246</sup>.

There are no reports to date of a two component system comprising a reductase and a compound P450 and FMN domain, possibly making the *xpl* gene cluster a novel type of P450. A comparison of XplA with all bacterial P450s has been performed by the P450 nomenclature committee, who have found it to be unique, and have given it a new family name of CYP177A1 (D. Nelson, personal communication).

A gene with homology to adrenodoxin reductase, *xplB*, is found 62 bp upstream of *xplA*. Adrenodoxin reductases perform electron transfer as part of the mitochondrial P450 system involved in steroid biosynthesis, generally transferring electrons between NADP and adrenodoxin, a 2Fe2S ferredoxin. Amino acid sequence analysis shows that two of the conserved residues of adrenodoxin reductases are not present in XplB (§5.3.9.3); these residues are implicated in the electron transfer mechanism from alignments and structural studies<sup>352</sup>, although the significance of this is not yet known. Adrenodoxin reductases differ significantly from ferredoxin reductases, which are more generally thought to be involved in Type I systems, in sequence and structure<sup>351</sup>, although both perform effectively the same function. Several mycobacterial proteins with homology have been identified, indicating that similar proteins are found in bacteria, but none have a proven function.

The function of the flavodoxin domain in XplA as a redox agent and the possible use of XplB as a reductase in the mechanism have not, as yet, been demonstrated.

Both *xplA* and *xplB* appear to possess ATG start codons. Initiation codons of GTG are commonly found in rhodococcal genes<sup>189</sup>, but do not appear to be utilized in *xplA* or *xplB*. There are no possible in-frame GTG initiation codons upstream of *xplB*, and the initial amino acid of XplA aligns directly with the methionine of the flavodoxin from *D. vulgaris*, indicating that it has been correctly assigned.

P450s which are involved in xenobiotic metabolism are commonly induced by their substrate. These include P450<sub>cam</sub> from *P. putida*, induced by an order of magnitude in the presence of the substrate camphor<sup>173</sup>, P450<sub>SU1</sub> and P450<sub>SU2</sub> involved in herbicide metabolism in *Streptomyces griseolus* which is absent from uninduced cells as assayed by HPLC<sup>221</sup>, and a rhodococcal P450 involved in the degradation of herbicide EPTC, whose induction is apparent by 2D gel electrophoresis<sup>210</sup>. There are also reports of constitutive bacterial P450s such as P450<sub>CON</sub> in *Streptomyces griseolus*, and the quantity of this protein appears not to change regardless of the addition of inducer<sup>221</sup>. RT-PCR demonstrated that both *xplA* and *xplB* are transcribed in cells grown in rich medium (LB) in the absence of RDX, which confirms the previous observation that activity against RDX was found in cells grown without RDX (§4.3.3). The activity was seen to increase three-fold in the presence of RDX, indicating a degree of inducibility.

The RT-PCR experiments give an indication of the positioning of the promoter of *xplA*. The expression of *xplA* from the subcloned fragment in plasmid pHSX1b demonstrated that a promoter of *xplA* must be present within the 697 bp upstream of the gene on pHSX1b. RT-PCR reaction E showed that at least 649 bp upstream of *xplA* are transcribed. Therefore, the putative promoter region can be narrowed down to a region of 48 bp between 697 bp maximum and 649 bp minimum upstream of the start codon.

Few studies have been performed on the regulatory regions of rhodococcal genes, and they seem not to have highly conserved sequences such as those in *E. coli*. Of those which appear in publications, some have homology to *E. coli*  $\sigma^{70}$  or *B. subtilis* promoters<sup>26, 118, 193, 297</sup>, although this is by no means the norm. Apart from these similarities there is no obvious consensus between the promoter sequences reported. The putative promoter of *xplA* appears to be significantly further upstream than has previously been reported, with known rhodococcal transcription start sites varying from 26 to 280 nucleotides upstream of the start codon<sup>80, 174</sup>.

The 48 bp putative promoter region is shown in Figure 5.20. Within this region no areas with homology to *E. coli* promoters could be found. Comparison with other rhodococcal promoters shows that the sequence TCGACG (shown in bold) is similar to the putative –35 promoter sequences of TTGACG/A<sup>26, 118, 297</sup>, and GGGTGC (in bold) is similar to the –10 GGGTGA promoter sequence reported from *Rhodococcus erythropolis dsz* gene<sup>193</sup>. In addition, the CTGGC and CGCCGA sequences (underlined) found 18 bp apart approximate to GTGGC and CGCGGA, usually found 12-26 bp apart, identified as promoter sequences in 15 genes from *Rhodococcus erythropolis*<sup>346</sup>. The region was also analysed for direct and inverted repeats using the Repeat and StemLoop programs in GCG, and none were found. Repeats have been identified previously around rhodococcal promoters and may be involved in protein binding or stemloop formation. Two direct repeats have been determined in *Rhodococcus* promoter regions, one of 9 bp (imperfect) located upstream of the transcription start site<sup>80</sup>, and another of 66 bp (imperfect) immediately upstream of the transcription start site<sup>73</sup>. Several inverted repeats varying from 6 to 21 bp in length, some being perfectly complementary and others with one or more base pairs lacking complementarity<sup>80, 174, 297</sup>, have been identified in the upstream regions of rhodococcal genes. The location of these may be within the transcribed region, or further upstream. Palindromic sequences of 16 to 36 bp<sup>73, 180</sup> have been identified upstream of rhodococcal genes, but as transcriptional start sites were not determined in these studies, it cannot be established whether or not they lie within the transcribed region.

```

1070          1080          1090          1100          1110
CGCACCTGGC GTCGACGGAG TTCCGCCTCG CCGATGCGGG GGTGCTCG

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**Figure 5.20: Putative promoter region of *xplA*.** The 48 bp region containing the *xplA* promoter. Sequences showing homology to known rhodococcal promoter sequences are shown in bold or underlined.

The next chapter describes efforts directed towards heterologous expression of the RDX degrading proteins.

## Chapter 6. Heterologous expression of XplA and XplB

### 6.1 Background

The genes which enable *Rhodococcus rhodochrous* strain 11Y to degrade RDX have been cloned, and the deduced amino acid sequence data has identified a P450 with flavodoxin domain at the N-terminus (XplA), and a reductase (XplB) immediately upstream. The potential novelty of this electron transfer system requires further investigation. The use of a flavodoxin as an electron donor to a P450 has not been documented previously, and would be of particular interest as a domain fused to the P450, but its use in this context within XplA remains to be established. The use of the reductase to complete the electron transfer system also remains to be determined. It is therefore necessary to obtain pure, active protein, and to reconstitute the activity *in vitro*. The first stage in the attempt to obtain activity *in vitro* is the heterologous expression of the two proteins, XplA and XplB.

For maximum expression in *E. coli*, pET vectors were chosen, as they put the gene under the control of the strong, inducible T7 promoter. The unique *Nde* I site found in the cloning site of many pET vectors contains the start codon ATG within its recognition site and is placed in an optimum position for expression, relative to the promoter and ribosome binding site sequences. Many of the pET expression vectors contain a *Bam*H I site within the multiple cloning site downstream of the *Nde* I site. This is a suitable site to engineer downstream of the stop codon of the gene to be expressed for ligation into the vector.

