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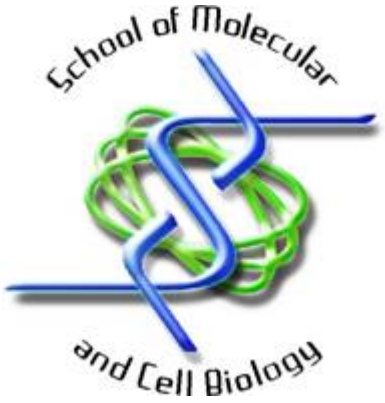
Biodegradation of 2,4,6-Trinitrophenol in Rhodococcus opacus and Nocardioides simplex : Developing a mutagenesis system and cloning of the nitrite eliminating enzyme

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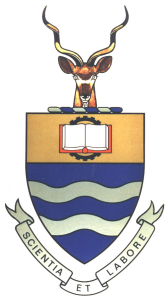
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Biodegradation of 2,4,6-Trinitrophenol in *Rhodococcus opacus* and *Nocardioides simplex*: Developing a Mutagenesis System and Cloning of the Nitrite Eliminating Enzyme



Ana Rebić



A thesis submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg, 2006

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

_____

Ana Rebić

19th day of October, 2006

ABSTRACT

Bioremediation tends to be an effective technology, used to remove pollutants and it involves relatively little capital. Microorganisms, especially bacteria, evolving into many types with exceedingly diverse metabolic capabilities, which can be applied in bioremediation. The two bacterial strains, *Nocardioides simplex* FJ2-1A and *Rhodococcus opacus* HL PM-1 are capable of biodegradation of 2,4,6-trinitrophenol (TNP, picric acid). In *N. simplex*, the biodegradation pathway is inducible while in *R. opacus* is constitutive. Some enzymes were identified in both strains and shown to be isofunctional.

In this work, a molecular characterisation of the *orfB* from the *npd* gene cluster was achieved. The mutant *R. opacus* AR1 strain deficient in the *orfB* gene was constructed by a gene replacement method using the expression of *sacB* as the selectable marker. The gene was also cloned as the His tag fusion protein and expressed in *E. coli.* Theoretical pI / Mw predictions for the protein sequence were 5.47 / 42536.23 Da. SDS-PAGE analysis showed the size of the purified fusion protein, which was calculated to be 42.5 kDa. The protein was purified by Ni-NTA metal affinity chromatography.

This study furthermore identified a DNA fragment, which encodes the nitrite-eliminating enzyme (NEE) activity. This was achieved by enriching the activity using the fast performance liquid chromatography (FPLC) and protein sequencing. The two internal peptide sequences were obtained: L P G D Y T D Q L L R and S G A I V G L G H A Q V D R. These were used to design the degenerate primers in order to pool a clone from nocardioform genome. Two different genomic libraries were constructed and screened using *R. opacus* HL PM-1 and *N. simplex* FJ2-1A total DNA, since the activity in crude extracts was reported with both strains. The partial DNA sequence of NEE was obtained (252 bp). The similarity with fatty acid hydroxylase from *Nocardioides* sp. JS6614 was detected.

DEDICATION

This is dedicated to my parents Dragan and Milunka Rebić

ACKNOWLEDGEMENTS

Many sincere thanks to:

- my parents, tata Dragan and mama Milunka. You have taught me that journeys we take will make us better people. This journey has been a hard one but very fruitful academically and personally. I could not have done it without your strength and patience;
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- my professors in Stuttgart, Prof. Dr. Hans-Joachim Knackmuss, for his invitation to Germany, and Prof. Dr. Georg Sprenger for his two years support that included the required laboratory space, the necessary funding, very modern and safe biotechnological facilities and critical suggestions during experimental design;
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ABBREVIATIONS

Amp	Ampicillin
APS	Ammonium persulphate
ATCC	American Type Culture Collection
BCIP	5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt
bp	Base pair
BSA	Bovine Serum Albumin
Cam	Chloramphenicol
cm	Centimetre
DIG	Digoxygenin
DIG-11-dUTP	Digoxygenin-11-deoxyuridine triphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNCH	Dinitrocyclohexanone
DNH	Dinitrohexanoate
DNP	2,4-Dinitrophenol
dNTPs	Deoxyribonucleoside triphosphate
dsDNA	Double stranded DNA
EDTA	Ethylenediaminetetraacetic acid
ESI^-	Negative-mode electrospray ionisation
FPLC	Fast performance liquid chromatography
g	Gram
h	Hour
H^--DNP	Hydride Meisenheimer complex of DNP
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonicacid]
HPLC	High performance liquid chromatography
HTI / II	Hydride transferase I / II
H^--TNP	Hydride δ-complex of 2,4,6-TNP (Hydride Meisenheimer complex of TNP)
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
kbp	Kilo base pair
kDa	Kilo Dalton
L	Litre
LB	Luria-Bertani
LC-MS	Liquid chromatography, coupled to mass spectroscopy
M	Molar
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
min	Minute
µg	Microgram
µl	Microlitre
ml	Millilitre
mm	Millimetre
mM	Millimolar
MW	Molecular weight

Nal	Nalidixic acid
NBT	Nitro blue tetrazolium chloride
NCH	Nitrocyclohexanone
NDFR	NADPH-dependent F_{420} reductase
NEE	Nitrite-eliminating enzyme
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometre
NMR	Nuclear magnetic resonance
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFGE	Pulsed field gel electrophoresis
pfu	Plaque forming units
PicA	Tetrabutylammonium hydrogensulphate
PMSF	Phenylmethylsulphonyl fluoride
Rif	Rifampicin
RNase	Ribonuclease
rpm	Revolutions per minute
s	Second
SDS	Sodium dodecyl sulphate
Str	Streptomycin
Suc	Sucrose
TE buffer	Tris-EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tet	Tetracycline
TNP	2,4,6-Trinitrophenol (picric acid)
U	Unit
UV	Ultraviolet
V	Volts
v/v	Volume/volume
W	Watt
w/v	Weight/volume
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1. Introduction

1.1. Phenols, nitrophenols and polynitrophenols

Phenols are defined as organic compounds with the chemical formula ArOH (Ar, "aromatic"= fragrant). The aromatic constituent is usually phenyl, substituted phenyl or some other aryl group, for example naphthyl. Phenols differ from alcohols in having the hydroxyl group attached directly to an aromatic ring. The essential quality of aromaticity lies in substitution reactions irrespective of functional groups. In addition, the reactivity of the aromatic rings is affected with these functional groups and *vice versa*. Most common chemical states of the simplest phenolic compounds are liquids or low-melting solids. This is due to hydrogen bonding. They also have relatively high boiling points in the range between 152 – 236°C, as seen with *ortho*-fluorophenol (lowest) and *ortho*, *meta*-bromophenol (highest). Phenols are colourless but they may carry some groups attached that are capable of producing colour. Many easily undergo oxidation and are coloured by the reaction products. The physical properties of the isomeric nitrophenols and polynitrophenols could be attributed to their intramolecular versus intermolecular hydrogen bonding (Morrison and Boyd, 1992). The hydroxyl (-OH) and nitro (-NO_2) groups in the *ortho* isomer are positioned in such a way to form intramolecular hydrogen bonding as oppose to *meta* or *para* isomers that have intermolecular bonding. *o*-Nitrophenol is the only structural conformation that could readily undergo steam distillation process because it is volatile in steam. Also it has the lowest boiling point and lowest water solubility compared to its isomers. Phenols are also considered as stronger acids than water and most of them have K_a value (the strength of acid) of about 10^{-10} (H_2O at 25°C has K_w value of 1.0×10^{-14}) (Atkins and Beran, 1992; Morrison and Boyd, 1992).

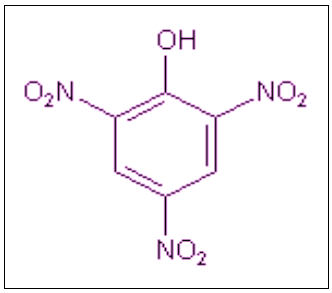
Table 1. Physical properties of the nitrophenols and polynitrophenols (Morrison and Boyd, 1992).

Name	***Melting point, °C***	***Boiling point, °C at 70 mm***	***Solubility, g/100 g H_2O at 25°C***	***K_a***
ortho-nitrophenol	45	100	0.2	600×10^{-10}
meta-nitrophenol	96	194	1.4	50×10^{-10}
para-nitrophenol	114	ND	1.7	690×10^{-10}
2,4-dinitrophenol	113	ND	0.6	1.0×10^{-4}
2,4,6-trinitrophenol (picric acid)	122	ND	1.4	very large

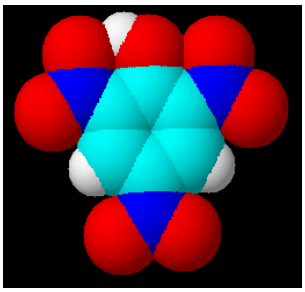
ND = not determined

1.1.1. Chemical properties of picric acid

Picric acid ($C_6H_3N_3O_7$) is a toxic yellow odourless crystalline solid that melts at 122ºC, has molecular weight 229.11 g/mol and is soluble in most organic solvents. It should be kept moist as it is highly reactive in dry conditions, highly flammable and shock sensitive. Picric acid is stored as a yellow paste or liquid. The presence of three nitro groups makes picric acid very unstable. The chemical bonding in the nitro group results in the nitrogen atom being positively charged and each oxygen atom has a partial negative charge. This is the reason why the nitro group has a powerful attraction for electrons. The electrophilic nitro group can be easily reduced by a number of reducing agents and the most useful of these is hypophosphorous acid, H_3PO_2 (Morrison and Boyd, 1992). In addition, the electron-withdrawing character of the nitro groups result in a highly electron deficient π-electron system. As a consequence, catabolic pathways initiated by oxygenation are unknown for these xenobiotics (Lenke *et al.*, 2000).



2D-structural formula



3D-structural formula

Figure 1. Molecular structure of picric acid indicating spatial arrangements of the functional groups. Red: oxygen atoms, white: hydrogen atoms, royal blue: nitrogen atoms and turquoise: carbon atoms.

1.2. Preparation of phenols and polynitrophenols

There are several methods used in the laboratory and industry to prepare phenols. For example, hydrolysis of diazonium salts or alkali fusion of sulfonates. The first method is very common and it is the final step in a synthetic pathway which usually begins with nitration. On a large scale it is also possible to perform hydrolysis of aryl halides. These compounds carry strong electron-withdrawing groups at *ortho* and *para* position to the halogen. The two extensively used polynitrophenols, 2,4-dinitrophenol (2,4-DNP) and 2,4,6-trinitrophenol (2,4,6-TNP, picric acid) are produced in this way. In addition, conversion of phenol by concentrated nitric acid into picric acid is manageable but the nitration reaction is accompanied by significant oxidative destruction of phenol. For instance, a treatment of 2,4-phenoldisulphonic acid with nitric acid has the advantage over the direct nitration of phenol. The oxidation is reduced and the yield is increased by applying firstly a mild sulphonation followed by electrophilic exchange of the sulphonyl versus the nitro group (Morrison and Boyd, 1992; Rieger, P.-G. pers. comm.).

1.3. Polynitroaromatic compounds in the environment and uses

Polynitrophenols, such as 2,4-DNP and 2,4,6-TNP have been introduced into the environment by anthropogenic activities. In industrial processes, nitroaromatics are important initial compounds for the synthesis of pesticides, azo dyes and explosives. Picric acid and 2,4-dinitrophenol are by-products during large scale synthesis of aniline and colour-fast dyes. TNP is the oldest nitro dye and was first obtained by Woulfe in 1771 and used for dyeing of wool, silk and leather (Elvers *et al.*, 1991). In addition, 2,4,6-TNP is used during production of picramic acid (2-amino-4,6-DNP), which is further important as a feedstock for the synthesis of azo dyes. In analytical chemistry 2,4,6-TNP serves as a test reagent to identify alkaloids. Dinitrophenols have a wide range of biocidal activities and are used as fungicides and herbicides. Major sources of DNP and TNP are industrial effluents, water systems and soil. These xenobiotics occur as contaminants in wastewater because of their high solubility. For example, trinitrophenols that are used as explosives are commonly found in ground water systems at specific military sites and former production sites (Ebert *et al*., 1999; Lenke *et al*., 2000; Rieger *et al*., 2002). Today, one of the principal sources of dinitrophenols and picric acid in industrial effluents is the production of nitrobenzene from benzene. During this process 0.1% of the benzene consumed ends up as picric acid and dinitrophenols (Russ *et al*., 2000).

1.4. Toxicity of TNP and DNP

There are multiple mechanisms which contribute to the toxicity of picric acid. In general, via external contacts it causes irritations, burns and allergies. If ingested, acute toxicity is most probably due to acidosis while on prolonged exposure it may cause damage to red blood cells, kidneys and liver (Wyman *et al.*, 1992). Picric acid may also be a carcinogen and suitable precautions such as gloves, eye and respiratory protection must be taken. Picric acid has much less of an uncoupling effect than 2,4-DNP because it has a higher acidity (seen as pKa of 0.3

of PA as oppose to pKa of 4.1 of 2,4-DNP). Therefore, DNP is more toxic than TNP. Long-term exposure can lead to the damage of skin, eyes, bone marrow, central nervous system and cardiovascular system. Occurrence of cataracts, skin lesions and increased basal metabolic rate have been reported also. The acute toxicity comes from the ability of dinitrophenol to act as an uncoupler. It is a weak acid and crosses the membrane in its protonated form acting as an H^+ carrier. This generates the electrochemical gradient across cell membranes and thus uncoupling the oxidative phosphorylation pathway (the energy production of the cell as ATP) without blocking oxygen consumption (cellular respiration). The uncoupling effect is dependent on the external pH and increase in external pH results in a decreased toxicity of 2,4-DNP (Michels and Bakker, 1981; Russ *et al.*, 2000).

1.5. Biodegradability of picric acid and degrading bacterial strains

Picric acid has been found to be degraded by a number of organisms. Microbial attack on picric acid was first reported by Erikson in 1941. He conducted studies on some lake-mud strains of *Micromonaspora*. Thereafter, Gundersen and Jensen (1956) illustrated the metabolism of picric acid by *Corynebacterium simplex* that was isolated from soil as a 4,6-dinitro-2-methylphenol-degrading bacterium. The formation of nitrite served as an indicator of picric acid bioconversion. In 1964 Tabak *et al.* reported a change of colour from yellow (2,4,6-TNP) to orange-red in enrichment cultures with picric acid. Detailed analysis of bacterial growth and degradation pathway was lacking. Picramic acid was identified in 1979 by Wyman *et al.* as the only metabolite of picric acid. In 1992 Lenke and Knackmuss reported that *Rhodococcus erythropolis* HL 24-2, initially isolated as a 2,4-DNP degrading organism, could also utilize picric acid as a nitrogen source after spontaneous mutation. This mutant, designated HL PM-1, can induce the enzymes of 2,4-DNP catabolism in the presence of picric acid. Later Heiss *et al.* proposed reclassification of the mutant as *Rhodococcus opacus* HL PM-1 after fatty acid and mycolic acid analysis indicating a chain length of 48-54 carbon atoms. In

addition, the complete 16S rDNA sequence of strain HL PM-1 showed 99% sequence identity to the 16S rRNA gene of *R. opacus* strain 1CP and *R. opacus* strain GM-29. The first 500 base pairs (bp) revealed 99% sequence identity to *R. opacus* RB1 and the type strain *R. opacus* DSM 43205 (Blasco *et al.*, 1999; Heiss *et al*., 2002; Klatte *et al*., 1994).

In 1996 Rajan *et al.* reported isolation of the four bacterial strains that use picric acid as their sole carbon and energy source. These four biodegraders were identified as close relatives of *Nocardioides simplex* ATCC 6946 based on their small ribosomal subunit (16S rRNA) gene sequences. The *Nocardioides* strains described by Rajan and his coworkers were able to grow on picric acid without dead-end product formation. They also observed a transient formation of 2,4-DNP and stoichiometric release of three moles of nitrite per mole of picric acid. More examples of picric acid biodegraders include *Nocardioides* sp. strain CB 22-2 (Behrend and Heesche-Wagner, 1999), *Nocardioides simplex* FJ2-1A (Ebert *et al.*, 1999; Ebert *et al.*, 2001). The strain CB 22-2 was the first isolation and characterisation of a bacterium that completely mineralizes picric acid with intermediate formation of the hydride-Meisenheimer complexes of TNP and DNP. It was also demonstrated experimentally with crude extracts that the hydride-Meisenheimer complex of DNP is a physiologically relevant step in picric acid metabolism (Behrend and Heesche-Wagner, 1999).