## 6.2 Materials and methods

### 6.2.1 Expression constructs

For *XplA* expression constructs, three PCR reactions were performed using primers: H35f/ H28r, H28f/ H29r and H29f/ H27r (Table 6.1) and pHSX1 as a template. Each reaction used an annealing temperature of 63 °C and 2 min extension time, and product sizes were confirmed by gel electrophoresis. The fragment of 236 bp was electrophoresed on a 2.5 % w/v agarose gel, and the fragments of 536 bp and 959 bp on a 1 % w/v agarose gel (§2.3.2); each was extracted and gel purified. A fourth PCR was performed using the primers H35f/ H27f, and the products of the previous three reactions as template. The PCR conditions were as described above. The resulting 1.7 kb fragment was observed by gel electrophoresis, extracted, gel purified, cloned into pCR<sup>®</sup>2.1-TOPO and transformed into an *E. coli* host. Plasmids were extracted and the inserts sequenced using M13F and M13R primers to the *lacZ* gene surrounding the insert site.

**Table 6.1: Primers used to amplify *xplA*, engineer in *Nde* I and *Bam*H I cloning sites and engineer out *Bam*H I sites.** Engineered sites are shown in bold.

Primer	Sequence	Tm (°C)
H35f	5' GGAGACATATGACCGACGTAAC <b>TGTCCTG</b> 3'	72.2
H28r	5' GAATTCAG <b>GGT</b> CGGACCACGGCACGAG 3'	68.5
H28f	5' CTCGTGCCGTGGTCC <b>GACCCT</b> GAATTC 3'	68.5
H29r	5' GTGTGATGCGGAG <b>GGT</b> CGGAACCCAG 3'	68.6
H29f	5' CTGGGT <b>TCCGACCCT</b> CCTCCGCATCACAC 3'	68.6
H27f	5' CGGAT <b>GGAT</b> CCTCAGGACAGGACGATCG 3'	68.4

For the *XplB* expression construct, PCR was performed with primers H34f/ H36f (Table 6.2) using an annealing temperature of 65 °C with 1 min 30 s extension. The reaction was visualized by gel electrophoresis and a band of the desired 1.3 kb size observed. This was cloned into pCR<sup>®</sup>2.1-TOPO and sequenced as above.

**Table 6.2: Primers used to amplify *xplB* and engineer in *Nde* I and *Bam*H I cloning sites.** Engineered sites are shown in bold.

Primer	Sequence	Tm (°C)
H34f	5' GAGTATTCATATGGACATCATGAGTGAAGTGG 3'	70.1
H36f	5' CCT <b>GGAT</b> CCTCAGCAGACCGATTC 3'	72.7

Each engineered PCR fragment was digested from pCR<sup>®</sup>2.1-TOPO using *Nde* I and *Bam*H I. The resulting fragments were subject to gel electrophoresis and the bands of the desired sizes excised and gel extracted. Both fragments were inserted into pET vectors also digested with *Nde* I and *Bam*H I. The two pET vectors used were pET-11a and pET-16b (Novagen).

### 6.2.2 Expression strains and growth conditions

Three *E. coli* expression strains were used: BL21(DE3) (Stratagene), B834(DE3) (Novagen) and Rosetta(DE3) (Novagen).

Strains were transformed with expression constructs through heat shock, recovered, and an aliquot plated onto selective medium to check for transformants. The remainder of the transformation reaction was used to inoculate 5 – 10 ml selective medium, which was grown at the temperature specified in the appropriate sections. Selective antibiotic used was 100 µg/ml carbenicillin with 40 µg/ml chloramphenicol added to Rosetta(DE3) lines for retention of the plasmid carrying the tRNAs. Trace elements (§2.2.3) were also added to 4 ml/l when expression of XplA was being attempted, to provide extra iron which might be required for the haem containing protein. When the cultures reached an OD<sub>600</sub> of approx. 1, a sample was taken (uninduced) and 1 mM IPTG added to induce expression. Samples taken after induction were split into two and harvested by centrifugation for 2 min at 13,000 rpm. One sample was resuspended in 100 mM Tris-HCl pH 8.0, 4M urea, to obtain total protein for determination of induction, as was the uninduced sample. From the other sample, crude extracts were prepared.

### 6.2.3 Preparation of crude extracts

Cells were resuspended in 100 mM Tris-HCl buffer pH 8.0. Sonication was performed using a Sanyo MSE Soniprep 150, while the sample was cooled in an ice-water bath, using a small probe operated at an amplitude of 12 µm for 10 cycles of 10 s followed by 30 s for cooling. Soluble and insoluble fractions were separated by centrifugation at 4 °C for 10 min at 40,000 rpm in a Beckman TLA-45 rotor using a Beckman Optima TLX ultracentrifuge. The supernatant was taken as the soluble fraction and the pellet resuspended in 100 mM Tris-HCl pH 8.0, 4M urea, to obtain the insoluble fraction.

#### 6.2.4 Protein concentration determination

Protein concentration was routinely assayed using the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, Illinois, U.S.A.) according to the manufacturer's instructions. Bovine serum albumin (Pierce) was used for the construction of standard curves.

#### 6.2.5 SDS-PAGE electrophoresis

SDS-PAGE was performed according to Laemmli<sup>183</sup> on a Bio-Rad Mini-Protean II system (Hemel Hempstead, Hertfordshire, U.K.). Samples for SDS-PAGE were denatured by heating to 100 °C for 5 min after dilution with 1/4 volumes of sample buffer consisting of 60 mM Tris-HCl, pH 6.8, 15 % v/v glycerol, 2 % w/v SDS, 5 % v/v β-mercaptoethanol and 0.15 % w/v bromophenol blue. The acrylamide mixture (Protogel) consisted of 30 % w/v acrylamide and 0.8 % w/v bis-acrylamide in solution (37.5 : 1 by weight) and was purchased from National Diagnostics (Hessle, Yorkshire, U.K.). Polyacrylamide gels consisted of a 1 cm 4 % w/v acrylamide stacking gel and a 5 cm resolving gel of 12 % w/v acrylamide. The gels were run at 140 V, stained with 0.1 % w/v coomassie blue R-250 in methanol: water: acetic acid (4: 5: 1 by volume) and destained with several changes of the same solvent. Markers used were low molecular weight range markers (Sigma).

#### 6.2.6 Activity assay

An aliquot (10 µl) of soluble fraction from *E. coli* expressing XplA was added to a 10 µl aliquot of soluble extract from *E. coli* expressing XplB, with 1mM cofactor (NADPH, NADP<sup>+</sup>, NADH or NAD<sup>+</sup>) and 0.25 mM RDX, made up to a final volume of 40 µl with 100 mM Tris pH 8.0. After incubation for 1 h at 30 °C, nitrite was assayed for using the Griess assay (§2.4.4).

#### 6.2.7 Electroblotting and N-terminal sequencing

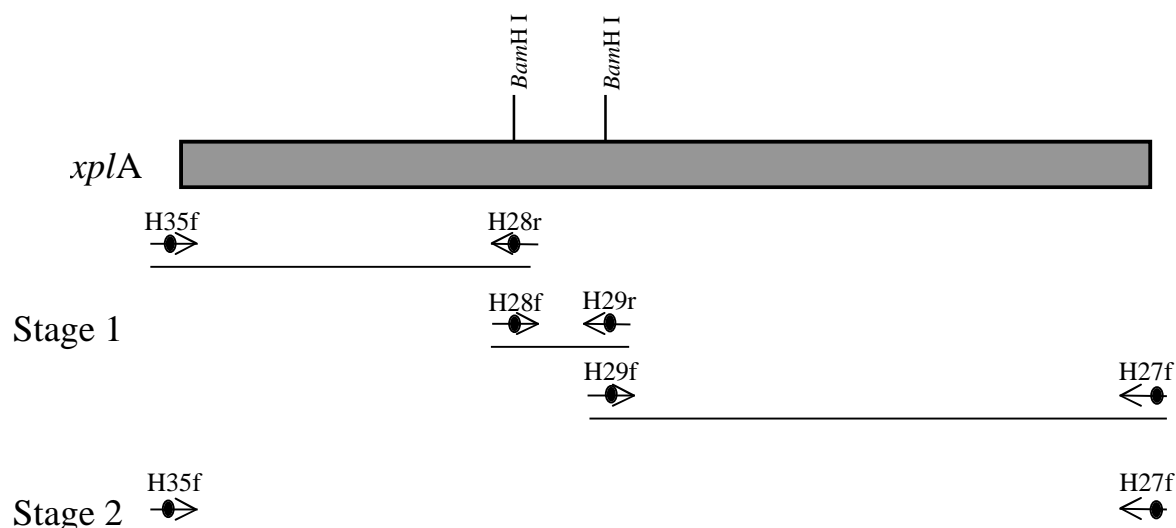
Crude extract was separated using a 12 % v/v SDS-PAGE gel, which had been allowed to polymerize overnight, and run using buffer containing 2 mM mercaptoacetic acid. The gel was blotted onto polyvinyl difluoride (PVDF) membrane (Boehringer Mannheim, Basel, Switzerland) using a Trans-Blot<sup>®</sup> SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The transfer buffer used was 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 5 mM dithiothreitol, 0.02 % w/v SDS and 10 % v/v methanol, pH 11. Electroblotting was performed at 0.04 A for 75 min. The blot was rinsed in water for 10 min, stained for 5 min in 0.1 % w/v coomassie brilliant blue (R-250), 50 % v/v methanol, 1 % v/v acetic acid, and destaining was achieved with four changes of 50 % v/v

methanol. The membrane was then washed in water twice for 5 min and airdried. The N-terminal sequence was determined by automated Edman degradation by the Protein and Nucleic Acid Chemistry Facility, Department of Biochemistry, University of Cambridge.

## 6.3 Results

### 6.3.1 Expression of *XplA* and *XplB* in *E. coli* hosts

Primers for the amplification of both genes to be expressed were designed, engineered to contain an *Nde* I site over the ATG start codon and a *Bam*H I site downstream of the stop codon for placement in the vector. The nucleotide sequences of *xplA* and *xplB* were studied and neither were found to contain *Nde* I sites, but two *Bam*H I sites were found within the reading frame of *xplA*. In order for *Bam*H I to be used as a downstream cloning site, it was necessary to create silent mutations at these two sites to prevent *Bam*H I recognition and avoid amino acid substitution. In both cases, the GGATCC *Bam*H I recognition sequence was exchanged for CGACCC and the following base was altered from C to T to avoid a run of 4 cytosines, which could cause problems in primer manufacture. All the mutations were designed to be at the third base position within the codons such that they should not alter the coding potential of the codon. The primers are shown in Table 6.1 and a schematic of the stages in cloning by PCR in Figure 6.1.



**Figure 6.1: Method used to engineer *Bam*H I restriction sites from *xplA*.** Labelled arrows represent primers used, with engineered sites represented by black spots. Stage 1 involved three separate PCR reactions using the primers indicated and pHSX1 as a template. Stage 2 used the resulting PCR products as template and the primers shown.

PCR was performed using pairs of primers: H35f/ H28r, H28f/ H29r, H29f/ H27r and finally H35f/ H27r (Table 6.1). The product, when sequenced, confirmed that the fragment had been engineered as required, as the sequence was identical to the desired sequence.

Primers were also designed to amplify the gene for the adrenodoxin reductase-like protein, *xplB*, again with an *Nde* I site over the start codon and a *Bam*H I site immediately after the stop

codon. These primers are shown in Table 6.2. Sequencing confirmed that the PCR fragment had been amplified as required, as the sequence was identical to the desired sequence.

Vectors pET-11a and pET-16b were chosen for expression of the two genes. Vector pET-11a, with a fragment inserted between the *Nde* I and *Bam*H I sites, carries no modifications to the protein, and pET-16b carries an N-terminal His tag which may be useful for future purification. A range of host strains were chosen, all lysogenic for a  $\lambda$  prophage containing an IPTG-inducible T7 RNA polymerase, denoted by (DE3). BL21(DE3) is the standard expression host cell line, and B834(DE3) its parental line which can result in higher expression levels. In addition, as gram positive bacteria often have codon biases different from those in *E. coli*, an expression strain carrying tRNAs with unusual anticodons was chosen. Rosetta(DE3) host strain was designed for the expression of eukaryotic, AT- or GC- rich genes in *E. coli*, and allows the recognition of codons AUA, AGG, AGA, CUA, CCC and GGA . The absence of these unusual tRNAs can restrict the translation of heterologous proteins in *E. coli*. Some of these codons are found frequently within *xplA* and *xplB* as indicated in Table 6.3.

**Table 6.3: Occurrence of rare codons in *xplA* and *xplB*.** These codons are recognized by rare tRNAs in Rosetta cell lines.

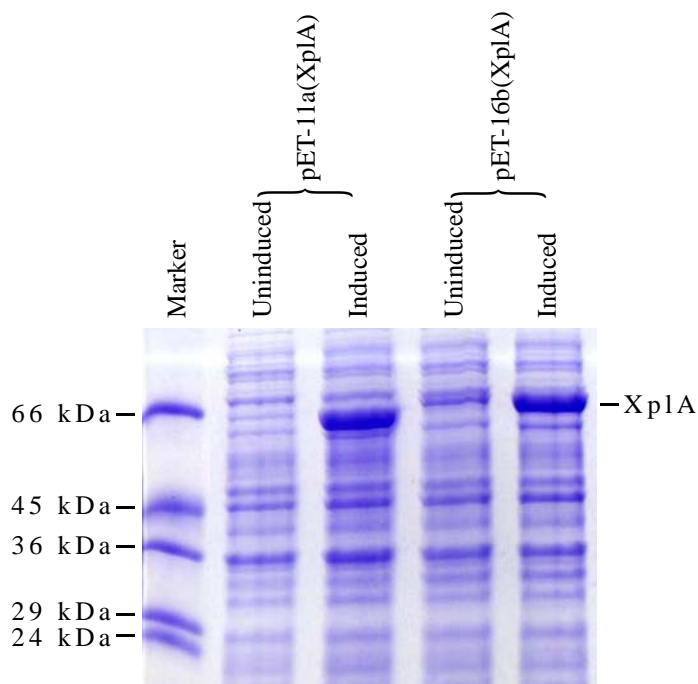
Rare codon	Occurrence in <i>xplA</i>	Occurrence in <i>xplB</i>
AUA	0	0
AGG	1	4
AGA	2	0
CUA	1	2
CCC	9	14
GGA	5	9

#### 6.3.1.1 Determination of predicted molecular weights

Predicted molecular weights of both *XplA* and *XplB* were calculated using [http://us.expasy.org/tools/pi\\_tool.html](http://us.expasy.org/tools/pi_tool.html). Deduced amino acid sequences were used to predict the molecular weight of *XplA* at 59.9 kDa and that of *XplB* at 45.9 kDa.