1.6. Picric acid degradation gene cluster and proposed degradation pathway

1.6.1. Identification of the TNP and DNP metabolic genes

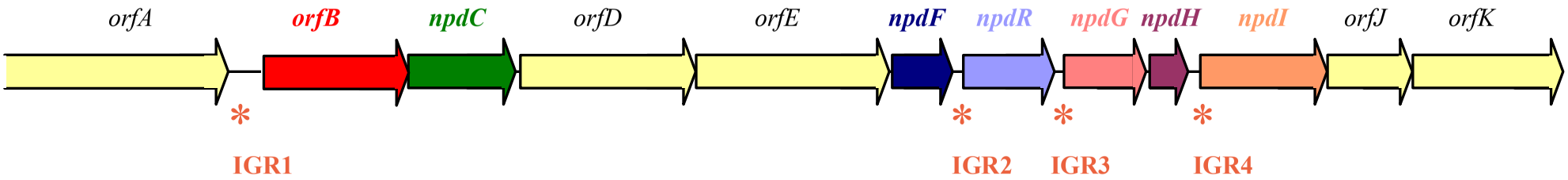
Identification of metabolic genes is a very laborious assignment based usually on two strategies. The first approach involves direct genetics and it relies on the expression of genes *in vivo*. This could be successfully achieved by the heterologous expression of metabolic genes (Suen and Spain, 1993). Alternative process consists of the isolation of specific mutants of the original strain, which

are defective in the genes of interest. The advantage of the direct genetic method is that it allows identification of DNA fragments which carry the genes of interest. However, it is only applicable to those organisms that have a developed genetic system or whose genes can be expressed in heterologous host. Successful identification of degradation genes by complementation of Gram-negative hosts is diminished if the source of the DNA is a high G+C Gram-positive organism like nocardioform bacteria. The regulatory components of gene expression differ and their high G+C content limits the recognition of promoter-like sequences in A+T rich regions. Lastly, it is unlikely that all the genes of a pathway can be successfully expressed to confer the degradative capabilities to the heterologous host (Maloy *et al.*, 1994; Russ *et al.*, 2000).

The second strategy in identification of metabolic genes is called reverse genetics. It is based on the purification of the enzyme of interest and subsequently identification of its gene. This method is applicable to any microorganism and it is very basic. The necessity to express a cloned fragment in the cloning host has many limitations. For example, if the enzyme activity cannot be assayed easily, if the enzyme is resistant to purification procedures, if the enzyme activity is labile, and finally if the enzyme is not abundant. Isolation of the metabolic genes responsible for a degradation pathway for many xenobiotic compounds, including the picric acid and 2,4-DNP, using direct or reverse genetic approaches have failed. Scientific community had to develop a new approach to gene discovery, which does not depend on a genetic system or on protein purification (Burden and Whitney, 1995; Russ *et al.*, 2000; Scopes, 1994).

Recently, Walters *et al.* (2001) showed that mRNA differential display is an important tool for the identification of metabolic genes in prokaryotes. In their investigation a long polycistronic mRNA was sampled repeatedly increasing the density of the mRNA population. Researchers had to modify the existing protocols for bacterial differential display. They used a large number of primers that allows for a high-density sampling of the mRNA population and the

identification of many differentially amplified DNA fragments. Furthermore, overlapping of these short sequences results in assembly of a long contiguous sequence which encodes several genes. They chose the strain *Rhodococcus opacus* HL PM-1 because the degradation pathway of picric acid and 2,4-DNP is inducible in contrast to *Nocardioides simplex* FJ2-1A, where the pathway is constitutive. This was important because in all differential display experiments, the RNA extraction was performed from the cultures where the induction had been thoroughly defined. Finally, a gene cluster of 12 open reading frames (ORFs) and about 12.5 kb was identified and sequenced. The sequence of the catabolic gene cluster was deposited in GENBANK under the accession number AF323606 (Russ *et al.*, 2000; Walters *et al*., 2001). Later, these genes were named *npd* for ***n***itro***p***henol ***d***egradation (Heiss *et al.*, 2002).



orfA	Transcription factor
orfB	**Carnitine dehydratase**
npdC	**Hydride transferase I**
orfD	Aldehyde dehydrogenase
orfE	Acyl CoA synthase
npdF	**2,4-Dinitrocyclohexanone hydrolase**
npdR	**Transcriptional regulator**
npdG	**NADPH-dependent F_{420} reductase**
npdH	**$2H^-$ - TNP tautomerase**
npdI	**Hydride transferase II**
orfJ	Enoyl CoA hydratase
orfK	Acyl CoA dehydrogenase
IGR	**Intergenic regions**

Figure 2. The nitrophenol degradation (*npd*) gene cluster of *Rhodococcus opacus* HL PM-1 showing the genes whose products have been functionally identified and predicted gene functions. Intergenic regions IGR1 and IGR4 contain potential promoters. In both, the putative -35 and -10 hexamers are found (five out of six nucleotides are identical) and possible Shine-Dalgarno sequences are also present (Dang *et al.*, 2004).

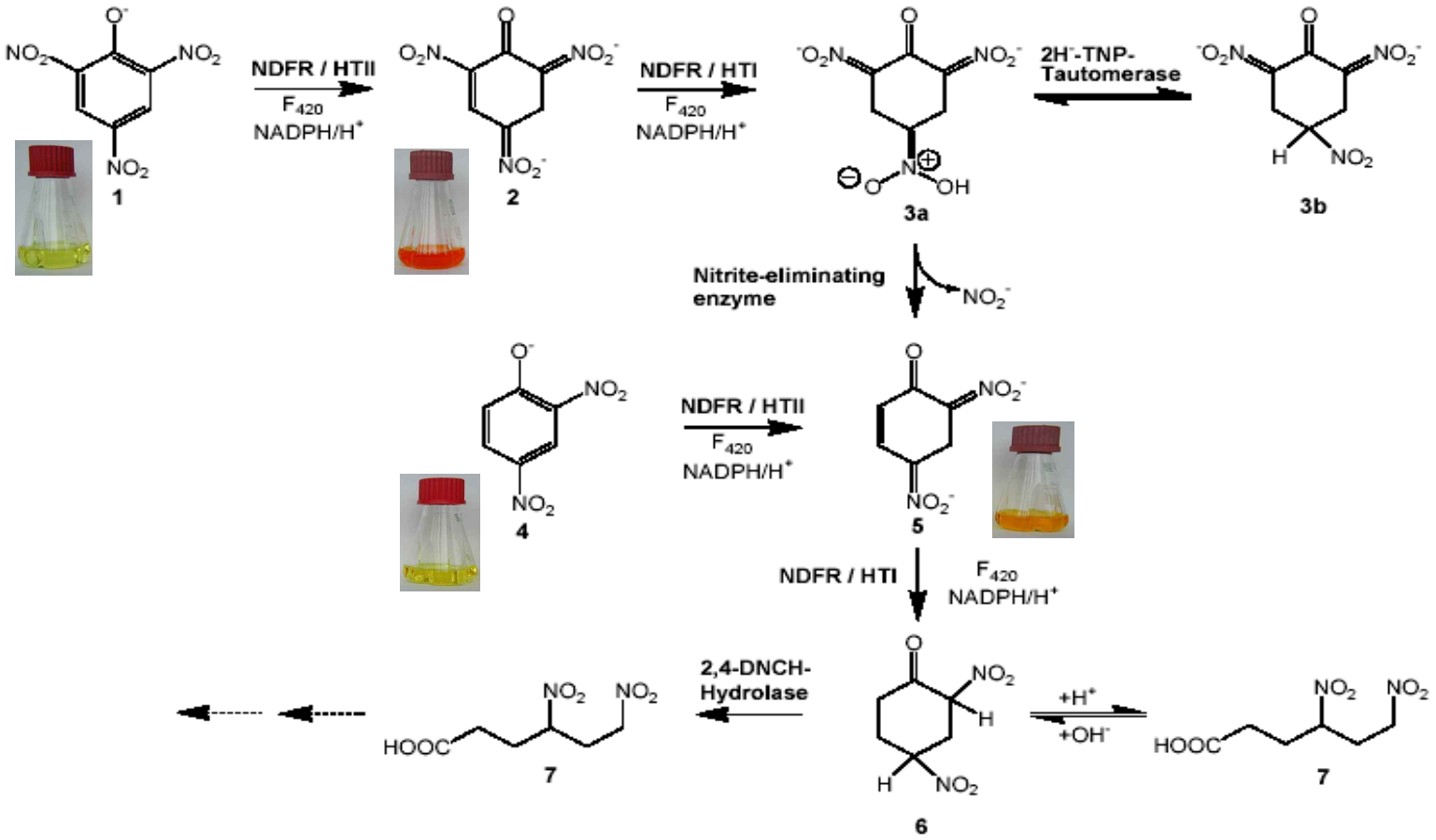


Figure 3. Proposed degradation pathway of TNP and DNP. (1) TNP = 2,4,6-trinitrophenol (picric acid) (2) H^--TNP = hydride σ-complex of 2,4,6-TNP (hydride Meisenheimer complex of TNP) (3a) *aci*-nitro form of $2H^-$-TNP (dihydride Meisenheimer complex of TNP) (3b) nitro form of $2H^-$-TNP (4) DNP = 2,4-dinitrophenol (5) H^--DNP (hydride Meisenheimer complex of DNP) (6) 2,4-DNCH (2,4-dinitrocyclohexanone) (7) 4,6-DNH (4,6-dinitrohexanoate). HTI = hydride transferase I, HTII = hydride transferase II, NDFR = NADPH-dependent F_{420} reductase.

1.6.2. Enzymatic degradation of the 2,4,6-trinitrophenol

1.6.2.1. Hydrogenation reactions of TNP

Under aerobic conditions, the first step of TNP biodegradation involves a hydrogenation reaction, also called the initial reduction level of the aromatic ring. Consequently, a formation of the hydride δ-complex of 2,4,6-TNP (hydride Meisenheimer complex of TNP) is detected as the orange-red extra-cellular metabolite. This has been observed in *R. opacus* HL PM-1 whereas in *N. simplex* FJ2-1A the metabolite is located intracellularly. Additional reduction of the aromatic compound takes place producing *aci*-nitro form of $2H^-$-TNP (dihydride Meisenheimer complex of TNP). The first hydrogenation step is catalyzed by hydride transferase II (HTII) while hydride transferase I (HTI) catalyzes the second hydride transfer. Both transfer reactions require a coenzyme NADPH-dependent F_{420} reductase (NDFR) to supply the hydride ions from NADPH to F_{420} in the form of $F_{420}H_2$. A two-component enzyme system is slightly different in *R. opacus* and *N. simplex*. In *Rhodococcus* strain there are two hydride transferases isolated and characterized while in the strain FJ2-1A only a single hydride transferring enzyme system has been shown to transfer hydride from reduced coenzyme F_{420} to the aromatic ring of the nitrophenols (Ebert *et al*., 1999; Ebert *et al.*, 2002; Heiss *et al*., 2002; Lenke and Knackmuss, 1992).

1.6.2.2. Tautomerisation reaction of $2H^-$-TNP

Additional reaction step in the TNP pathway was accelerated by a small enzyme called tautomerase, which catalyzes a proton shift between the *aci*-nitro and the nitro forms of $2H^-$-TNP. It has been shown that under alkaline conditions (pH 8) the dihydride Meisenheimer complex of TNP exists as a double-charged anion (*aci*-nitro form), with a retention time of 1.86 min after High Performance Liquid Chromatography (HPLC) analysis. In addition, HPLC illustrated the tautomeric nitro form that was eluted after 2.28 min. The structures of the tautomers were

established using Nuclear Magnetic Resonance (NMR) spectroscopy. The proton-shift tautomerisation was catalyzed by the tautomerase speeding up the rate of reaction between 300-600 fold. Also, the tautomers were in equilibrium at pH 8.0 and the ratio of 20:80 (*aci*-nitro**:**nitro form) was estimated from the HPLC peak areas at A_{390} because this wavelength results in the ultraviolet (UV) maximum for either form. It was also demonstrated that the time period required to reach the equilibration ratio was dependent on the pH. At pH 7.0 the time needed to reach the equilibrium in the presence of the tautomerase was approximately 0.1 min while at pH 8.0 it was roughly 1 min (Hofmann *et al*., 2004).

1.6.2.3. Nitrite elimination from the *aci*-nitro form of $2H^-$-TNP

The nitrite elimination reaction from the *aci*-nitro form of $2H^-$-TNP during the biodegradation pathway was of the particular interest for this work. Nitrite-eliminating activity has been reported in the crude extracts of *R. opacus* HL PM-1 and *N. simplex* FJ2-1A by Hofmann *et al.* (2004). During the reaction stoichiometric amounts of nitrite from $2H^-$-TNP were released. The nitrite-eliminating enzyme (NEE) was purified from *N. simplex* and a molecular mass was estimated to be 35.3 kilo Daltons (kDa) using the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In addition, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) measurements gave a signal at *m/z* 30.50 kDa while the molecular mass of the purified NEE as determined by gel filtration has been predicted to be 42 kDa. The protein is a monomer and N-terminal amino acid was sequenced but a comparison to sequences in databases showed no similarity to any known protein. The researchers also confirmed a conversion of the *aci*-nitro form (and not the nitro form) of $2H^-$-TNP to the hydride Meisenheimer complex of DNP (H^--DNP) by the NEE.

1.6.2.4. Hydrogenation reaction of H^--DNP

Heiss *et al.* (2002) showed that HTI catalyzes a conversion of H^--TNP to $2H^-$-TNP. Thereafter, Hofmann *et al*. (2004) decided to investigate a substrate diversification for the same enzyme and the assays were conducted with H^--DNP, F_{420}, NADPH and HTI. Metabolites were detected and quantified by HPLC. The turnover of H^--DNP by the HTI exhibited a reduction in absorbance at 441 nanometre (nm) and 306 nm. An associated increment in absorbance at 340 nm illustrated the propagation of a new metabolite with a retention time of 3.2 min. Furthermore, Hofmann and his colleagues reported the two absorbance maxima at 232 and 340 nm after UV-visible spectrum analysis. The metabolite was also confirmed by a coupling of HPLC, negative-mode electrospray ionization (ESI^-) and mass spectra (MS) giving the intense signal at m/z 187 to the molecular anion of the dihydride Meisenheimer complex of DNP ($2H^-$-DNP). Protonation of $2H^-$-DNP to 2,4-dinitrocyclohexanone (2,4-DNCH) occurs at pH 7.5 and the specific activity of the HTI for H^--DNP has been calculated to be 29.6 U mg^{-1}.

1.6.2.5. Hydrolytic ring fission of 2,4-DNCH

The activity that converts the 2,4-DNCH, the protonated form of $2H^-$-DNP, has been assayed for in crude extracts. Availability of the substrate either by chemical or biochemical synthesis is usually an obstacle for detailed kinetic studies. Hoffman *et al.* had to use the analogous compound 2-nitrocyclohexanone (2-NCH), commercially accessible, for new investigations. *N. simplex* FJ2-1A and *R. opacus* HL PM-1 exhibited growth on solidified mineral medium supplemented with 2-NCH as the sole nitrogen source. Crude extracts of both organisms, grown with DNP and 2-NCH, demonstrated hydrolase activity for 2-NCH, producing 2,4-DNCH biologically. The enzyme was purified from the FJ2-1A strain, using 2-NCH as the experimental substrate. Moreover, SDS-PAGE showed a sole protein band at 15.3 kDa, purity greater than 98% and the specific activity for 2-NCH has been calculated to be 24.4 U mg^{-1}. MALDI-TOF measurements gave a

signal at *m/z* 16.989 kDa and the molecular mass of the purified hydrolase as determined by gel filtration has been predicted to be 63 kDa. This led scientists to assume that the enzyme is composed of four identical subunits. They further sequenced N-terminus of the purified protein using automated Edman degradation. Data analysis revealed 78% sequence identity to the product of *orfF*, encoding a putative lyase in *R. opacus* HL PM-1, so *orfF* has been renamed as *npdF* (Russ *et al.*, 2000; Walters *et al*., 2001).

Furthermore hydrolysis of 2,4-DNCH to 4,6-dinitrohexanoate (4,6-DNH) is pH dependent. Under alkaline conditions, pH > 8, the reaction blend had only 2,4-DNCH while 4,6-DNH have been predominant compound in acidic solution, pH < 5. The HPLC peak areas at A_{210} have been determined in the presence of 2 micrograms (μg) of hydrolase because the substrate could not be prepared in uncontaminated form. Nevertheless, a ring cleavage picked up to a 15-fold increase in the peak areas corresponding to 4,6-DNH after 10 min. This result confirmed that the enzyme converts 2,4-DNCH to 4,6-DNH. After 4,6-DNH was incubated at pH 7.5, HPLC analysis showed chemical unsteadiness as noted in the past also. Although it is still unclear how 4,6-DNH is digested, they hypothesize that a further degradation of the compound could yield an acetic acid derivative. Subsequently, the two nitro groups would be cleaved, resulting in a carboxylic acid being the substrate for the tricarboxylic acid cycle (Hofmann *et al*., 2004, Lenke and Knackmuss, 1992).