## 6.3.1.2 Comparing expression vectors

XplA expression was determined in both vectors pET-11a and pET-16b. Host strain BL21(DE3) was used to express pET-11a(XplA) and pET-16b(XplA) and was grown in LB at 37 °C. Total protein samples (uninduced and induced) were analysed by SDS-PAGE. After induction, both vectors were seen to be expressing a band at approx. 60 kDa, when compared to markers of known molecular weight, which is in agreement with the predicted value of 59.9 kDa (Figure 6.2).



**Figure 6.2: Expression of XplA in pET-11a and pET-16b.** A band at approx 60 kDa appeared after induction using vectors pET-11a and pET-16b.

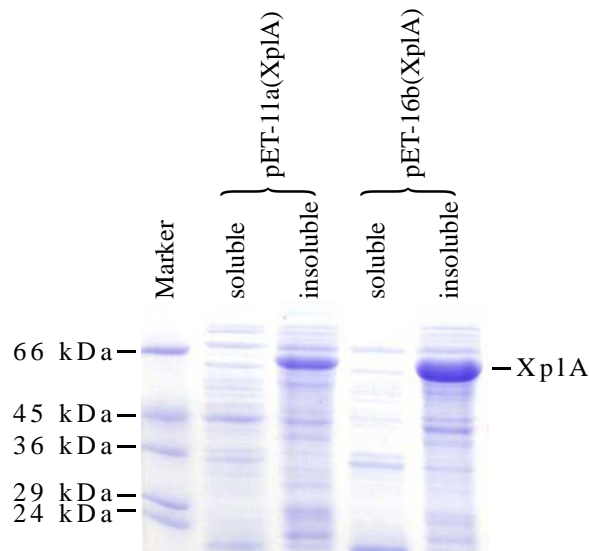
When sonicated to separate the proteins into soluble and insoluble fractions, XplA expressed using either vector was found in the insoluble fraction when visualized by SDS-PAGE (Figure 6.3). No band of the correct size was present in the soluble fraction in quantities which could be visualized by staining with coomassie.

XplB expression was determined in the same way, and again both vectors showed the appearance of a band of the predicted molecular weight (46 kDa) in induced samples, which was not present in uninduced samples (data not shown). XplB expressed using either vector was found in the insoluble fraction (Figure 6.4), with no expressed protein apparent in the soluble fraction. Therefore both vectors can be seen to be expressing proteins of the correct size, but neither protein

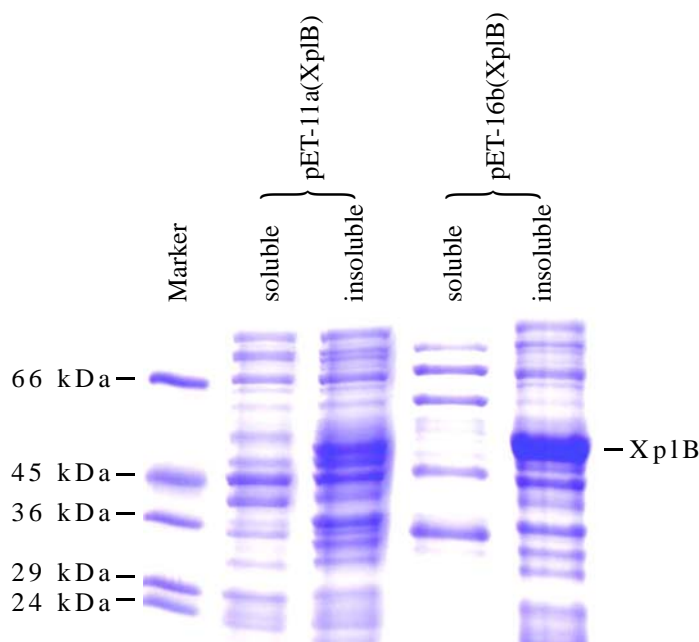
is found in the soluble fraction. Both vectors will continue to be used, as both may later be useful for different purification methods.

An activity assay was performed on the soluble fractions to determine whether there was sufficient protein present to release nitrite from RDX. Soluble fractions from cells expressing both XplA and XplB were added together in the presence of RDX and one of the cofactors: NADPH, NADP<sup>+</sup>, NADH or NAD<sup>+</sup>. Nitrite presence was assayed for after 1 h. No activity was apparent, as there was no increase in absorbance at 540 nm above background levels in the absence of cell extracts.

The insolubility of the expressed proteins therefore presents a problem in terms of reconstituting activity. To obtain soluble protein various changes in growth conditions of the host were attempted.



**Figure 6.3: Soluble and insoluble fractions of XplA expressed using pET-11a and pET-16b.** After sonication and centrifugation the recombinant XplA is found only in the insoluble fraction.



**Figure 6.4: Soluble and insoluble fractions of XplB expressed using pET-11a and pET-16b.** After sonication and centrifugation the recombinant XplB is found only in the insoluble fraction.

#### 6.3.1.3 Comparing expression hosts

All four constructs (pET-11a(XplA), pET-16b(XplA), pET-11a(XplB) and pET-16b(XplB)) were transformed into each of the three hosts BL21(DE3), B834(DE3) and Rosetta(DE3) and grown and harvested as above. All three strains showed similar levels of expression of each protein in each vector (data not shown). When soluble and insoluble fractions were visualized by SDS-PAGE, it was again seen that all strains produced proteins which segregated into the insoluble fraction (data not shown). It was therefore not possible to distinguish between the strains on the grounds of higher expression levels or ability to produce soluble protein. Strains BL21(DE3), which is the standard line used for expression, and Rosetta(DE3), which is useful in the expression of genes with codons not usually found in *E. coli*, were used in future experiments.