1.7. Regulation of the *npd* genes expression

Usually, soil bacteria grow and divide in environments that change rapidly. The number of proteins assembled satisfying the needs of a particular cell and most of the time avoiding wasteful synthesis. Some of the enzymes may be useless at one moment or even counterproductive soon afterwards. Also, a sudden requirement for an enzyme whose presence was previously unnecessary is possible. For some genes, the different rate of gene expression is due to the efficiency of transcription

and translation. These genes are under control of regulatory mechanisms, which act like an on-off switch allowing high levels of gene expression only when signals are received from outside. The compounds that transmit these signals are called inducers and repressors control inducible enzyme synthesis (Maloy *et al*., 1994; Watson *et al*., 1992).

The regulation of polynitroaromatic degradation pathway at the molecular level is still unclear. Walters *et al.* (2001) illustrated an inducibility of the picric acid biodegradation by the 2,4-dinitrophenol. Earlier, Russ *et al.* (2000) noticed the two transcriptional regulators, encoded by *npdR* and *orfA* within the TNP degradation gene cluster. Lastly, Dang *et al*. (2004) demonstrated that NpdR is a repressor, which negatively regulates the expression of the *npd* genes. Heterologous expression of the *npdR* showed the presence of an isopropyl-β-D-thiogalactopyranoside (IPTG) induced polypeptide and the size of the purified fusion protein was about 28 kDa. In addition, the repressor binds to two intergenic regions (IGRs) in the *npd* gene cluster. Both intergenic regions have been cloned upstream of a promoterless reporter gene (*xylE*) in the vector pK4. The first one, 275 bp between *orfA* and *orfB*, and the fourth one, 102 bp between *npdH* and *npdI*, contain potential promoters (Figure 2). In both, the putative -35 and -10 hexamers are found (five out of six nucleotides are identical) and possible Shine-Dalgarno sequences are also present. *In vitro* binding studies confirmed that the NpdR is the repressor involved in picric acid degradation. It is also a helix-turn-helix type regulator, which belongs to the IclR family of transcriptional regulators.

1.8. Significance and aims of this work

Bioremediation is generally defined as a pollution treatment technology, which is based on the utilization of biological systems in order to catalyze the destruction or transformation of various chemicals to less harmful forms. It is also a cost-effective mean of restoring environmental quality (Atlas and Unterman, 1999).

Synthetic chemicals, usually referred to as xenobiotics, do not or uncommonly exist as natural products. In addition, these compounds may contain structural elements that not known to be synthesized biochemically. In the course of natural selection of catabolic enzymes and pathways, microorganisms were not exposed to these building blocks and therefore have not evolved the capability to use these structures as sole source of carbon and energy (Rieger *et al*., 2002). Understanding behaviour of a single bacterial genus in the terrestrial environment and a pathway by which the organism degrades a xenobiotic are the prerequisites of a successful bioremediation strategy.

In this study I worked with two bacterial genera, *Nocardioides* and *Rhodococcus*, notably, *N. simplex* FJ2-1A and *R. opacus* HL PM-1. Both are Gram-positive Actinomycetes, which degrade DNP and TNP by the same initial mechanism. While working with these organisms, intriguing questions arose since the *npd* gene cluster identified could not provide all the answers. During initial purification procedures, Hofmann isolated the nitrite-eliminating activity by FPLC from cell extracts of *N. simplex* FJ2-1A. Following native polyacrylamide gel electrophoresis (PAGE), the gel was stained in a solution containing 1 mM $2H^-$-TNP and the dark yellow band with the NEE was cut. Furthermore, it was separated by SDS-PAGE and bloted on a PDF-membrane. Although pure, the sequencing of the enzyme was not possible. The N-terminus was chemically bloked during the electrophoresis or blotting, which further hindered the attack of phenyl isothiocyanate during the Edman degradation. Only after improved growth conditions, the protein expression was promoted and purification possible. In addition, a gene encoding the activity is not a part of the *npd* cluster. A $2H^-$-TNP tautomerase was purified from *N. simplex* as a His-tag fusion protein. Comparison to the isofunctional NpdH from *R. opacus* showed a DNA sequence identity of 63% and a protein sequence identity of 59% (Hofmann, 2004). Knowing the nitrite-eliminating activity only, one could further attempt to answer the following questions:

- Where does the gene come from and is there a parallel pathway switched on simultaneously during the DNP and TNP degradation?
- Is it a plasmid borne or is there a single chromosomal copy of the gene?
- If the activity is plasmid localized, how many cellular copies of the same gene are present?
- Is there a unique regulatory mechanism different from the one already explained in *R. opacus* HL PM-1 because the enzyme was purified from *N. simplex* FJ2-1A?
- Does the enzyme catalyze release of the two remaining nitrite groups from 4,6-DNH since no activity was reported for this reaction?

There were three major **aims** of my research. The first one was to investigate a function of the *orfB* contained in the *npd* gene cluster. The second one was to clone a DNA fragment encoding nitrite eliminating enzyme activity using the reverse genetics approach. Finally, to suggest a bioremediation system since the pathway is proposed and not confirmed. This would deepen our knowledge with respect to biodegradability of nitroaromatic compounds. Furthermore, it would facilitate accomplishment of environmental biotechnology in establishing highly efficient biological processes, which use the natural catabolic potential in order to eliminate and detoxify these xenobiotics.

2. Materials and methods

2.1. Bacterial strains, culture conditions, vectors and their plasmids

Maintenance was accomplished by sub-culturing organisms on a routine basis, twice a month, by streaking microbes aseptically onto agar plates containing a selective agent(s). With solutions of recombinant DNA molecules, multiple freeze-thaw cycles were avoided.

2.1.1. Bacterial strains

Table 2. *Escherichia coli* strains used to construct a cosmid library.

Bacterial strain	Relevant characteristics	Reference or source
EPI100™	F^- mcrA Δ(*mrr-hsd*RMS-*mcr*BC) φ80δ*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G Plating strain for a cosmid library; also for amplification and storage of the library.	Epicentre®, Madison
LE392MP	F^- e14$^-$ ($McrA^-$) Δ(*mcr*C-*mrr*) (Tet^R) *hsd*R514 *sup*E44 *sup*F58 *lac*Y1 or Δ(*lac*lZY)6 *gal*K2 *gal*T22 *met*B1 *trp*R55 λ^- Plating strain for the MaxPlax™ Lambda Packaging Extracts (MaxPlax Control DNA packaging reactions)	Epicentre®, Madison

Table 3. *Escherichia coli* strains used to construct a phage library.

Bacterial strain	Relevant characteristics	Reference or source
BM25.8	*supE thi* Δ(*lac-proAB)* [*F' traD36 proA*+ *B*+ *lacI*^{q}*Z*Δ*M15*] λimm^{434} (Kan^{R}) P1(Cam^{R}) *hsdR* ($r_{K12}^{-}m_{K12}^{+}$) Used for automatic subcloning. Lysogenic for phages λ and P1. Maintained on 34 μg/ml Cam plus 50 μg/ml Kan plates.	Novagen®, Merck Biosciences
ER1647	F^{-} *fhuA*2 Δ(*lacZ*)*r1 supE44 recD1014 trp31 mcrA1272*::Tn*10*(Tet^{R}) *his-1 rpsL 104*(StrR) *xyl7 mtl2 metB1*Δ(*mcrC-mrr*) *102*::Tn*10*(Tet^{R}) *hsdS* ($r_{K12}^{-}m_{K12}^{+}$) Plating strain for a phage library; also for amplification, titering and screening of the library. Maintained on 12.5 μg/ml Tet.	Novagen®, Merck Biosciences
LE392	F^{-} e14^{-} (*mcrA*) *hsdR514* ($r_{K12}^{-}m_{K12}^{+}$) *supE44 supF58 lacY1* or Δ(*laclZY*)6 *galK2 galT22 metB1 trpR55* Plating strain for the PhageMaker® Extracts (Control DNA packaging reactions)	Novagen®, Merck Biosciences

Table 4. *Escherichia coli* strains used for DNA manipulations, overexpression and transformations.

Bacterial strain	Relevant characteristics	Reference or source
DH5α™	F⁻ φ80Δ*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 ($r_K^- m_K^+$) *pho*A *sup*E44 λ-*thi*-1 *gyr*A96 *rel*A1 Used for general cloning with α complementation, blue/white selection and plasmid DNA preparations. Maintained on 50 μg/ml Nal plates.	Invitrogen
MM294-4	*endA1, hsdR17, gyrA96* Used for routine cloning and plasmid DNA preparations. Maintained on 50 μg/ml Nal plates.	Dabbs, E.
Rosetta 2(DE3)pLysS	F⁻ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3) pLysSRARE2 (Cam^R) Used for heterologous overexpression. Enhances expression of proteins having codons rarely used in *E. coli*; supplies tRNAs for seven rare codons: AUA, AGG, AGA, CUA, CCC, GGA, and CGG. Maintained on 34 μg/ml Cam plates.	Novagen®, Merck Biosciences
S17-1	*thi pro hsdR*⁻ *hsdM*⁺ *recA* res⁻ mod⁺ RP4-2-(Tc::Mu)-(Km::Tn*7*) Mobilizing donor strain, has an RP4 derivative integrated into the chromosome; used for conjugation experiments.	Simon *et al*. (1983)

Table 5. *Nocardioides* and *Rhodococcus* strains used in this work.

Bacterial strain	Relevant characteristics	Reference or source
N. simplex FJ2-1A	Ability to grow on TNP as a sole nitrogen source	Rajan *et al.* (1996)
R. erythropolis SQ1	Highly transformable derivative of ATCC 4277, Rif^R Maintained on 50 μg/ml Rif plates.	Quan and Dabbs (1993)
R. opacus HL PM-1	Ability to grow on TNP or DNP as a sole nitrogen source	Lenke and Knackmuss (1992)
R. opacus AR1	Derivative of *R. opacus* HL PM-1; deletion in *orfB*	This work
R. rhodochrous ATCC[a] 12674 (Ri8-R)	Inability to grow on TNP and DNP as a sole nitrogen source, Rif^R Maintained on 50 μg/ml Rif plates.	Dabbs, E.

[a] ATTC = American Type Culture Collection

2.1.2. Culture conditions, strain storage and revival

Recombinant *E. coli* strains were grown with agitation at 37°C in Luria-Bertani (LB) medium [10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl] supplemented with ampicillin (100 μg/ml) and kanamycin (30 μg/ml) for the maintenance of plasmids. All auxotrophs required 40 μg/ml of the appropriate amino acid for growth on minimal medium. For gene expression, freshly transformed overnight cultures of *E. coli* Rosetta 2(DE3)pLysS(pARS3 / pARS4 / pARS5) were inoculated into LB (1/10 v/v inoculum) and further incubated for 1.5–3 h (till OD_{600} reached 0.6–1.0) at 37°C. Thereafter, the cultures were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) following incubation for 4-5 h at 30°C.

N. simplex FJ2-1A was grown on a shaker in conical flasks at 30°C in 50 mM phosphate buffer (pH 7.5) containing 0.7 mM picric acid (PA), 20 mM sodium acetate, 1:100 diluted R2A medium (0.5 g/L yeast extract, 0.5 g/L proteose peptone, 0.5 g/L casamino acids) and mineral salts. Mineral salts without nitrogen contained 20 mg/L of Fe(III)-citrate, 1 g/L of $MgSO_4$ x $7H_2O$, 50 mg/L of $CaCl_2$ x $2H_2O$ and 1 ml of SL6 trace element solution (100 mg/L $ZnSO_4$ x $7H_2O$, 30 mg/L $MnCl_2$ x $4H_2O$, 300 mg/L H_3BO_3, 200 mg/L $CoCl_2$ x $6H_2O$, 10 mg/L $CuCl_2$ x $2H_2O$, 20 mg/L $NiCl_2$ x $6H_2O$, 30 mg/L Na_2MoO_4 x $2H_2O$). After consumption of initial PA, additional amount of 0.35 mM PA was added and the cells were harvested by centrifugation immediately after decolourization of the medium.

R. opacus strains were cultured at 30°C in LB medium or in minimal medium [50 mM KH_2PO_4-Na_2HPO_4 buffer (pH 7.4) containing 0.45 mM $CaCl_2$ x $2H_2O$, 40 mM CH_3COONa and mineral salt solution as described above for *N. simplex*] supplemented with 0.5 mM picric acid or $(NH_4)_2SO_4$ as a sole source of nitrogen. For induction of 20 ml overnight pre-culture in order to detect a colour change, 0.5 mM DNP was added at an optical density at 600 nm of 0.5–1.0 and culturing was continued for 3–4 h. Plasmids in *Rhodococcus* were selected for on kanamycin (100 μg/ml). Sucrose sensitivity was tested on LB agar containing 10% (w/v) sugar.

R. erythropolis SQ1 and *R. rhodochrous* ATCC 12674 were cultured at 30°C in LB medium containing 50 μg/ml rifampicin. All strains were also grown on a solid medium as described above with the addition of 1.5% (w/v) agar. Gram positive cells were frozen in liquid nitrogen and stored at −20°C till used for further experimentation. For short-term storage, the bacterial strains were kept on agar plates at 4°C up to two months. Alternatively, for long-term storage, 2 ml of a mid-log culture was harvested and resuspended in the appropriate fresh liquid medium supplemented with 70% (v/v) glycerol and stored at −70°C. The stored cells were revived by scraping off splinters of solid ice with a toothpick or sterile pipette and streaking onto the suitable agar plate.

2.1.3. Vectors and plasmids

Table 6. Phagemid and vectors used.

Plasmids/Vectors	Relevant features	Reference or source
pBluescript II SK (+)	Amp^R; 2961 bp phagemid; *rep* (pMB1); *lacZ* fragment allows blue/white screening; P_{lac} General cloning and sequencing vector	Gibco Life Technologies, BRL
pET-11a*	Amp^R; phage T7*lac* promoter; ColE1 origin; contains a polylinker with additional restriction sites: *Sma*I, *Kpn*I, *Xho*I, *Sal*I; expression vector, 5701 bp	Novagen®, Merck Biosciences
pET-22b(+)	Amp^R; phage T7*lac* promoter; ColE1 origin; expression vector, 5493 bp, with C-terminal hexahistidine affinity tag	Novagen®, Merck Biosciences
pET-28a(+)	Kan^R; phage T7*lac* promoter; ColE1 origin; expression vector, 5369 bp, with N-terminal hexahistidine affinity tag	Novagen®, Merck Biosciences
pK18*mobsacB*	Contain *oriT* of pRP4; mobilisable vector; $Suc^S Kan^R$; used for selection of a double-cross over event in *R. opacus*; ~5.7 kb	Schäfer *et al.* (1994)

Table 7. Plasmids constructed in this work.

Plasmids/Vectors	Relevant features	Reference or source
pARS2	*Eco*RI - *Bam*HI fragment (carrying a Δ285 bp in *orfB*) ligated into *Eco*RI and *Bam*HI sites of pK18*mobsacB*	This work
pARS3	*orfB* cloned into *Nde*I and *Xho*I sites of pET-11a*	This work
pARS4	*orfB* cloned into *Nde*I and *Xho*I sites of pET-22b(+), lack a stop codon within *orfB* insert	This work
pARS5	*orfB* cloned into *Nde*I and *Xho*I sites of pET-28a(+)	This work
pARS6	252 bp NEE probe inserted into pBluescript II SK (+) via T/A cloning	This work
pARJ1	~15 kb FJ2-1A insert in λBlueSTAR™ vector	This work
pARJ2	~10 kb FJ2-1A insert in λBlueSTAR™ vector	This work

2.2. Molecular techniques

Standard protocols were used for manipulation of DNA according to Ausubel *et al*. (2001), Burden and Whitney (1995) and Sambrook *et al*. (1989). Plasmid DNA was isolated using the FlexiPrep™ Kit (Amersham Pharmacia Biotech) or GFX™ *Micro* Plasmid Prep Kit (Amersham Pharmacia Biotech) and visualized using Pellet Paint® Co-Precipitant (Novagen®, Merck Biosciences). *E. coli* was transformed according to Inoue *et al.* (1990). DNA fragments were purified from an agarose gels using QIAquick® Gel Extraction Kit or QIAquick® PCR Purification Kit (QIAGEN). DNA sequencing was performed by GATC Biotech AG (Konstanz). Internal sequencing of a protein was performed by TopLab (Martinsried).

2.3. Isolation and preparation of nucleic acid

2.3.1. Genomic DNA extraction

For preparation of total DNA from *Nocardioides* and *Rhodococcus*, the strains were pre-cultured in their selective picric acid medium as described previously (section 2.1.2.). Thereafter, the strains were cultured in 5 ml LB overnight at 30°C with constant shaking. The cells were then harvested at 5 000 rpm for 10 min at 4°C (Sigma 3K-1, Deisenhofen) and washed twice with 50 mM Tris-HCl pH 8.0. The pellet was resuspended in 3 ml lysis buffer (50 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 10% (w/v) sucrose, 10 mg/ml lysozyme) and the suspension was incubated overnight at 37°C. The following day, the mixture was pelleted by centrifugation and the supernatant was decanted. The pellet was resuspended in 5 ml of TE buffer (10:1 buffer; 10 mM Tris-HCl, 1 mM EDTA pH 8.0) containing 100 µg/ml proteinase K and 500 µl of 10% (w/v) SDS. This was further incubated for 2–4 h at 37°C till clearing occurred. The lysate was extracted twice with 5 ml of phenol, twice with 5 ml phenol/chloroform/isoamyl alcohol [25:24:1 (v/v/v)] and once with 5 ml of chloroform/isoamyl alcohol (24:1, v/v). The DNA was precipitated with 0.7 volume of isopropanol and 0.1 volume of 3 M sodium / potassium acetate pH 5.2, and centrifuged at 14 000 rpm for 10 min (Eppendorf 5417C, Hamburg). The pellet was washed once with 70% (v/v) ethanol and once with 95% (v/v) ethanol, air dried, finally resuspended in TE buffer (10:0.1 buffer; 10 mM Tris-HCl, 0.1 mM EDTA pH 8.0) and stored at 4°C.