#### 6.3.1.4 Comparing temperatures of expression

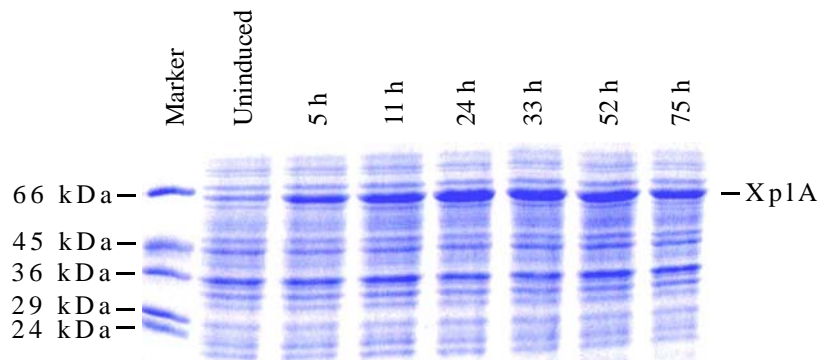
In some cases, the problem associated with production of the proteins in the insoluble fraction can be solved by growing the cells at a lower temperature (§6.4). Strains BL21(DE3) and Rosetta(DE3) were each transformed with all four expression vectors (pET-11a(XplA), pET-16b(XplA), pET-11a(XplB) and pET-16b(XplB)), and grown in LB at 20 °C. This was used as a starter culture to inoculate a further culture in LB, grown at 20 °C. This extra stage was introduced

to avoid the possibility of identifying proteins expressed as a result of the change of temperature from the post-transformation recovery at 37 °C to the cooler incubation temperature.

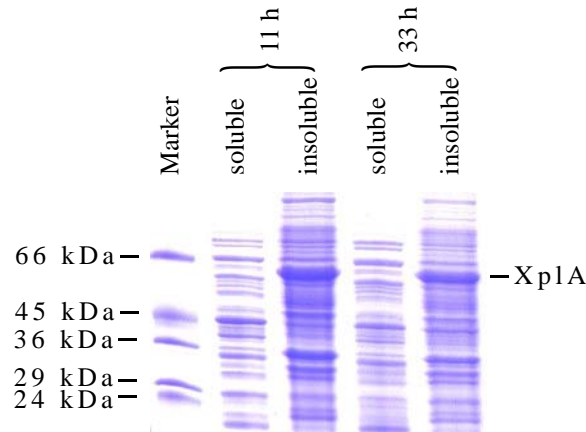
Uninduced and induced total protein samples were visualized by SDS-PAGE and showed that all samples were expressing proteins of the appropriate sizes (data not shown). Although a higher degree of soluble protein was expected at this lower temperature, when soluble and insoluble fractions were compared, all the protein was present in the insoluble fraction, as before (data not shown).

### 6.3.1.5 Comparing growth stage at harvesting

The stage of the growth cycle at which the cells are harvested, post induction, may alter the solubility of the protein. The growth of expression host BL21(DE3) carrying both pET-11a(XplA) and pET-16b(XplA) in LB at 20 °C was tested over time. The cultures were induced after approx. 48 h, when the cultures were at an OD<sub>600</sub> of 1, and samples were taken at 5, 11, 24, 33, 52 and 75 h post induction. All samples showed induction, with approximately the same degree of expression from all samples (Figure 6.5). Soluble and insoluble fractions were compared for the presence of a band of the correct molecular weight. Samples from all timepoints showed only insoluble protein, indicating that the stage at which the cells were harvested does not affect the solubility of the XplA gene product (Figure 6.6).



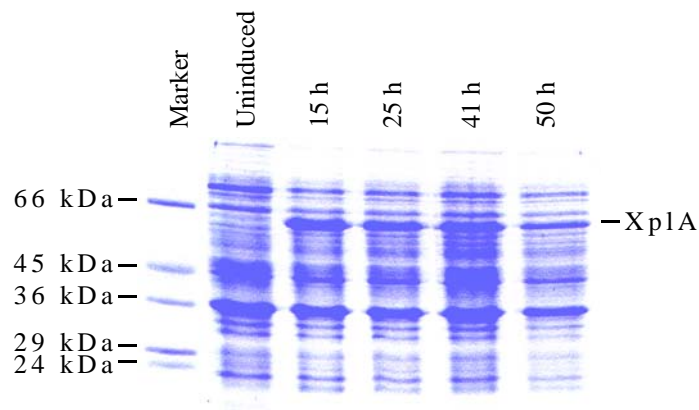
**Figure 6.5: Induction of XplA using pET-11a throughout the growth of the host.** Production of recombinant XplA was apparent at all stages post-induction.



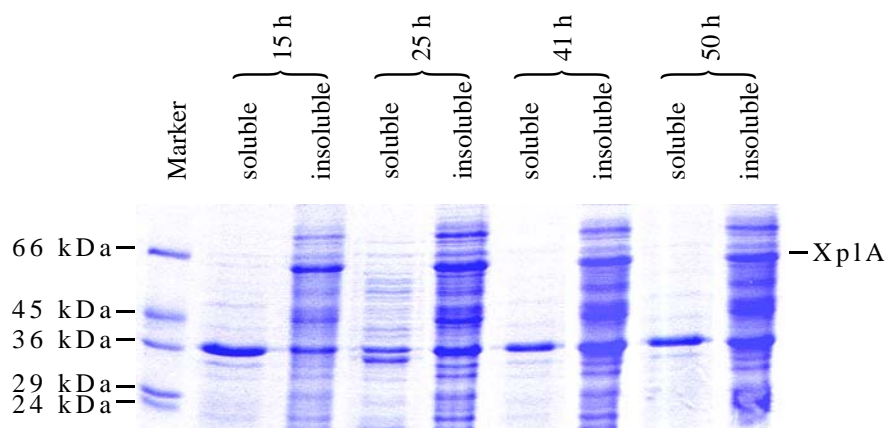
**Figure 6.6: Expressed XplA remains in the insoluble fraction throughout the growth of the host.** At all timepoints XplA expressed from pET-11a was found in the insoluble fraction. Representative timepoints 11h and 33h are shown.

### 6.3.1.6 Comparing medium used for growth

Minimal medium was used for the growth of Rosetta(DE3) carrying pET-11a(XplA) at 20 °C and again, although induction was seen (Figure 6.7), no protein was observed in the soluble fraction (Figure 6.8).



**Figure 6.7: Induction of XplA throughout the growth of the host in minimal medium.** Production of recombinant XplA from pET-11a was apparent at all stages post-induction.



**Figure 6.8: Expressed XplA remains in the insoluble fraction when grown in minimal medium.** At all timepoints XplA expressed from pET-11a was found in the insoluble fraction.

### 6.3.2 Identity analysis of expressed proteins

To ensure that the correct proteins were being expressed, N-terminal sequencing was performed. Vector pET-11a was used for this experiment, as pET-16b has an N-terminal His tag, and the expression host used was Rosetta(DE3), grown in LB at 20 °C.