2.3.2. Rapid isolation of plasmid and cosmid DNA

FlexiPrep™ Kit was used for the rapid extraction and purification of plasmid DNA from *E. coli* cultures ranging in volume from 1.5 ml to 500 ml (miniprep and large scale). The kit is a simple alternative to CsCl density gradients, employing a standard alkaline cell lysis procedure, including RNase treatment and isopropanol precipitation. The DNA was visualized using Pellet Paint® Co–

Precipitant. Plasmid DNA was subsequently purified using a novel glass matrix – Sephaglas FP. GFX™ *Micro* Plasmid Prep Kit was used for the fast extraction and purification of plasmid and cosmid DNA from *E. coli* cultures ranging in volume from 1–3 ml (minipreps only). The kit employs a modified alkaline cell lysis procedures and a glass fibre matrix (in GFX Column) to give high yields of the recombinant molecules. Matrix-bound DNA is eluted from the column in a buffer. Both kits were used following recommendation by the manufacturer. Isolated DNA was used for PCR amplifications, enzymatic manipulations, subcloning, DIG labelling transformations and automated sequencing.

2.3.3. Estimation of DNA concentrations

Concentration of DNA was estimated using qualitative and quantitative methods. Firstly, an estimate was made by qualitatively comparing the fluorescence of DNA bands in an agarose gel, in the presence of ethidium bromide, to a standard – FastRuler™ DNA Ladders or MassRuler™ DNA Ladder (Fermentas). The comparison was made following the electrophoresis of the standard and the unknown sample. Bands of DNA below 5 ng were not detected by this method. Secondly, the concentration of DNA was determined quantitatively with a Cary 50 Biospectrophotometre controlled by Cary WinUV Biopackage software (Varian, Mulgrave, Australia). The spectrophotometre was used to measure the absorbances at 260, 280 and 325 nm of the samples and water served as a blank to zero the instrument. The A_{325} reading indicated contamination by particulate matter or a dirty cuvette. 1:100 dilutions of each sample were made and quartz cuvettes with one cm detection path were used. The absorbance at 260 nm allowed calculation of the concentration of DNA in the sample. An A_{260} of 1 corresponds to 50 µg dsDNA per ml water, which was used as diluent. The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provided a very rough estimate of the purity of the nucleic acid. In addition, the ratio of a pure DNA solution is between 1.8 and 2.0. The concentration of DNA (µg/ml) was calculated based on the following relationship: A_{260} x dilution factor x 50 µg/ml = [DNA].

2.3.4. Cleaving DNA with restriction endonucleases

Restriction endonucleases used in this work were purchased from Fermentas, New England BioLabs or Roche and used according to the guidance of the supplier. Generally, a digestion reaction was set up in a final volume of 20 µl and contained 0.5–1.0 µg DNA in 1x restriction buffer and 3–5 U of enzyme. A mixture was incubated at the optimal temperature for 1–2 h. Genomic digestions were performed using 10 µg DNA in 100 µl 1x buffer supplemented with 20 U of enzyme with overnight incubation. The digested samples were further analysed by agarose gel electrophoresis.

2.3.5. Dephosphorylation of 5' ends from DNA

Shrimp alkaline phosphatase (Fermentas or Roche) was used to catalyze the dephosphorylation of terminal 5'-phosphates from DNA. After a vector digestion, restriction endonuclease was inactivated by heating at 65°C for 15 min and in the same reaction mixture dephosphorylation buffer to 1x final concentration and 1 U phosphatase were added. Following incubation for 15 min at 37°C, the enzyme was heat inactivated and a ligation reaction was performed in the same tube. Alternatively, the vector DNA was purified using phenol (removes the enzyme) or agarose gel extractions for the subsequent ligation.

2.3.6. The ligation reaction

The formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA with blunt or cohesive ends was catalyzed using T4 DNA ligase (Fermentas or Roche). The ligations were performed in a 20 µl reaction volume with 1x ligation buffer, 2 U ligase (for sticky ends) or 5 U ligase (for blunt ends + 50% PEG 4 000 solution) and a 3 **:** 1 molar ratio of insert DNA termini to vector DNA. The ligated mixture was used for bacterial transformation.

2.3.7. Phenol extraction of DNA

Protein contaminants or solute molecules were removed from aqueous solution using phenol extraction. This technique was also used when DNA solutions needed to be concentrated for reasons of convenience. An equal volume of phenol/chloroform/isoamyl alcohol [25:24:1 (v/v/v)] mixture was added to the DNA solution to be purified. The tube was vortexed vigorously for 10 s and microfuged for 15 s with maximum speed at room temperature. The top aqueous phase containing DNA was carefully removed and transferred to a new Eppendorf tube. To prevent carryover of residual phenol, the aqueous phase was re-extracted with 24:1 (v/v) chloroform/isoamyl alcohol. The DNA solution was further concentrated with ethanol or isopropanol.

2.3.8. Precipitation of DNA

Concentration of DNA from aqueous solutions was achieved by precipitation with either ethanol or isopropanol. In the case of ethanol, 1/10 volume of 3 M CH_3COONa (pH 5.2) was added to the solution of DNA, the tube was mixed by flicking, following by addition of 2 to 2.5 volumes (calculated after salt addition) of ice-cold 100% ethanol. The precipitation step was done in a −20°C freezer for at least 30 min. In the case of isopropanol, equal volumes of alcohol and DNA solution were used in precipitation. Thereafter, the tube was microcentrifuged for 5 min at maximum speed, the supernatant was removed and pellet was washed with 1 ml ambient 70% (v/v) ethanol. Additional microfugation step was performed, following the removal of the supernatant and drying the pellet in a Speedvac evaporator. The dry pellet was dissolved in an appropriate volume in water or TE buffer (10:0.1), pH 8.0.

2.4. Constructions of genomic libraries

Two different genomic libraries were constructed in this work. The first gene

bank was made with *R. opacus* HL PM-1 total DNA using the pWEB™ Cosmid Cloning Kit (EPICENTRE®). The second one was made with *N. simplex* FJ2-1A total DNA using λBlueSTAR™ Vector System (Novagen®). General considerations and construction protocols were followed as explained in the supplied manuals.

2.4.1. Cosmid library

After purification of *R. opacus* genomic DNA, the DNA was sheared into approximately 40 kb fragments by passing it through a syringe needle. The sheared DNA was end-repaired in order to generate blunt ends and size selected on a low-melting point agarose gel by comparison with a 40 kb standard. Lastly, the size-selected DNA was ligated into the blunt-ended Cloning-Ready pWEB cosmid vector, packaged using ultra-high efficiency MaxPlax™ Lambda Packaging Extracts (>10^9 pfu/µl for phage lambda) and plated on the *E. coli* EPI100™ strain. The optimal number of clones in the library was determined using the following formula:

$$\mathbf{N = \ln(1-P) / \ln(1-f)}$$

where **P** is the desired probability (expresses as a fraction); **f** is the proportion of the genome contained in a single clone (the average size of the genomic fragment / the size of the genome); and **N** is the required number of cosmid clones. The number of clones required to ensure a 99% probability of a given DNA sequence of *R. opacus* being contained within a cosmid library composed of 40 kb inserts was 461.

$$N = \ln(1-0.99) / \ln(1-[4 \times 10^4 \text{ bases} / 6.5 \times 10^6 \text{ bases}]) = -4.61 / -0.01 =$$
$$= 461 \text{ clones}$$

The library was further amplified and stored in LB containing sterile glycerol to a final concentration of 15% using a 96–well microtiter plate. A single bacterial colony was inoculated into 100 µl of the storage medium and mixed well. Plate was incubated at 37°C for 6–8 h and stored at −70°C.

2.4.2. Phage library

Following purification of *N. simplex* genomic DNA, to prepare for cloning, the DNA was partially digested with *Bam*HI. Afterwards, the desired size range of fragments (>10 kb) was obtained performing small scale digestions. Once the conditions were optimized to give a maximum yield of fragments in the appropriate size range, the chosen reaction was scaled up to digest 10–20 μg DNA. The next step was to optimize insert **:** vector ratios for maximum cloning efficiency and highest efficiency was obtained using 3 **:** 1 molar ratio. Successful ligations to λBlueSTAR arms were packaged using PhageMaker® extracts and plated on the *E. coli* ER1647. Blue/clear screening was achieved by the presence of the complete *E. coli lacZ* gene and control region within the 13 kb stuffer fragment. Nonrecombinants were identified as blue plaques on plates containing X-gal, whereas phage containing genomic DNA inserts produced clear plaques. Furthermore, plasmid subclones were obtained by plating λBlueSTAR phage on the *E. coli* BM25.8 strain expressing Cre recombinase in the presence of ampicillin (50 μg/ml). The colonies obtained carried the genomic insert in a plasmid excised from the lambda vector. The number of clones required to ensure a 99% probability of a given DNA sequence of *N. simplex* being contained within a phage library composed of 10 kb inserts was 2305. The library was amplified and the phages were eluted with autoclaved SM buffer [5.8 g/L NaCl, 2 g/L $MgSO_4$ x $7H_2O$, 50 ml of 1 M Tris-HCl (pH 7.5), 5 ml of 2% gelatin]. A titre was determined and stored in aliquots over 7% DMSO at −70°C.

2.5. Introducing recombinant molecules into *E. coli* and *Rhodococcus*

2.5.1. Preparation of *E. coli* competent cells

A single colony of the *E. coli* strain was cultured in 5 ml LB and incubated overnight at 37°C on a shaker. The following day, 2 ml of pre-culture was aliquoted into 200 ml SOB medium [2.0% (w/v) tryptone, 0.5% (w/v) yeast

extract, 0.05% (w/v) NaCl, 2.5 mM KCl, 10 mM $MgCl_2$] and the cells were grown at room temperature or 30°C until an OD_{600} reading reached 0.4–0.6. The cells were cooled on ice/water slurry for 5 min and harvested by centrifugation at 4°C, 5 000 rpm for 10 min (Sigma 3K-1, Deisenhofen). The pellet was resuspended in 80 ml ice-cold TB buffer (10 mM HEPES, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$) and left on the slurry for 15 min. The cells were centrifuged once more under the same conditions and resuspended in 20 ml of ice-cold TB buffer containing 7% DMSO. The suspension was cooled for additional 15 min and aliquoted into prechilled Eppendorf tubes. The competent cells were used immediately or frozen in liquid nitrogen and placed at −70°C for long-term storage.

2.5.2. Transformation of *E. coli*

The competent cells stored at −70°C were thawed slowly on ice before use. A small aliquot, 1–5 µl (10 pg – 10 ng) of plasmid DNA was added into 50–100 µl of competent cells and incubated on ice for 15–30 min. Afterwards, the cells were heat-shocked at 42°C between 30–60 s and 900 µl of SOC medium (SOB medium supplemented with glucose, 20 ml filter sterilized 1 M glucose / 1 L SOB) was added. The tube was incubated at 37°C with moderate shaking for 30–60 min. Finally, a desired portion of the transformed cells were streaked on LB plates containing the appropriate selection chemical and incubated till satisfactory growth of bacterial colonies reached.

2.5.3. Conjugation by filter mating

The recombinant plasmid pARS2 was transformed into the mobilizing donor strain *E. coli* S17-1 and selected for on LB medium supplemented with 30 µg/ml kanamycin. Then, a single colony was inoculated into 5 ml of the fresh medium and left overnight at 37°C with agitation. *R. opacus* HL PM-1 was cultured in 5 ml LB and grown on a shaker at 30°C for 24 h. Both cultures were harvested by

centrifugation (4°C, 5 000 rpm for 10 min) and washed twice with salts solution [0.9% (w/v) NaCl, 0.01% (w/v) Triton X-100]. After washing, the strains were resuspended separately in 500 µl of the fresh LB. The donor and recipient cells were further mixed in 3 **:** 1 ratio and 200 µl of the mixture were spotted onto a sterile filter (25 mm in diametre, Sartorius) placed in the middle of LB plate. The plate was incubated at 30°C for 2 days. Thereafter, the conjugation membrane was placed into 2 ml Eppendorf tube and washed with 1 ml of the same salts solution by vortexing. The cells were pelleted and resuspended in 700 µl of the salts solution. Finally, 70 µl of the suspension was taken and plated on the LB medium supplemented with 100 µg/ml kanamycin and 20 µg/ml nalidixic acid. Total of 10 plates from a single membrane were used in conjugation experiments. The plates were incubated at 30°C for 3–5 days and restreaked at least twice till a pure *Rhodococcus* transconjugant was obtained.

2.6. Electrophoresis of nucleic acids

2.6.1. Agarose gel electrophoresis

Agarose gel electrophoresis is the standard method applied to analyze DNA molecules, check the purity, determine the size, and also in recovery of the target DNA from a PCR or restriction digestions. High quality agarose was employed during electrophoresis. For example, SeaKem® LE agarose (BioWhittaker Molecular Applications) was used for general analysis and retrieval, SeaKem® GTG agarose (Biozym) was employed to recapture DNA by freeze–squeeze method, SeaPlaque® GTG® low melting temperature agarose (Cambrex Bio Science) was used to perform in-gel ligations. The DNA samples containing 1/10 volume of loading buffer, were applied to an appropriate agarose gel. Agarose gel concentration used during casting of the gel was dependent on the sizes of DNA fragments to be separated. The agarose was melted by boiling or in a microwave oven in 1x TAE buffer or 0.5x TBE buffer, the gel was then cooled down to 55°C and supplemented with 0.5 µg/ml ethidium bromide. Electrophoretic separation

was carried out in the same buffer used to make the gel at 10 V/cm. The electrophoresis units used were either from the Bio-Rad or Amersham Pharmacia Biotech. Following the separation, the DNA samples were detected using IDA Gel Documentation System based on the FujiFilm DS-7 camera (IDA system, Raytest, Straubenhardt, Germany). The DNA fragments were sized and quantified using Fermentas broad selection of DNA Ladders and Markers.

2.6.2. Pulse field gel electrophoresis (PFGE)

Initial and necessary step for a successful detection of megaplasmids by PFGE was a preparation of whole-cell DNA from *Rhodococcus*. The cells were cultured in 20 ml LB medium for 24 h. The culture was harvested by centrifugation and washed twice with a solution containing 1 M NaCl and 10 mM Tris-HCl (pH 7.6). Afterwards, the pellet was resuspended in 500 µl EC buffer [1 M NaCl, 100 mM EDTA, 6 mM Tris-HCl (pH 7.6), 0.5% (w/v) Brij 58, 0.2% (w/v) deoxycholate, 0.5% (w/v), 0.5% (w/v) N-lauroylsarcosine]. An equal volume of 1% (w/v) InCert Agarose (BMA, Rockland, ME, USA), prepared in EC buffer, was melted at 95°C in a thermomixer at 900 rpm and maintained at 50°C (Comfort, Eppendorf). The agarose was thoroughly mixed with the cell suspension avoiding the formation of air bubbles. Immediately in order to prevent in-tube agarose solidification, 100 µl of the mixture was transferred to a plug moulder (Bio-Rad) allowing plugs to cool down on ice for 15 min. Solidified plugs were then removed from the moulder into 2 ml Eppendorfs containing 1 ml EC lysis buffer (EC buffer complemented with 20 µg/ml RNase and 1 mg/ml lysozyme). The tubes were incubated in the thermomixer at 300 rpm and 37°C until the plugs became transparent indicating complete cell lysis. Subsequently, the plugs were further incubated overnight at 50°C without shaking in ES buffer [1% N-lauroylsarcosine, 0.5 M EDTA (pH 8.0)] containing 1 mg/ml proteinase K. The enzyme was inactivated by incubating the plugs (45 min, 37°C, 400 rpm) in 1 ml of freshly prepared 1 mM solution of PMSF in TE buffer (10**:**1). Furthermore, the plugs were incubated twice under the same conditions in TE buffer only to remove PMSF. Prepared plugs were stored

at 4°C for at least two weeks in the Eppendorf tubes. Finally, 2–3 mm gel slices were cut out from the plugs and placed individually on a comb (Bio-Rad) including the Lambda Ladder PFG Marker (New England BioLabs). The comb was then placed into 14 x 14 cm gel chamber (Bio–Rad). The agarose (Molecular Biology Grade, Eurogentech, Seraing, Belgium) was prepared as 100 ml 1% (w/v) solution in 0.5x TBE, cooled down to 50°C and poured slowly into the gel chamber to prevent removal of the plugs from the comb. After the solidification of the gel the comb was removed leaving the gel plugs embedded in the agarose. The total DNA was separated by electrophoresis in 0.5x TBE buffer employing a contour-clamped homogeneous electric field apparatus (CHEF Mapper, Bio-Rad) at 6 V/cm and linearly increasing pulse times from 7.23–24 s for 26 h. After a completion of the electrophoretic run, the gel was stained with ethidium bromide for 20 min and the image was taken with the IDA Gel Documentation System.

2.7. Screening for clones and a chromosomal mutation

The confirmation of cloned DNA by PCR, further fragmentation and ligation of remaining pieces, and lastly the subsequent transformation were all the steps necessary to generate a mutant *Rhodococcus* strain. A 285 bp deletion mutation in *orfB* was detected and confirmed using the PCR and Southern hybridization.

2.7.1. Polymerase chain reaction (PCR)

PCR was performed with a T Gradient Thermocycler 96 (Biometra GmbH, Göttingen, Germany). Primers were purchased from Eurogentec, Biomers or Inqaba. Reaction mixtures were prepared in a final volume of 25 µl and contained 25 pmol of each primer, 1.5 mM $MgCl_2$, 4% DMSO, 0.2 mM each deoxynucleoside triphosphate, 0.5 to 1.0 U of *Taq* polymerase (Eppendorf) or iTaq™ DNA Polymerase (Bio–Rad), 1x PCR buffer, 10 ng of genomic DNA or 100 ng of plasmid DNA. Reactions performed with *Pwo* DNA polymerase (Peqlab Biotechnologie GmbH, Erlangen, Germany) were set up the same way

but 1.0 U of the enzyme and 2.0 mM Mg_2SO_4 were used. The cycling parametres were as follows: initial denaturation at 95°C for 3 min, followed by 20–30 cycles consisting of denaturation at 95°C for 30 s, annealing at 55–64°C for 30 s, and elongation at 72°C between 2–7 min. After purification from an agarose gel, the PCR products were cloned directly via T/A cloning or ligated into an appropriate cloning site within a vector.

2.7.2. Southern blotting – alkali transfer gel treatment protocol

Southern blotting involved several steps: (a) cutting purified DNA with a restriction endonuclease, (b) separating fragments by gel electrophoresis, (c) transferring (blotting) the DNA from the gel to a positively charged nylon membrane, (d) hybridizing a nucleic acid probe with the target, and (e) visualizing the probe. This technique was used to detect a DNA sequence and locate the fragment in which the homologous sequence resides. Blotting has many generalized features: transfer and linking DNA to the membrane, denaturing the DNA to single strands, neutralization, prehybridization of the membrane, hybridization of a probe to the bound DNA and visualization of the probe.

The DNA samples were separated on a suitable neutral agarose gel. Following electrophoresis, the DNA was visualized with UV light and photographed. Thereafter, the DNA was transferred to the membrane using Bio–Rad vacuum blotter model 785 at 500 – 600 mm Hg for 2-3 hours. Firstly, the gel was depurinated with 0.25 M HCl for approximately 10 min until the bromophenol blue turned yellow and xylene cyanol changed to green. This step was not required for DNA fragments <10 kb in size. The acid solution was then removed and replaced with denaturation solution. Further denaturation of the DNA to single strands was achieved by a NaOH/NaCl solution which separates the double helix utill the dye reverted back to the original colours (~20 min). The gel was subsequently neutralized in a buffered salt solution (~20 min). Afterwards the gel was treated with 20x SSC for 1 h. After blotting the membrane was air dried.

Finally, the DNA was covalently linked to the membrane. This was accomplished by UV cross-linking at 254 nm (Min UVIS, Desaga, Heidelberg) for 3 min, laying the membrane DNA side down. The membrane was washed briefly with 2x SSC and stored dry between Whatman filter paper for several months at room temperature.

2.7.3. DNA dot blotting

After DNA isolation, 50–300 ng of samples were denatured at 100°C for 10 min in 0.4 M NaOH and 10 mM EDTA. The samples were placed on ice and an equal volume of 20x SSC was added to each sample. The DNA was spotted onto the membrane in successive 3 µl aliquots. Each spot was dried before the next one was applied. The membrane was UV cross-linked at 254 nm for 3 min, washed with 2x SSC and dried between Whatman filter paper.

2.7.4. Colony lifting

The agar plates with colonies were cooled down at 4°C for at least 30 min. The membrane disc, placed on the surface on the plate carefully, was marked in three asymmetric locations around the edge by poking through it with a needle into the agar below. Additional marks were made with waterproof ink or pencil for easier positioning of the hybridization disc. The membrane was removed with tweezers after 1 min on the plate and briefly blotted with colonies facing up side on a Whatman 3MM paper. Then, it was soaked with 10% (w/v) SDS stock solution for 1 min and air-dried on Whatman 3MM paper briefly. Following treatment with denaturation solution for 15 min, neutralization solution for 15 min and 2x SSC for 10 min always facing colonies in the upright position, the membrane was air-dried and UV cross linked at 254 nm for 3 min.

2.7.5. Southern hybridization

Southern hybridization was performed using the nonradioactive DIG system for Nucleic Acid Labeling and Detection as per manufacturer recommendations (Roche Diagnostics GmbH, Germany). The process included respective steps:

1) Labeling DNA probe with DIG-11-dUTP

Standard labeling assay was performed after the probed DNA was denatured in a boiling water bath for 10 min at 95°C and chilled quickly in an ice/water bath. The reaction was set up as per following table, incubated for 1 h to overnight at 37°C and stopped by adding 2 µl 0.2 M EDTA (pH 8.0).

Reagent	Volume (µl)
DNA (10 ng – 3 µg)	15
Hexanucleotide mix, 10x conc.	2
DIG DNA labeling mix, 10x conc.	2
Klenow enzyme	1

2) Hybridization

The membrane was prehybridized in hybridization buffer (20 ml/100 cm^2 of filter) at 68°C for at least 1 h. This buffer was then replaced with 10 ml (for 100 cm^2) of hybridization buffer containing 10-50 ng/ml of freshly denatured DIG labeled probe. The hybridization was carried out for 16 h at 60-68°C. Afterwards post-hybridization washes were performed: low stringency wash with 2x SSC, 0.1% (w/v) SDS solution at room temperature twice for 5 min and twice for 15 min at 68°C with 0.1x SSC, 0.1% (w/v) SDS (high stringency wash).

3) Immunological detection

After stringent washing, the membrane was submerged in buffer H1 for 1 min and incubated for 30 min in 100 ml buffer H2. Afterwards, the membrane was incubated with 20 ml buffer H2 containing 4 µl of Anti-Digoxigenin-AP, Fab fragments (1:5000 dilution) positioning DNA side up for further 30 min on Bio-Rad Ultra Rocking Platform at 30 rpm. The unbound antibody conjugate was removed by washing the membrane twice with washing buffer for 15 min. This was followed by equilibration for 2 min with buffer H3. Finally, the immunological detection was started by adding 50 ml buffer H3 containing 150 µl NBT/BCIP stock solution without agitation in a dark box for several hours or left overnight. After detection of desired bands or spots, the reaction was stopped by washing membrane thoroughly with water, membrane was dried and stored.

2.7.6. Stripping Southern blots

When the Southern hybridization needed to be optimized, the same blot was used 2–3 times. The membrane was made wet firstly with distilled water and then stripped. It was incubated in heated dimethylformamide at 50–60°C until the colour was removed. Then, it was rinsed thoroughly with distilled water and washed twice in a solution containing 0.2 M NaOH and 0.1% (w/v) SDS at 37°C for 20 min to remove DIG labeled probe. Finally, the membrane was washed in 2x SSC, air-dried and directly used for Southern hybridization.

2.8. DNA sequencing and sequence analysis

Sequencing was performed commercially by GATC Biotech AG (Konstanz, Germany) using Run24 Supreme (http://www.gatc.de/home_flash.htmusing). Homology searches were performed using BLASTN, BLASTP, and BLASTX. In addition, NCBI ORFfinder was used to detect possible open reading frames. Also ExPASy and FASTA softwares were utilized for the protein predictions. Pairwise

and multiple alignments were carried out by using the search tools BLAST2 (http://www.ncbi.nlm.nih.gov/gorf/bl2.html) and CLUSTALW (http://www.ebi.ac.uk/clustalw/) (Altschul *et al.*, 1990; Tatusov *et al.*, 1997; Tatusova and Madden, 1999). Translations were achieved by employing the Translation Machine (EMBL Outstation European Bioinformatics Institute, http://www2.ebi.ac.uk/). Conserved domain searches were performed with the CD-search tool (http://www.ncbi.nlm.nih.gov). Sequence analysis and editing were conducted with DNA Star (DNASTAR, Inc).

2.9. Analytical methods for proteins characterisation

2.9.1. Preparation of crude extracts

Common methods of cell disruption involved enzymatic cell lysis or shear techniques such as French press or sonication. *E. coli* strains that carry pLysS plasmid were lysed using freezing and thawing. The cells were harvested by centrifugation at 4°C, 6 000 rpm for 20 min, washed and resuspended in 1-2 ml of lysis buffer. After the suspension was frozen at −20°C, the cells were incubated at 30°C water bath for 20 min until cell lysis occurred. The lysate was further treated with DNase II and RNase at room temperature for additional 20 min, followed by sonication 2–3 times for 30 s on ice (Sonicator W-385, Heat Systems Ultrasonics, New York, USA). The cellular debris was removed by centrifugation at 40 000 rpm, 4°C for 30 min (Ultracentrifuge Beckman optima LE-80K, California, USA). The small aliquots were further analysed on 12% SDS-PAGE.

Rhodococcus opacus strains were grown to exponential phase and harvested by centrifugation at 4°C, 6 000 rpm for 20 min. The pellet was frozen in liquid nitrogen and resuspended in 5 ml of 50 mM KH_2PO_4 / K_2HPO_4 buffer pH 7.5. Then the hydrodynamic shear by French press (Amico, Illinois, USA) was applied twice to disrupt cells. The cellular debris was removed by centrifugation as explained above with *E. coli* strains.

The crude extract from *Nocardioides simplex* FJ2-1A for the FPLC protein purification involved the following. Approximately 20 g wet cells were resuspended in 35 ml 50 mM Tris-HCl. The cells were passed twice through the French press. The lysate was then centrifuge using TFT 70.38 rotor (Optima™ LE-80K ultracentrifuge) at 40 000 rpm, 4°C for one hour. The crude extract was further treated with DNase II and RNase at room temperature for additional 20 min. Afterwards it was filtered with Millipore membrane filters 0.45 μm and 0.2 μm. Protein concentration was determined using the Bradford assay and an aliquot was further analysed on a denaturing polyacrylamide gel.

2.9.2. Quantitation of proteins using the Bradford assay

The unknown protein samples were diluted in deionised water 10x or 100x fold. The BSA standard calibration curve was prepared as explained in Appendix. 100 μl of prepared samples or BSA were mixed with 900 μl of the Bradford reagent. After 5 min incubation at room temperature, the absorbances of the samples were measured at 595 nm. 1 cm disposable plastic cuvettes were used for the reaction mixture and Cary WinUV Biopackage software (Varian Carry 50 Bio Spectrophotometre, Victoria, Australia). The protein amounts were then determined using the standard protein curve and subsequently the protein concentrations were calculated (Bradford, 1976).

2.9.3. SDS–PAGE (Laemmli, 1970)

SDS–PAGE is an example of a denaturing electrophoresis technique used to exploit molecular weight / subunits of unknown proteins. The proteins are denatured and reduced by heating them in a sample buffer containing SDS detergent and a thiol reducing agent such as 2–mercaptoethanol (β–ME). A standard curve was generated from a mixture of known proteins of various molecular weights. This was further used to determine the molecular weight of

unknown proteins. Bio-Rad Mini–PROTEAN® 3 Cell electrophoresis system was used for casting and running the gels. The way of casting the mini gels and assembly of the electrophoresis chamber was performed as per instruction manual. After a gel apparatus has been set up, the separating and the stacking gels were made. Gel solutions were prepared per table below. Volumes given here were sufficient for two small gels (8 x 7.3 cm). Prior to pouring the gel APS and TEMED were added. Gels were poured using a Pasteur pipette and a rubber bulb.

	12% separating gel (ml)	**4% stacking gel (ml)**
Distilled H_2O	5.04	6.04
1.5 M Tris-HCl, pH 8.8	3.75	-
0.5 M Tris-HCl, pH 6.8	-	2.5
30% Acrylamide/Bis-acrylamide Solution, 37.5:1 mixture	6.0	1.3
10% (w/v) SDS	0.150	0.100
10% (w/v) APS	0.100	0.050
TEMED	0.010	0.010
Total volume	15.05	10.00

Once the separating gel was placed in the chamber, isopropanol or distilled water was added in the remaining space. It was allowed to polymerize for 45 min to 1 h depending on the freshness of stock solutions of APS and TEMED. Afterwards, the overlay was decanted and the area in between the glass plates was dried with a piece of filter paper. The stacking gel was poured and the comb was inserted between the glass plates. It was allowed to polymerize for 30-45 min. The comb was removed gently and the wells were rinsed carefully with running buffer or distilled water. Preparation of denatured protein samples for loading involved a dilution of sample at least 1 **:** 2 with sample buffer in a total volume of 20 µl, followed by heating at 95°C for 5 min. Afterwards the samples were centrifuged for 30 s at 14 000 rpm and loaded with a Hamilton syringe or a pipette using gel loading tips. Electrophoresis was performed at 100–120 V, room temperature till the tracking dye had reached the bottom of separating gel. 10 µl of low molecular weight protein mixture (Amersham Biosciences) or Precision Plus Protein™

(Bio–Rad) were used as standards. After electrophoresis was completed, the stacking gel was removed and the separating gel was stained with Coomassie Brilliant Blue staining solution for 30 min and destained until the background gel is transparent, 1–2 hours with frequent changes of destaining solution. Some gels were destained overnight and dried on a Whatman 3MM paper sheets using Bio–Rad gel drier.

2.9.4. 2-D Electrophoresis

The crude extract from *N. simplex* was prepared using French press, precipitated and resuspended in the IPG rehydration solution [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 30 mM DTT, 2 % (v/v) Pharmalyte pH 3–10, 0.002 % bromophenol blue]. For 18 cm IPG strip pH 3–10, 340 µl of the rehydration solution including 400 µg of the sample was applied over the dry strip in the reswelling tray. The IPG strip was placed in well of reswelling tray with gel side facing down avoiding air bubbles. In addition, 1 ml mineral oil was used to cover the strip and left overnight for rehydration. The following day, the first-dimension step, isoelectric focusing (IEF) – separation of proteins according to their isoelectric points (pI) was performed. The electrophoresis was employed using a gradient mode as follows:

Phase	Voltage (V)	Current (mA)	Duration (h:min)
1	500	10	0:01
2	3500	30	1:30
3	3500	30	6:20

Then, the second-dimension step (SDS-PAGE) was conducted to separate proteins according to their molecular weights. Firstly, the IPG strip was equilibrated in 10 ml SDS-equilibration buffer [4% (w/v) SDS, 50 mM Tris–HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 0,002% bromophenol blue] containing 100 mg DTT in a screw cap tube for 15 min on a rocker. Thereafter, the strip was placed on top of

12% polyacrylamide gel cross-linked with bisacrylamide and was fixed with 0.5% (w/v) agarose. It was important to avoid air bubbles between the stripe and the agarose. The electrophoresis was run overnight at 4°C, 18 mA for large gels. Finally, the gels were stained with colloidal Coomassie solution [40 g ammonium sulphate in 400 ml 2% (v/v) phosphoric acid, 8 ml Coomassie G-250 (5% in water), 100 ml methanol] overnight and destained with water.

2.9.5. Expression and purification of His-tag fusion protein

OrfB was amplified from *R. opacus* HL PM-1 genomic DNA with specific primers. The resulting PCR product was ligated into the pET vectors and expressed in *E. coli* Rosetta 2(DE3)pLysS. His-tag fusion proteins were purified by Ni-NTA metal affinity chromatography (QIAGEN) as instructed by the supplier. Purified fractions were desalted by utilizing pD10 Desalting Columns (Amersham Pharmacia Biotech) and thereafter concentrated with a Vivaspin 2 ml concentrator (Vivascience AG, Hanover, Germany).

2.9.6. Protein precipitation

Precipitation of protein was a step used in preparation of sample for 2-D electrophoresis. It was employed to prepare a concentrated sample from a crude extract after French Press. Acetone was used during the precipitation. The procedure started by addition of 3 volumes of ice-cold acetone to the extract. Proteins were allowed to precipitate at −20°C for 4 h and were pelleted by centrifugation. Residual acetone was removed by air drying of the sample.