Crude extract from both samples was electroblotted onto a PVDF membrane. The recombinant proteins were identified on an SDS-PAGE gel by comparison with uninduced protein and approximate size. The N-terminal sequences of the proteins were determined by automated Edman degradation and were found to be:

XplA: **Thr-Asp-Val-Thr-Val-Leu-Phe-Gly-Thr**

XplB: **Met-Asp-Ile-Met-Ser-Glu-Val-Asp-Val-Ala**

These experimentally determined amino acid sequences are consistent with those deduced from the nucleotide sequences. The N-terminal sequences confirm that XplA and XplB are expressed in *E. coli*. The N-terminal Met appears to have been cleaved off XplA but not XplB. This residue is generally removed when followed by residues Ala, Gly, Pro, Ser, Thr or Val, but is retained when the second amino acid is Arg, Asp, Asx, Glu, Gln, Ile, Leu, Lys or Met<sup>61</sup>. The cleavage of the N-terminal Met from XplA and not XplB is consistent with this pattern.

## 6.4 Discussion

Both XplA and XplB are expressed in *E. coli*. Expressed proteins of the correct size are visible using SDS-PAGE, and N-terminal sequencing has identified the proteins, compared to the amino acid sequence deduced from the gene sequences. That XplA and XplB express in *E. coli* indicates that the high G+C content of the rhodococcal genes, and the unusual codon usage, do not affect expression. The sequenced N-terminal region of XplA contains one of the rarely expressed codons, the eighth amino acid being a glycine encoded by GGA (Table 6.3), and this appeared to be expressed satisfactorily. Despite the high levels of expression observed, all the expressed protein is found to accumulate in the insoluble fraction. These insoluble aggregates of protein, or inclusion bodies, are thought to arise due to excessive levels of expression leading to misfolding of the proteins and hence aggregation. The insoluble protein is not in an active form. To enhance the production of soluble protein, several parameters can be altered: host strain, temperature, media and vector<sup>61, 147</sup>. The success of most of these alterations can be rationalized to act through reducing the rate at which the polypeptide is synthesized, thereby allowing each protein to fold correctly without aggregating with surrounding unfolded proteins.

Several conditions have been varied in the attempt to produce soluble XplA and XplB. The use of different host strain did not appear to influence the formation of inclusion bodies. In cases where this approach has led to the production of soluble protein, up to 11 strains have been tested<sup>147</sup>, and there does not appear to be one particular strain which allows production of all proteins in a soluble form. Reduction of temperature has been successful in several cases<sup>61, 147, 326</sup>, but expression of XplA and XplB at 20 °C, which is a recommended lower temperature<sup>61</sup>, produced no more soluble protein than expression at 37 °C. To determine whether growth stage influences the rate of protein production and the formation of inclusion bodies, samples throughout the growth stages were assayed, and the level of soluble protein present was not seen to increase at any of the stages. The medium in which the recombinant *E. coli* are grown has been suggested to play a role, perhaps to alter the rate at which the protein is expressed. Growth of the host in minimal medium produced no more soluble protein than growth in rich medium (LB). None of the techniques described above were successful in producing soluble, or therefore active, protein.

The expression of rhodococcal proteins in *E. coli* has varied success. The production of active protein from expression vectors, driven by lac or T7 promoters, has been reported in many cases<sup>13, 40, 43, 78, 123, 134, 164, 169-171, 178, 208, 230, 241</sup>, and in a few cases successful expression has been achieved under the indigenous promoters<sup>52, 134, 169</sup>. However, in some cases the production of active protein

from rhodococcal genes in *E. coli* has not been possible to achieve<sup>13, 152, 200, 272, 285</sup>. Although expression of the proteins is apparent, through visualization on SDS-PAGE, the protein present is not active<sup>152, 285</sup>. In some cases, activity can be detected in low levels when the protein sample is treated with urea and dialysed<sup>152, 169</sup>. The problems associated with expression in *E. coli* can sometimes be avoided by expression in other strains, such as *Pseudomonas putida*<sup>200</sup> or *Streptomyces lividans*<sup>285</sup>, but expression in alternative strains does not always work<sup>272</sup>. Therefore there do not appear to be any foolproof methods for the heterologous expression of rhodococcal proteins, and a pragmatic approach appears to be necessary.

Both microsomal and microbial P450s have been expressed in *E. coli*. Microsomal P450s are membrane-bound (<http://drnelson.utmem.edu/PIR.P450.description.html>), and the hydrophobic N-terminal domain commonly requires modification for expression in *E. coli* and targeting to host membranes<sup>119</sup>. The production of soluble microbial proteins has been achieved under various conditions. P450s from *Streptomyces* species, *Bacillus subtilis* and *Citrobacter braakii* have been expressed using pET vectors or pCW<sup>307</sup> and several *E. coli* host strains<sup>19, 113, 140, 141, 185, 186, 295</sup>. Growth in rich medium at temperatures between 25 and 37 °C results in the production of soluble and, in most cases, active protein. The haem precursor  $\delta$ -aminolaevulinic acid can be added to increase the levels of active protein<sup>181, 186</sup>, although soluble protein must be available in the first place. There are very few examples of the expression of rhodococcal P450s in an *E. coli* host. Heterologous expression of *thcB*, a P450 from *Rhodococcus* sp. involved in herbicide degradation, was achieved, as assayed by SDS-PAGE, although no activity was reported<sup>210</sup>. A further study with this gene showed that only very low levels of expression were achieved in *E. coli* from its own promoter<sup>273</sup>. A novel type of P450 has been expressed in *E. coli* using pET3a, and was produced in the soluble fraction when the host was grown at 30 °C<sup>246</sup>. This protein was active and produced a peak at 450 nm when a carbon monoxide difference spectrum was performed. There appear to be no inherent problems associated with the expression of recombinant P450s in *E. coli*, although the expression of rhodococcal P450s may prove more difficult.

Active forms of both bovine and rat adrenodoxin reductase have been produced in *E. coli*<sup>255, 256</sup>, as has human ferredoxin reductase, at a low yield<sup>39</sup>, and a homologue from *Mycobacterium tuberculosis*, FprA, although soluble protein was only obtained at temperatures as low as 15 °C<sup>92</sup>. However, a similar protein from *M. tuberculosis*, FprB, which has a ferredoxin-like domain at the N-terminus, was not expressed in the soluble fraction even at this reduced temperature<sup>92</sup>. The

amount of soluble bovine adrenodoxin reductase produced in *E. coli* was increased greatly by the co-expression of chaperone proteins, such as HSP60<sup>319</sup>.

There are yet more approaches to attempt with regard to the heterologous expression of XplA and XplB. The production of inclusion bodies can aid the purification of a protein, as soluble proteins are eliminated within one step. However, it is not always straightforward to obtain active protein this way. Resolubilization of the protein involves total denaturation, commonly using urea or guanidine, and refolding to obtain native protein. The refolding protocol varies for each protein and must be determined empirically, and is not always successful<sup>61</sup>. The incorporation of the relevant prosthetic groups (haem, FMN and FAD) during refolding may make the procedure more complex.

Alternative approaches to obtaining soluble protein include the use of weaker promoters, changing the pH of the growth medium, encouraging secretion of the protein to the periplasm and producing the protein in a fusion construct<sup>61</sup>. Altering the pH of the growth medium can have an effect on the solubility of the protein: in the case of salmon growth hormone a higher pH aided solubility<sup>147</sup>. Another idea would be to clone the *xplA* and *xplB* genes under promoters which are not as strong as the T7 promoter of the pET vectors. Recombinant proteins previously have been expressed from *lac* promoters in vectors such as pUC19 and pBluescript, using a non-DE3 expression host with pBluescript to ensure correct induction. This was not attempted in this study as alternative cloning sites would have to be engineered upstream and downstream of the genes, as the sites which allow cloning into pET vectors are not compatible with those required for cloning into either pBluescript or pUC19, and the time remaining on the project was limiting.

Secretion can be used as a way of obtaining soluble protein and also separating it from cytoplasmic proteins, which can be a useful step in purification. A secretory tag can be engineered to the N-terminus of the protein, and which targets the protein for periplasmic export. One example of this is the OmpT leader sequence which is present in pET-12 vectors. There are specific protocols which allow the separation of periplasmic proteins from the rest of the cellular proteins<sup>61</sup>. Fusion of the protein to be expressed to a carrier protein has also been used successfully. A survey of some of the purifications performed in this way is given in Coligan et al.<sup>61</sup>. Commonly used carrier proteins include maltose binding protein, glutathione S-transferase and thioredoxin, all of which encourage the accumulation of protein in a soluble form. Constructs exist for all of these, containing sites to allow the subsequent cleavage of the carrier. Overexpression of *E. coli* chaperone proteins, to encourage correct folding of the recombinant protein, can also help in the

production of soluble protein<sup>147, 326</sup>, as described above for the production of adrenodoxin reductase. Exposing the host cells to heat shock, cold shock or osmotic shock can improve expression and activity of P450s, which may also act through the production of molecular chaperones<sup>158</sup>.

The expression of XplA and XplB using these methods should be attempted, in order to produce soluble protein. Haem incorporation can then be determined by 3,3',5,5'-tetramethylbenzidine-peroxide staining of native gels<sup>304</sup>, and flavin presence determined using HPLC, TLC or spectrophotometric assays<sup>94, 107, 231, 233, 299</sup>. The presence of an active P450 can be determined by performing a reduced carbon monoxide difference spectrum, when a peak at approx. 450 nm should be produced<sup>224</sup>. Activity against RDX can be assayed by reconstituting a system using crude extracts containing each expressed protein, and purification attempted subsequently. A system consisting of purified XplA and XplB will give information on the use of the flavodoxin domain in electron transport, and if the system is not active, commercially obtained ferredoxin may be useful in completing the system. If the flavodoxin domain is active in this role, it would represent a novel P450 system. When a fully purified, operational system is obtained, more accurate information regarding the products of RDX degradation can be obtained, including mass balance analysis. Alternative substrates can be tested with the enzyme system, which may lead to insights regarding the degradation of the nitramine explosives HMX and CL20.

## Chapter 7. Final discussion

This study aimed to isolate bacteria capable of degrading RDX from contaminated sites, to isolate the gene required for the ability to break down RDX, and to investigate this activity. Nineteen bacterial strains were isolated from contaminated material through their ability to degrade RDX as a sole source of nitrogen. These were characterized and identified; all were found to be members of the genus *Rhodococcus*, with thirteen of the strains being most closely related to *Rhodococcus erythropolis*, four of the strains having a closest match to RDX degrading bacterium *Rhodococcus* sp. strain DN22<sup>59</sup> and two matching a further *Rhodococcus* species. Further characterization compared the strains to each other and *R. rhodochrous* strain 11Y, a previously isolated RDX degrader, and found them all to be distinct strains. Comparisons of the activities of all isolates against RDX in resting cell incubations showed that the activities varied widely between the strains, from 0 % to 100 % degradation of 250  $\mu$ M RDX over 60 min.

Based on these comparisons one strain was selected for further study. It was chosen to have a high rate of RDX degradation, antibiotic susceptibilities compatible with a range of rhodococcal plasmids and a non-mucoid phenotype to enable ease of transformation and manipulation. The chosen isolate was *Rhodococcus rhodochrous* strain 11Y.

Comparisons of the RDX degrading rate of strain 11Y found it comparable to, or faster than, other characterized aerobic strains<sup>27, 59, 157, 338</sup>. Strain 11Y appears to use three of the six nitrogens from RDX, as do other reported strains<sup>27, 59</sup>. Resting cell incubations demonstrated that the activity in strain 11Y is present in cells grown with ammonium provided as the sole source of nitrogen, but is increased when grown in the presence of RDX. This indicates that the gene is expressed in the absence of RDX, but is upregulated in its presence. This experiment also showed that the nitrite reductase of strain 11Y appears to be upregulated in the presence of either RDX or the nitrite produced from its breakdown. Despite the ability of strain 11Y to degrade RDX, it is not able to degrade the closely related nitramine explosives HMX and CL20. It is possible that the uptake of these compounds by the cells is not possible, and that use of a purified enzyme system would allow degradation of these compounds, or it may be the case that they are sterically hindered from binding in the active site.

A preliminary study of the products formed from RDX degradation by strain 11Y was performed, analysing the products of resting cell incubations over time using HPLC, ion chromatography and a colorimetric test for formaldehyde. Many of the products proposed to form

from RDX degradation would be detectable using these techniques. The products detected were nitrite, formate and formaldehyde, with no indication of ammonium or nitrate presence, or any dead end product accumulation. The mechanism proposed for RDX degradation by *R. rhodochrous* strain DN22 invokes an initial denitration step, followed by ring cleavage to form formaldehyde (eventually carbon dioxide), ammonium and a dead end product<sup>95</sup>. The products of the reaction using strain 11Y are consistent with a mechanism of denitration of RDX, followed by spontaneous decomposition of the molecule although the products are not entirely consistent with those determined using strain DN22. The different products can be explained by different competing reactions within the bacteria. It is also conceivable that different products were observed as a result of the different techniques used in determining them. To eliminate this possibility, the group who studied the pathway using DN22 are to perform the same experiments using strain 11Y, which may provide a useful and interesting comparison. The only way to unequivocally establish the initial step and the resulting compounds would be to follow the reaction using a purified enzyme system.

Using a gene library from *Rhodococcus rhodochrous* strain 11Y DNA, the ability to degrade RDX was conferred to a non RDX degrading strain of *R. rhodochrous*. The fragment of DNA which conferred this ability was sequenced and found to contain three open reading frames: an adrenodoxin reductase-like gene, a P450-like gene and a gene with homology to acetyl CoA synthase. Subcloning experiments showed that the genes with homologies to acetyl CoA synthase and adrenodoxin reductase were not required for RDX degrading activity in the rhodococcal host strain. The gene coding for the P450-like protein, which was necessary for RDX degrading activity, contains a flavodoxin domain at the N-terminus, and was designated *xplA*. The involvement of a P450 in the degradation of RDX in strain 11Y was strongly inferred, as the P450 inhibitor metyrapone inhibited the degradation of RDX in resting cells.