3.0. High performance liquid chromatography (HPLC)

Metabolites were investigated at 210, 340, 390 or 420 nm by HPLC analysis. The entire chromatographic system was composed of Dionex P 580 pump, Dionex DG 1210 degas system, Dionex UV / Vis UVD 170S/340S detector, Dionex Gina50

autosampler and Chromeleon Chromatography Data System 4.3 software. Cells at exponential phase (resting-cell experiments) were resuspended in 50 mM KH_2PO_4–K_2HPO_4 buffer (pH 7.5) to obtain an OD_{595} of 2.0. PA or 2.4-DNP was added to a final concentration of 0.5 or 0.7 mM. Cells were grown at 30°C and culture supernatants were analysed by HPLC at regular time intervals. The samples were resolved on Gromsil 100 Octyl-4 columns (125 x 4 mm or 250 x 4, particle size 5 μm; Grom) using 30 or 40% (v/v) methanol and 5 mM PicA, pH 7.8 as mobile phase. The pre-column was a Gromsil 100 Octyl-4 column (20 x 4 mm, particle size 5 μm; Grom). Enzyme reactions were terminated by freezing the samples in liquid nitrogen prior to HPLC analysis.

4.0. Fast performance liquid chromatography (FPLC)

The nitrite-eliminating enzyme (NEE) enrichment, needed to obtain an internal peptide sequences, was achieved by FPLC (LCC 500 controller, 500 pump, UV-1 monitor, REC–482 recorder and FRAC autosampler; Pharmacia, Uppsala, Sweden). The cell extract from *N. simplex* was passed through a Q Sepharose column (HP HR 16/10; Pharmacia) pre-equilibrated with basic buffer [50 mM Tris-HCl (pH 7.5)] at a flow rate of 1 ml/min. The activity was eluted from the column with a linear gradient of 0 to 1 M NaCl (200 ml) in basic buffer at 0.25 M NaCl. Ammonium sulphate (1 M) was added to the active fractions and applied to a Phenyl Superose column (HR 10/10; Pharmacia) pre-equilibrated with the same buffer. The enzyme was eluted with a linear gradient (65 ml) of 1 to 0 M ammonium sulphate in basic buffer at 0.41 M $(NH_4)_2SO_4$ and a flow rate of 0.5 ml/min. The activity of the FPLC fractions was assayed by repeated recordings of UV-visible spectra between 280 nm and 600 nm in 1 min cycles. The test was conducted with 50 mM Tris-HCl (pH 8.0) containing 0.1 mM $2H^-$-TNP and 6 μg of the NEE. Phenyl Superose active fractions were run on SDS-PAGE, NEE band was excised and sent for sequencing to TopLab.

5.0. Chemicals used in this work

The chemicals used in this work were of the highest available purity and were purchased from Fluka (Neu-Ulm Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Sigma-Aldrich (Taufkirchen, Germany). The complex media components were supplied by Oxoid (Wesel, Germany), Difco (Augsburg, Germany) and Roth (Karlsruhe, Germany).

3. Results

3.1. Investigation of the *orfB* gene function

The first aim in this work was to investigate the function of *orfB* contained in the *npd* gene cluster. This was achieved by cloning and heterologously expressing the gene in *Escherichia coli*, and followed by the purification and characterisation of the protein. Also a mutant strain, carrying a deletion in *orfB* region was engineered and a comparison between the wild type and mutant strains was made.

3.1.1. Heterologous overexpression of *orfB* in *E. coli*

The gene encoding OrfB protein was cloned into pET vectors [pET-11a*, pET-22b(+), pET-28a(+)] using *Nde*I and *Xho*I restriction sites. The resulting recombinant plasmids were designated pARS3, pARS4 and pARS5 respectively and verified by a double digestion (Figure 4) and DNA sequencing. In cultures of *E. coli* Rosetta 2(DE3)pLysS (pARS3 / pARS4 / pARS5), induced with 1 mM IPTG, heterologous gene expression was evident (Figure 5). After an SDS-PAGE gel comparison, more than half of the protein resided in the cells as inclusion bodies (Figures 5 and 6). In non-induced cultures, no protein band of the expected size was detected. OrfB-His, with the His tag at the either end (pARS4 and pARS5), was purified from cellular extracts of Rosetta 2(DE3)pLysS. Furthermore, SDS-PAGE analysis showed the size of the purified fusion protein, which was calculated to be 42.5 kDa. Figure 5 also shows one additional protein band slightly smaller than the 25 kDa molecular weight marker, which was induced with 1 mM IPTG. It could be a degradation product or some other artifact.

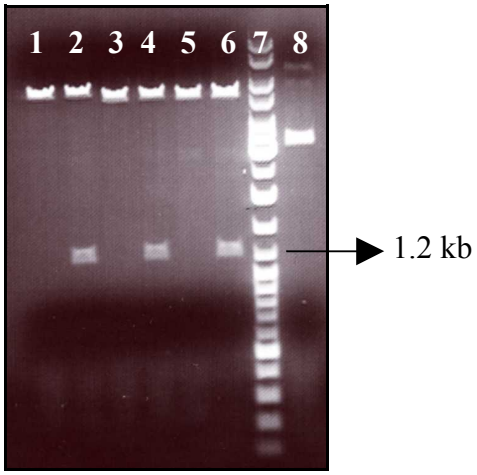


Figure 4. 0.8% agarose gel electrophoresis (1x TAE buffer, 100 V) results showing *Nde*I and *Xho*I digestions of vectors and their respective recombinant plasmids. Lane 2: pARS3, lane 4: pARS4, lane 6: pARS5, lane 7: GeneRuler™ DNA Ladder Mix, lane 8: uncut control.

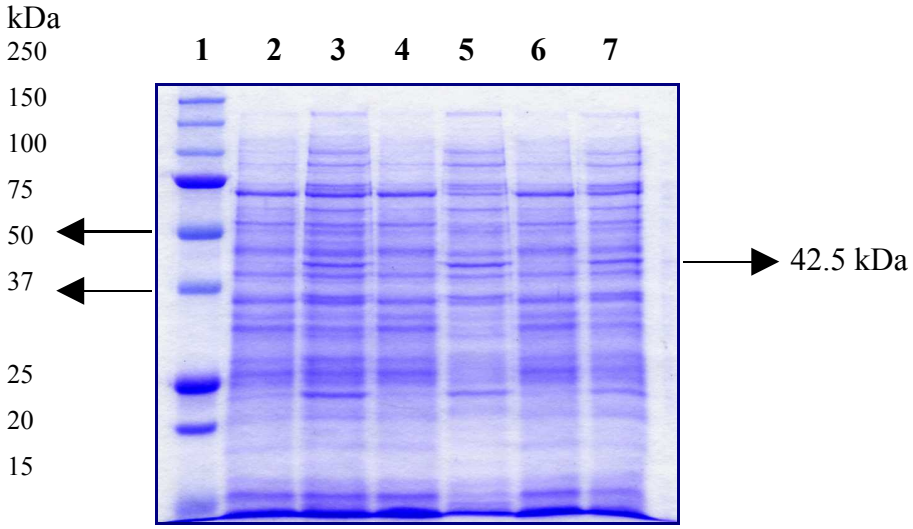


Figure 5. 12% SDS-PAGE (1x SDS running buffer, 100 V) results showing heterologously expressed product of *orfB*. Lanes 2, 4 and 6: cell lysates from uninduced *E. coli* cultures; 3, 5 and 7 cell lysate from induced cultures using 1 mM IPTG. Precision Plus Protein™ standards (lane 1).

3.1.2. Characterisation of OrfB protein

After analysis of *orfB* (1203 bp, accession no. AAK38096) using bioinformatics, BLAST and ExPASy tools, it was found that the OrfB protein, composed of 400 amino acids, showed a 52% sequence similarity and 34% identity to L-carnitine dehydratase (*Archaeoglobus fulgidus*). Theoretical pI / Mw predictions for the protein sequence were 5.47 / 42536.23 Da. This was consistent with calculated size from SDS-PAGE taking fusion of the vector-encoded His-tag into account (approximately 42.5 kDa). The protein was purified by Ni-NTA metal affinity chromatography using 250 mM imidazole during the elution step.

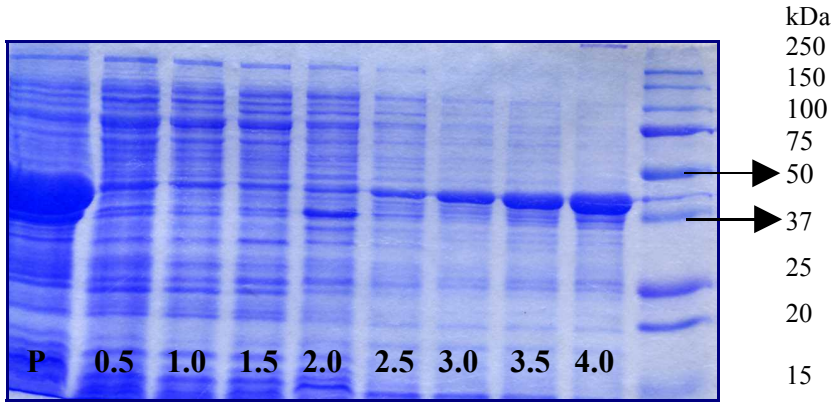


Figure 6. 12% SDS-PAGE (1x SDS running buffer, 100 V) presenting solubilized *orfB* proteins from inclusion bodies using urea. Lane 1: P = pellet sample; lanes 2-9: 0.5 – 4.0 M urea concentrations; lane 10: Precision Plus Protein™ standards.

It was suggested by Russ *et al.* (2000) that the protein encoded by *orfB* may be involved in the enzymatic hydrolysis of 2,4-dinitrocyclohexanone if the non-enzymatic reaction is too slow under physiological conditions. Chemical synthesis of the 2,4-DNCH and enzyme-substrate studies can be intended for the further research.

3.1.3. *orfB* gene deletion

To delete *orfB* from *Rhodococcus opacus* HL PM-1, the recombinant plasmid

pARS2, carrying the *Eco*RI – *Bam*HI fragment with deleted *orfB*, was constructed (Figure 7). Two sets of primers were used during the PCR amplification of the homologous region. The first set used carried *Eco*RI and *Kpn*I restriction sites (underlined) was 5'-CGGAATTCTGGCGCGTGCGTCTTCGGAC-3' and 5'-CCGGTACCGCCGTACCCGCTGACATTGAGG-3'. The second set of primers was: 5'-CAGGTACCGGTGGGCAACGGCAGCAATTTC-3' and 5'-GGGGATCCTTGGTCGCCGATGACGAGGGG-3' (*Kpn*I and *Bam*HI sites are underlined). The PCR products were ligated, creating a 2640 bp fragment with a deletion in *orfB*. *E. coli* S17.1 was transformed with the recombinant DNA and then pARS2 was conjugated into *R. opacus*. PCR (Figure 8) and Southern hybridization (Figure 9) of sucrose-resistant colonies showed the gene replacement via homologous recombination event. Also, both expected types of single crossover events were illustrated (Figure 10). The gene deletion mutant was named *R. opacus* AR1.

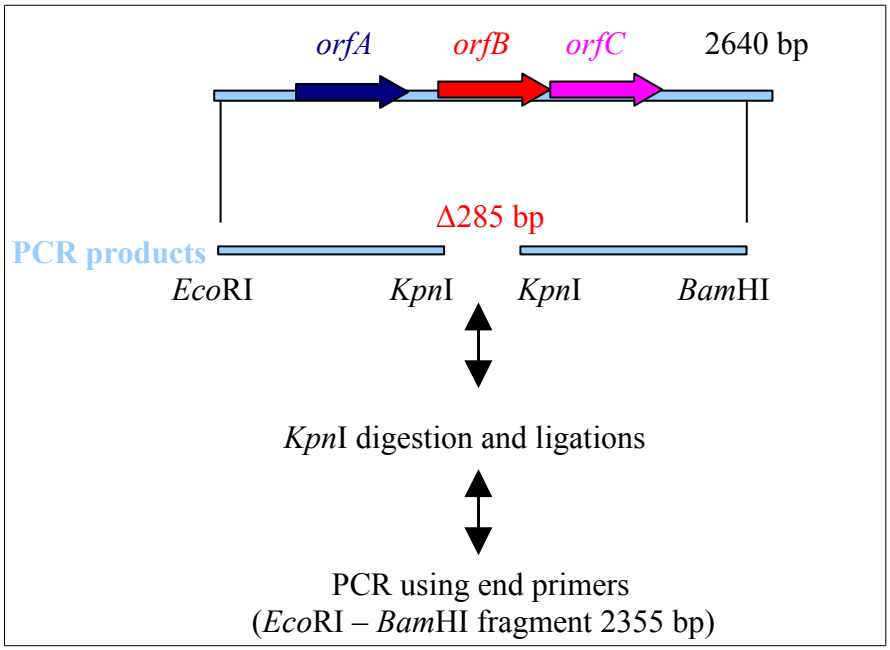


Figure 7. Diagrammatic representation of DNA manipulations of the homologous region used to construct the recombinant plasmid pARS2.

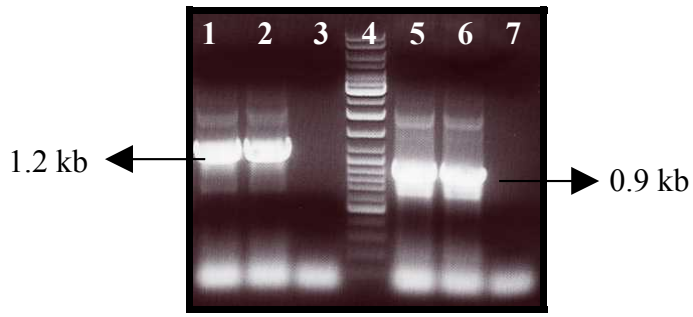


Figure 8. PCR analysis of *R. opacus* HL PM-1 and AR1 total DNA. Lanes 1&2: *orfB* amplified from HL PM-1 strain, lanes 5&6: deleted *orfB* from AR1 strain, lanes 3&7: primers only controls, lane 4: GeneRuler™ DNA Ladder Mix.

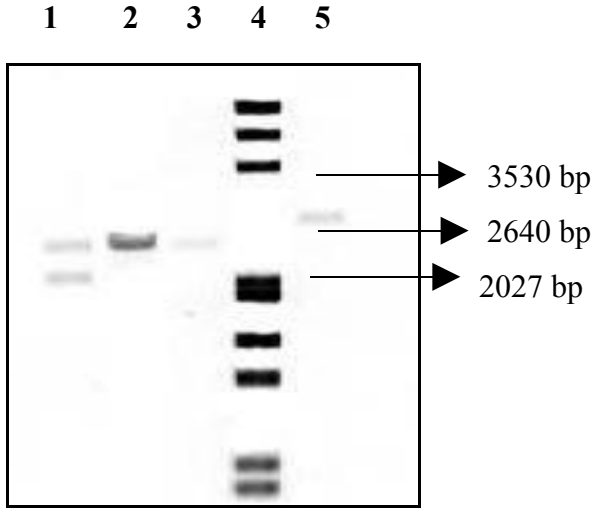


Figure 9. Southern hybridization analysis of *R. opacus* AR1 and HL PM-1 total DNA digested with *Eco*RI and *Bam*HI. Lane 1: mutant type 2 (single crossover), lanes 2&3: mutant (double crossover), lane 4: DNA molecular weight marker III (DIG-labeled), lane 5: wild type *orfB*.

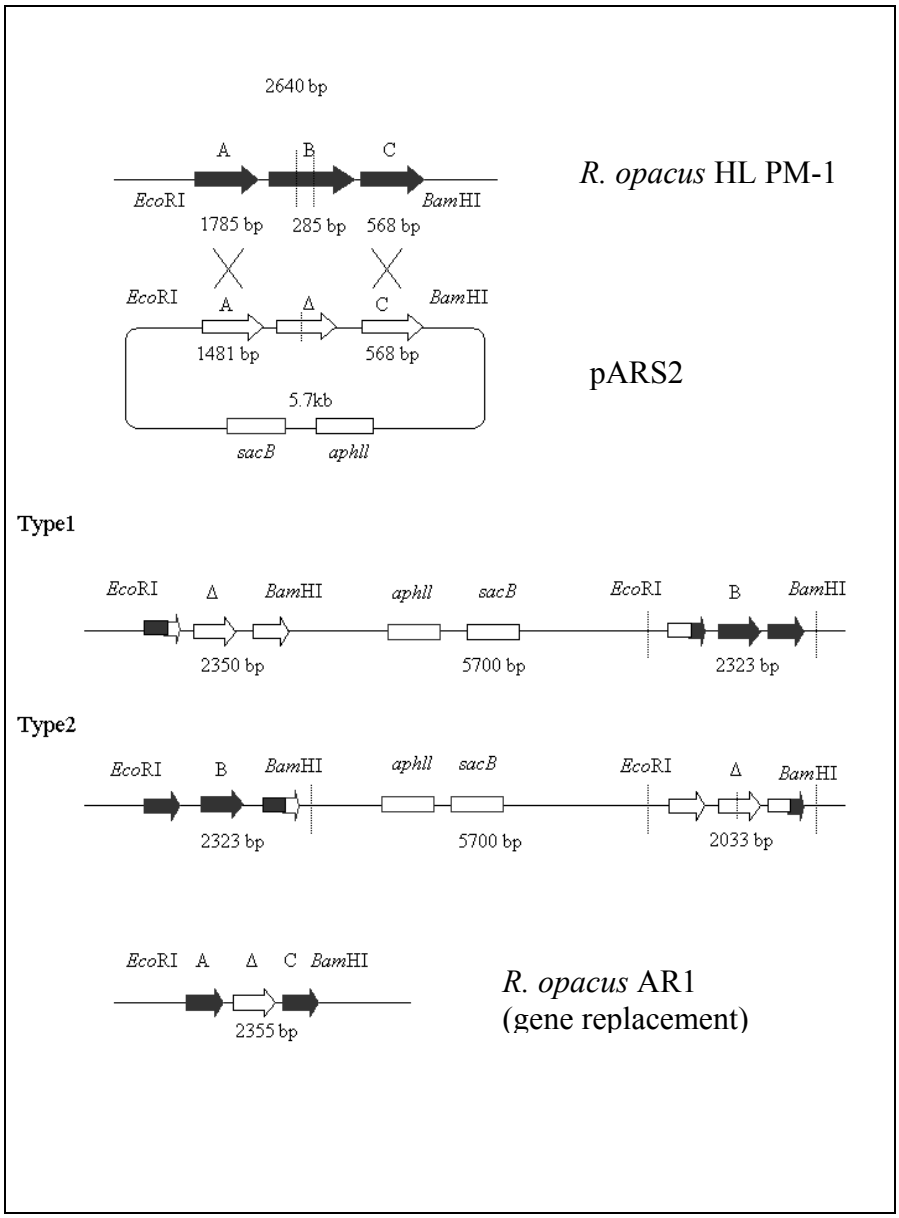


Figure 10. Schematic representation of the expected homologous recombination events used in gene deletion experiments. *aphII* – aminoglycoside phosphotransferase II gene, conferring kanamycin resistance.

After conjugation experiments, sucrose sensitivity was tested on LB agar containing 10% (wt/vol) sucrose. This resulted in clones that were wild-type, after a single cross-over event (*sacB* inactivated by random integration) and double cross-over (Δ*orfB*). Further selection on plates supplemented with kanamycin

resulted in clones that were only wild-type and with deleted *orfB*. Very low frequency (33.3%) of recombinants was detected (data not shown). Most probably the conjugation rate between *E. coli* and *Rhodococcus* was low.

3.1.4. Effect of *orfB* gene deletion on *R. opacus* growth

In the *npd* gene cluster *orfB* is localized next to the first intergenic region, which contains a promoter (Figure 2). To confirm that a mutation in the *orfB* did not affect expression of the downstream *npd* genes, the following growth experiments were conducted. In addition, both polynitrophenols, DNP and TNP, were tested for their ability to induce the expression of the *npd* genes. Figure 11 shows the growth curves and the expected extremely long lag phase, 48 h for *R. opacus* HL PM-1 and 96 hours for the mutant AR1. The two curves indicate that the strain AR1 required a longer adjusting period to the picric acid environment. Consequently, its cellular metabolism, the rapid biosynthesis of cellular macromolecules, in preparation for the next phase of the cycle was prolonged.

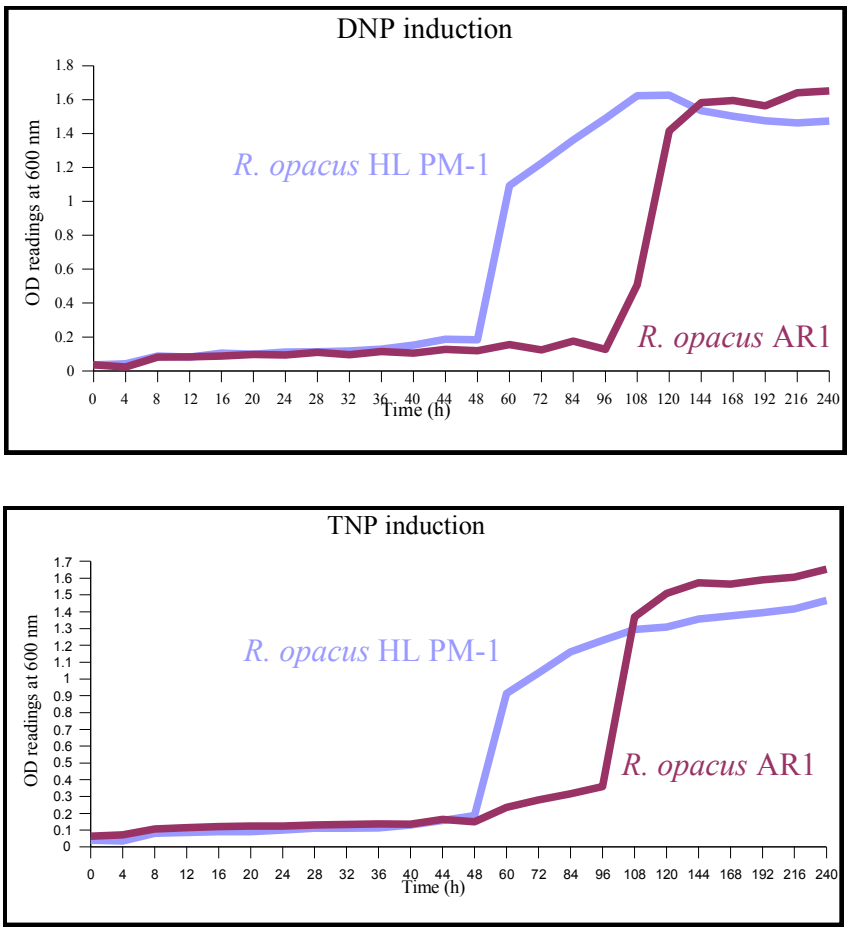


Figure 11. Growth curves of *R. opacus* strains using different inducing agents. The inducing agent was added at t=0. Growth was achieved with 0.5 mM picric acid as a sole nitrogen source. Increase in cell density was determined photometrically at 600 nm.

3.1.5. Picric acid conversion by resting cells of *R. opacus*

Two independent experiments of picric acid conversion by resting cells of *R. opacus* were conducted and the average values were taken. The rhodococcal cultures were grown in minimal media and exposed to 0.5 mM DNP for 3 hours. Then, the cells were harvested and resuspended in phosphate buffer containing 0.5 mM 2,4,6-TNP. The samples were collected at regular time intervals and analysed by HPLC. Detection was done based on the upper intermediates from the pathway. The results indicate the entire utilization of the picric acid molecule by

both strains. The TNP was converted to its hydride Meisenheimer complex in 2.5 h by the HL PM-1 strain, whereas the mutant AR1 took 30 min longer for the conversion (Figure 13). Furthermore, the highest concentration of the Meisenheimer complex was reached after 2 hours with HL PM-1 and 2.7 hours with AR1. The transient accumulation of the Meisenheimer was noticed with the mutant strain also. As the graph indicates the mutant requires two hours longer to convert the complex into the next intermediate during the biodegradation of picric acid.

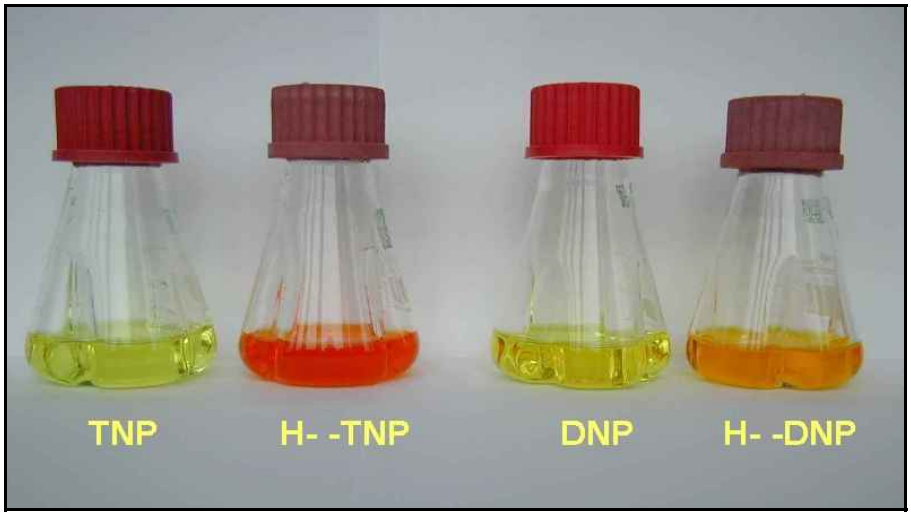
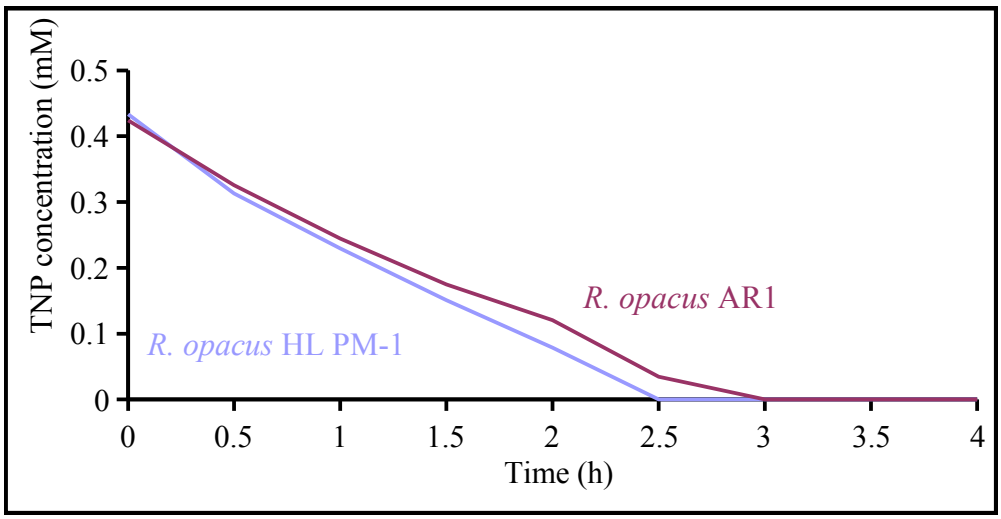
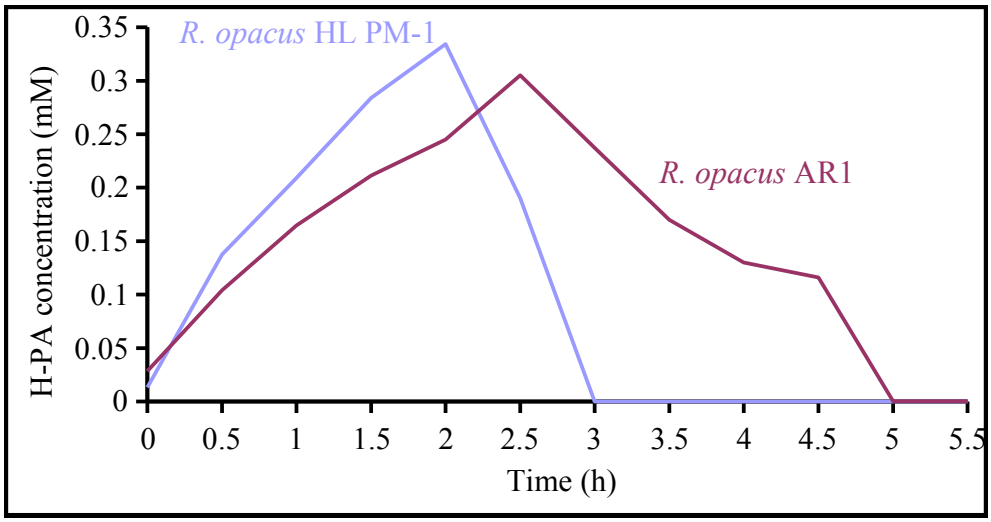


Figure 12. Metabolites released into the environment by *R. opacus* during picric acid biodegradation. TNP = 2,4,6-Trinitrophenol (picric acid), H^--TNP = Hydride Meisenheimer complex of TNP, DNP = 2,4-Dinitrophenol, H^--DNP = Hydride Meisenheimer complex of DNP.

A.





B.

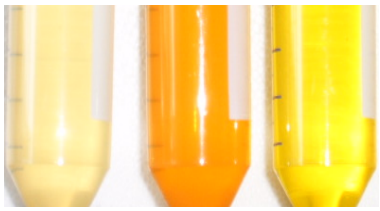


Figure 13. A. Conversion of TNP and hydride Meisenheimer complex of TNP (H-PA) by resting cells of *R. opacus* analysed using the HPLC. **B.** Transient accumulation of the hydride Meisenheimer complex by the mutant strain.

3.1.6. Growth of *R. opacus* under C- and N- limited conditions

To further investigate the growth yield between the two strains of *R. opacus*, the biomass (dry weight) of the cultures was determined. The cultures were grown in different flasks containing mineral medium and increasing concentrations of carbon source while keeping the nitrogen source constant and vice versa. The molar mass of picric acid is 229.11 g and this was used to follow the stoichiometry of reactions in solution. The dry weight of cells of a culture was measured directly and the numbers are the average of duplicated experiments. The cells were harvested by centrifugation, washed twice with distilled water to remove adhering components of the medium, allowed to dry at 105°C and weighed on a scale. There appears to be a difference between *R opacus* HL PM-1 and the AR1 mutant in the degree of utilization of picric acid as N-source. The yield of strain HL PM-1 at 23 mg picric acid and 820 mg sodium acetate is 81 mg (Table 8). The respective data in Table 9 gives a yield of 92 mg of biomass and there was no significant biomass difference 4.6 and 9.2 mg picric acid were used. In addition, *R. opacus* AR1 showed no significant increase in biomass when 4.6 – 23 mg were utilized. The difference is less with the AR1 strain, 67 mg and 69 mg.

Table 8. Biomass of *R. opacus* if N-source kept constant (TNP at 23 mg).

Na-acetate (mg)	Biomass (mg)	
	HLPM-1	AR1
0	63	37
164	83	51
328	83	75
820	81	67

Table 9. Biomass of *R. opacus* if C-source kept constant (820 mg Na-acetate).

PA (mg)	Biomass (mg)	
	HLPM-1	AR1
0	58	43
4.6	59	66
9.2	61	64
23	92	69

Table 10 below shows the biomass of control *Rhodococcus* cultures. Initial inoculum was measured after the cells were grown overnight in 20 ml mineral medium. Furthermore, the growth was monitored with and witout picric acid as a sole nitrogen source.

Table 10. Biomass of *R. opacus* control cultures.

Controls	Biomass (mg)	
	HLPM-1	AR1
0 mM PA 10 mM Na-acetate (164 mg)	7	7
0.5 mM PA 10 mM Na-acetate (164mg)	49	51
Initial inocula (20 ml pre-cultures)	70	52

3.2. Cloning of nitrite-eliminating enzyme

The second aim in this work was to identify a gene(s) which encode the nitrite-eliminating enzyme (NEE) activity. This was achieved by enriching the activity using the fast performance liquid chromatography (FPLC). Then, a set of internal peptide sequences was obtained (L P G D Y T D Q L L R and S G A I V G L G H A Q V D R) and used to design the degenerate primers in order to pool a clone from nocardioform genome, which may carry the gene for the NEE. Two different genomic libraries were constructed and screened using *R. opacus* HL PM-1 and *N. simplex* FJ2-1A since the activity in crude extracts was reported with both strains. Also, the amino terminus sequence of the NEE was known.N-terminal amino acid sequencing of the purified enzyme from *N. simplex* FJ2-1A revealed the sequence M K N L E L A Y V G L (G) V H E X L V Y Y A A (Q/T) (H) L D (L) Y R, showing uncertain amino acids in parentheses. Only M K N L E L A Y V G L (G) V H E X L V Y Y A A fragment was used to select the codons with highest frequency within the *R. opacus* genome. Since the N-terminus sequence from the NEE and the genomic library were from two different strains, this could be the reason for no detection of positive clones was possible. The hybridization probe, DIG – labeled, was more suitable for screening of *N. simplex* genomic

library.

3.2.1. *Rhodococcus opacus* HL PM-1 cosmid library

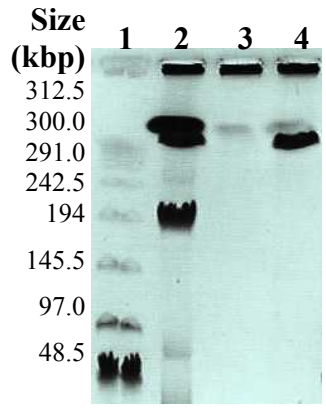


Figure 14. A gel of total DNA from different *Rhodococcus* strains showing linear megaplasmids. Lane 1: Lambda Ladder PFG marker, lane 2: *R. opacus* HL PM-1, lane 3: *R. rhodochrous* Ri8-R, lane 4: *R. erythropolis* SQ1.