Bacterial, type I, P450s are only one part of a multicomponent system consisting of a haem containing P450, an FAD containing reductase and an iron sulphur ferredoxin, whereas type II, eukaryotic, P450 systems comprise a P450 and a reductase containing both FAD and FMN domains, with no ferredoxin type protein<sup>74</sup>. As a reductase is necessary in the system, it is possible that the protein encoded by the reductase-like gene found directly upstream of *xplA*, designated *xplB*, is involved in the RDX degrading reaction. *XplB* may be able to be substituted by a similar protein in the rhodococcal host, explaining why a vector carrying only *xplA* is able to confer RDX degradation upon it. In addition, it is a distinct possibility that the flavodoxin domain at the N-terminal end of *XplA* is able to function in the role normally performed by ferredoxins, in shuttling electrons from

the reductase to the P450. Flavodoxins are able to perform the same electron transport functions as ferredoxins in some situations<sup>309</sup>, and several P450 systems containing fused components have been reported. P450<sub>BM-3</sub> contains the FAD, FMN and haem domains in one polypeptide<sup>212</sup>, and a new class of self-sufficient P450 contains FeS, FMN and haem domains, again in one polypeptide<sup>246</sup>. It is not unlikely that further rearrangements and fused systems will be discovered in the future. It is also common to find genes involved in xenobiotic degradation in gene clusters containing other genes required in the pathway. This is true of P450 systems and other genes involved in degradative pathways<sup>53, 66, 96, 97, 104, 210</sup>. The function of the reductase-like XplB and the flavodoxin domain in the transport of electrons to XplA during RDX degradation has not been proved, and cannot be until pure proteins are available to reconstitute the system *in vitro*. However, XplA has already been described as unique by the P450 nomenclature committee, who have given it the new family name of CYP177A1.

Although mechanisms have been proposed for many of the reactions catalysed by P450s, including dealkylation, epoxidation, dehydrogenation, dehalogenation, sulfoxidation and aromatization<sup>262</sup>, the degradation of RDX is not obviously comparable to any of them. Some less common reactions of P450s have been reviewed<sup>120</sup>, and some of these may be applicable to the breakdown of RDX. The denitration of the nitrate ester GTN, catalysed by a P450, has been reported to occur under anaerobic conditions<sup>75</sup>. The reaction required the presence of NADPH and was inhibited by oxygen, carbon monoxide, and specific P450 inhibitors. The proposed reaction mechanism involves donation of 2 electrons sequentially to the substrate, forming first a radical with the release of nitrite, and subsequently reduction of the radical<sup>75</sup>. However, the nitrate ester group cannot be considered equivalent to a nitramine group in the context of this reaction, and the denitration of RDX by this mechanism would be very unlikely to occur.

There are reports of the action of P450s on nitrogen-nitrogen compounds, including azo compounds, where the N=N bond is reduced to N-N and further reduction leads to cleavage of the N-N bond<sup>145, 334</sup>, as is seen in RDX degradation. These reactions were most efficient in anaerobic conditions, and it was proposed that reductions can only occur in the absence of oxygen<sup>108</sup>. More recently it has been suggested that the binding of particular substrates may inhibit oxygen binding, allowing the substrate to be reduced by the P450 in place of oxygen<sup>120</sup>. However, the denitration of the P450 appears not to be a reductive process, so a comparison to this reaction may not be relevant.

P450s are monooxygenase enzymes, and their general mechanism involves the incorporation of one atom from O<sub>2</sub> into a substrate with the reduction of the other atom by two electrons provided

by the associated ferredoxin<sup>262</sup>. Thus the typical reaction is one of substrate hydroxylation. The catalytic cycle involves binding of the substrate followed by reduction of the iron from Fe<sup>III</sup> to Fe<sup>II</sup>. Oxygen binding to the iron and a further reduction results in the production of a peroxo intermediate. Water is released by cleavage of the O-O bond, the substrate is oxygenated and the enzyme recycled.

One of the steps proposed in the oxidation of substrates is the abstraction of a proton by a hypothesized FeO<sup>3+</sup> species, which is shortly followed by substrate oxygenation<sup>120</sup>. The proton abstraction reaction is the same as that proposed to occur during the alkaline hydrolysis of RDX, forming an unstable intermediate which spontaneously decomposes in alkaline conditions<sup>148</sup> (Figure 1.4). However, the decomposition is not likely to occur at a neutral pH, and the hydroxylation of one of the carbons of RDX is the more likely result. Hydroxylation of a carbon would be necessary to create the products formate and formaldehyde, but the subsequent liberation of nitrite is not easily rationalized unless the RDX is significantly destabilized by the hydroxylation. To have a chance of determining the precise nature of the degradation mechanism a pure protein system is required.

The heterologous expression of XplA and XplB in *E. coli* has produced proteins of the predicted size, and N-terminal sequences have confirmed their identities. However, both are found in the insoluble fraction, forming inclusion bodies. Several of the growth conditions, including host strain, growth temperature, growth medium and stage of harvesting, were altered, with no observed increase in the production of soluble protein. For future expression of the proteins, a number of approaches can be attempted to obtain active recombinant protein. Refolding of the insoluble proteins may form active protein, if the relevant prosthetic groups are included. Factors which may be further altered to aid the expression of soluble proteins include: vectors, expression strains, co-expression of chaperone proteins, heat, cold or osmotic shock, targeting to the periplasmic space and construction of fusion proteins<sup>61, 147, 158</sup>.

The purification of the enzymes XplA and XplB are central to the continuation of this project. Reconstitution of the system *in vitro* will give information on the involvement of the reductase enzyme and flavodoxin domain within XplA. This could then provide information on a new class of P450. A pure enzyme system should also be used to thoroughly elucidate the products of RDX degradation. Until this is performed, the possibility of undesirable products remains, and RDX mineralization cannot be proven. This would also give information on how the reaction is catalysed by the P450, whereas currently the enzymatic mechanism can only be postulated. Crystallization of the enzyme, with and without substrate, would give information on the reaction

occurring within the active site. Substrate range should also be investigated. The possibility that pure enzyme could catalyse the degradation of other nitramine explosives and related compounds might provide information on the reason for the lack of degradation *in vivo*, and would make the enzyme system of even more value with respect to bioremediation.

Possibly the most exciting work that could follow on from this thesis, is also the most applied, and an obvious next step would be to engineer the genes isolated here into plants, to allow breakdown of contaminating RDX. Transgenic plants engineered to express bacterial genes have been very successful in the phytoremediation of other groups of explosives. Although RDX is not toxic to plants at concentrations up to 21 mg/l<sup>306</sup>, it is taken up by plants<sup>20, 21, 132, 245, 306</sup> and accumulates in the leaves<sup>20, 21, 132, 306</sup>. Here it does not appear to be transformed or degraded significantly although there are reports of small amounts of bound forms of RDX found intracellularly<sup>22, 237</sup>. This accumulation of RDX leads to issues regarding its movement through the food chain, as it is found at high concentrations in the edible portions of the plants. Therefore if phytoremediation of explosive contaminated land is to proceed using transgenic plants, the plants should be engineered to be able to break down the RDX, instead of accumulating it. The transgenic plants should be able to decontaminate soil and groundwater to significant depths through their root systems. In particular the use of the fast growing and deep rooted yellow poplar is being considered for this type of research, and transformation techniques have already been performed using this plant, demonstrating proof of principle<sup>253</sup>.

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