The complex genome of *R. opacus* is shown in the above figure. Using a calibration curve, the sizes of the megaplasmids were estimated to be 190 kbp, 300 kbp and 312.5 kbp. Cloning included isolation, fragmentation and ligations of the genomic DNA with a cosmid vector. The number of clones required to ensure a 99% probability of a given DNA sequence of *R. opacus* being contained within the cosmid library composed of 40 kb inserts was calculated to be 461. This library was screened using a 66 bp probe derived from the amino acid terminus of the NEE but no positive clones were detected. Primers were designed using *R. opacus* codon usage table and tested on the HL PM-1 genomic DNA (Figure 16 below). The primer sequences were 5'-atg aag aac ctg gag ctg gcc tac gtc ggc ctg-3' (forward) and 5'-ggc ggc gta gta gac cag ggc ctc gtg gac gcc-3' (reverse).

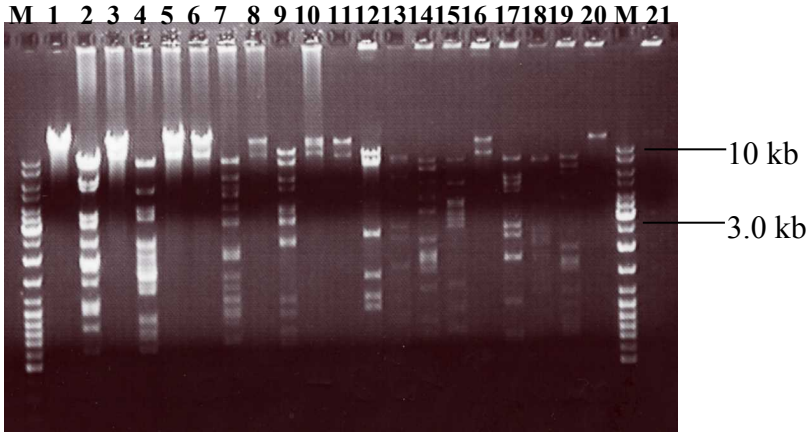


Figure 15. *Bam*HI digestions of recombinant cosmids from the *R. opacus* library electrophoresed on 0.8% agarose gel, 100 V. M = GeneRuler™ DNA Ladder Mix. The control cosmid in lane 21, lanes 1-20: different recombinants.

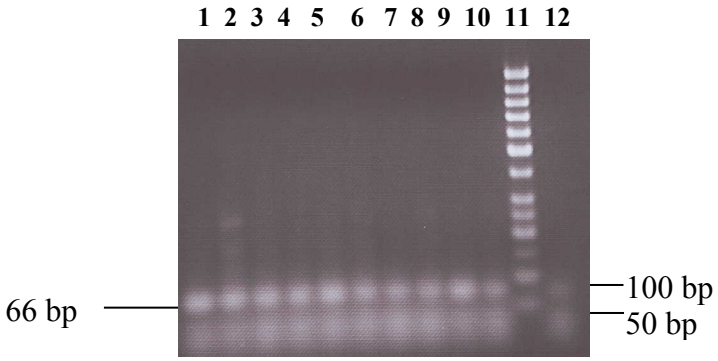


Figure 16. 3% agarose gel in 1x TAE, 60 V, showing the probe used for screening of the cosmid library. GeneRuler™ 50bp DNA Ladder (lane 11), no template control (lane 12). Lanes 1-10: PCR results using the HL PM-1 genomic DNA as the template.

3.2.2. FPLC enrichment of the NEE and peptide sequencing

The nitrite-eliminating enzyme was purified from *N. simplex* FJ2-1A by Hofmann *et al.* (2004). In this work, I have performed an FPLC enrichment using a Q Sepharose column (a strong anion exchanger, pH 2 – 12) and a Phenyl Superose column (hydrophobic separation). This was done in order to prepare the NEE

sample for internal sequencing of a protein. Collected fractions were tested for their activity by measuring the increase in absorbance of H^--TNP at 450 nm or repeated recording of UV-visible spectra between 280 and 600 nm in 1 min cycles and separated by SDS-PAGE.

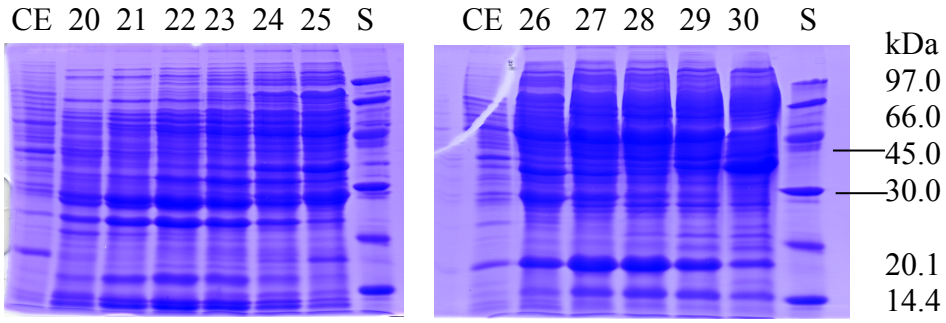


Figure 17. 12% SDS-PAGE electrophoresis showing active fractions (21-28) after Q Sepharose. CE = *N. simplex* crude extracts, S = Low Molecular Weight Calibration kit.

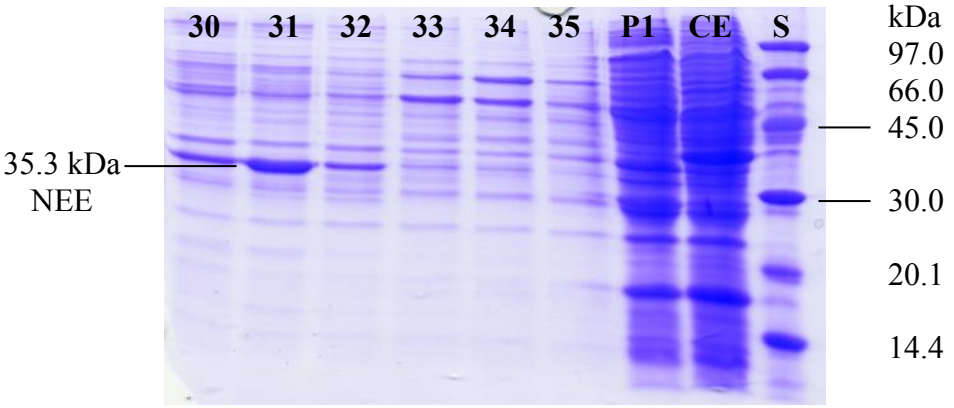


Figure 18. 12% SDS-PAGE electrophoresis showing active fractions (30-32) resulting from the hydrophobic separation. P1 = Q Sepharose pool of active fractions, CE = *N. simplex* crude extracts, S = Low Molecular Weight Calibration kit.

Once a prominent band of the NEE has been detected, the protein was excised from the gel (Figure 18) and 2 peptide sequences were achieved. Firstly, the SDS-PAGE band of the NEE sample was reduced by dithiothreitol,

carboxamidomethylated, and digested with trypsin. After in-gel digest, the resulting peptides were separated on a 320 µm x 150 mm capillary HPLC column (Figure 19). A total of 42 fractions were collected. Fraction 19 gave the sequence S G A I V G L G H A Q V D R and fraction 25 gave the sequence L P G D Y T D Q L L R.

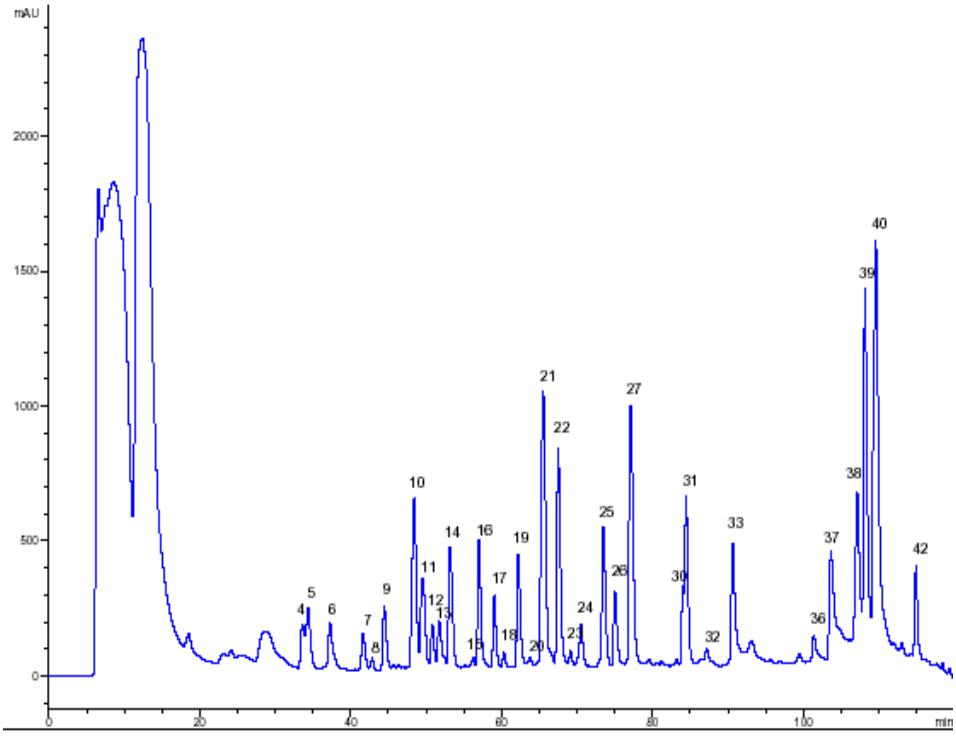


Figure 19. Nitrite-eliminating enzyme trypsin digest separated by HPLC.

3.2.3. Molecular screening using degenerate PCR technique

The amino acid sequences from the N-terminus (M K N L E L A Y V G L) and the fraction 19 (S G A I V G L G H A Q V D R) allowed the design of PCR primers using a codon usage table for the genus *Nocardioides*. No evolutionary conserved regions in either sequence and no similarity to any known protein sequences were detected using GenBank and National Center for Biotechnology Information. The forward primer was design using the highest frequencies from

the codon table and the reverse primer was design using a degenerate alphabet. The forward primer sequence was 5'-atg aag aac ctg gag ctg gcc tac gtc ggc ctg-3' and the reverse was 5'-**wss** ccv cgs tag cas ccv gas ccv gtr cgs gtc cas ctr gcs-3' (**w** = A, T; **s** = C, G; **v** = A, C, G; **r** = A, G). After optimization of PCR conditions, this set of primers resulted in a product that was approximately 350 bp in length. It was necessary to perform 2 steps PCR due to different annealing temperatures of the primers. The PCR product was purified, cloned and only one out of four clones that has been sequenced carried the respective amino acid sequence (in Figure 21 clones 1, 5, 6, 10).

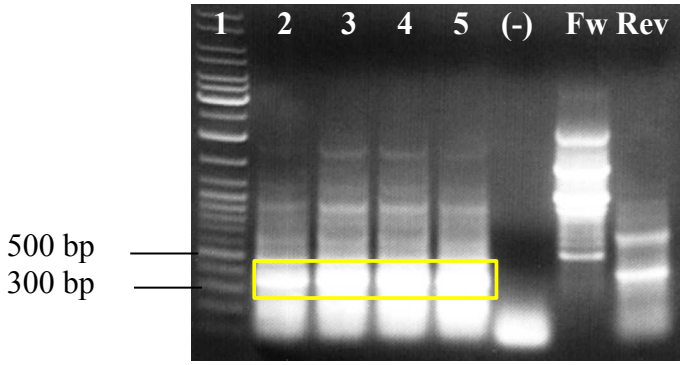


Figure 20. 0.8% agarose gel in 1x TAE, 100 V showing degenerate PCR result (lanes 2-5). (-) no template control, Fw = forward primer control, Rev = reverse primer control. Lane 1: GeneRuler™ DNA Ladder Mix.

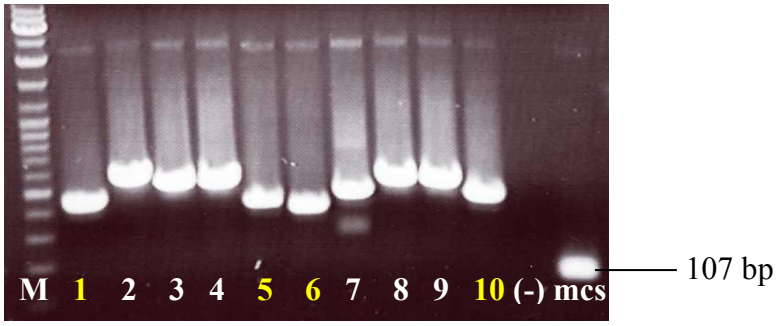


Figure 21. 0.8% agarose gel in 1x TAE, 100 V showing standard PCR results using T3 and T7 sequencing primers. M = GeneRuler™ DNA Ladder Mix, 1 – 10: clones in pBluescript II SK (+), (-) no template control, mcs = amplified multiple cloning site of the vector.

Table 11. Partial DNA and amino acid sequences of the putative nitrite-eliminating enzyme.

Clone #1 (NEE)
252 nucleotides ATG AAG AAC CTG GAG CTG GCC TAC GTC GGC CTG GTC GCG ATC CTG CCG GTC GTG GAG TGG GTC GTC CAC GTG TTC ATC CTG CAC TGG AAG CCG CGC CGC CTC GGC CCG CTC ACC CTC GAC CCG CTG CTG GCC CGC AAG CAC CGC GCC CAC CAC GCC GAC CCG CGC GCG ATC CCG CTC GTC TTC ATC CCC TGG CAG GCC CAG CTG TGG CTC GCG CCA GGT GGA CGC GTA CCG GCT CGG GGT GCT ACC GTG GGG
84 amino acids **Met K N L E L A Y V G L V** A I L P V V E W V V H V F I L H W K P R R L G P L T L D P L L A R K H R A H H A D P R A I P L V F I P W Q A Q L W L A P G G R V P A R G A T V G

Partial gene sequence encoding the putative nitrite-eliminating enzyme was further analysed. Homology searches were performed with BLASTN, BLASTP and BLASTX. The similarity with fatty acid hydroxylase (246 amino acid residues) from *Nocardioides* sp. JS614 was detected.

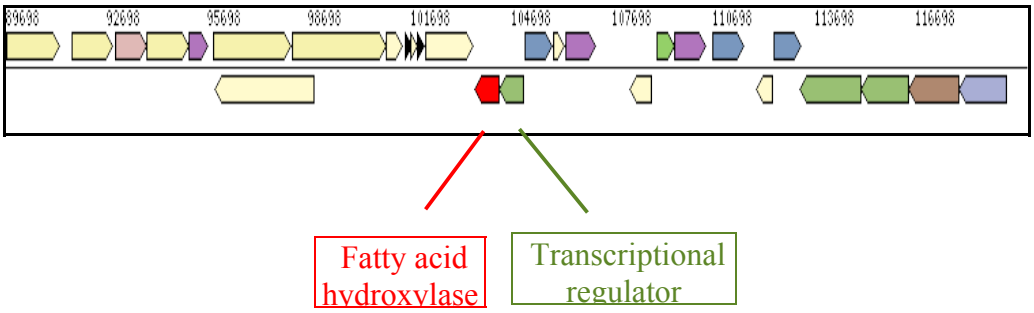


Figure 22. Chromosomal view of the *Nocardioides* sp. JS614 indicating a location of the putative NEE gene.

MTKSQSPIASGEVERLAAQRVAADEQRITGSRRTNVSLGQAWRSFWRH
PSPWMMASCIVASLLARVLVGGGAWWELLVPVGLVATLPVVEWVVH
VGILHWRPRHVGPVTLDPLLARKHRAHHADPRAIPLVFIPWQVELWLFP
SYVAIAWLAMPTTSSALTLLVAVYAIMSGYEWTHYLLHSDYRPRSRWY
RSVWRNHRLHHYKSEHYWFTVTTAGTADRLFGTYPDPAAVETSPTVR
ALHSRERL

Figure 23. The entire fatty acid hydroxylase peptide sequence showing the middle region showing sequence similarity to the putative nitrite-eliminating enzyme.

Table 12. Homologous search using BLAST. NEE = putative nitrite-eliminating enzyme in pARS6. FAH = fatty acid hydroxylase.

```
 gi|71365816|ref|ZP_00656366.1| Fatty acid hydroxylase [Nocardioides sp.
JS614]
 gi|71158521|gb|EAO08898.1| Fatty acid hydroxylase (FAH) [Nocardioides sp.
JS614]

 Length=246
 Score = 124 bits (311), Expect = 1e-27
 Identities = 56/67 (83%), Positives = 60/67 (89%), Gaps = 0/67 (0%)

NEE LAYVGLVAILPVVEWVVHVFILHWKPRRLGPLTLDPLLARKHRAHHADPRAIPLVFIPWQAQLWLAP
FAH L VGLVA LPVVEWVVHV ILHW+PR +GP+TLDPLLARKHRAHHADPRAIPLVFIPWQ +LWL P
FAH LVPVGLVATLPVVEWVVHVGILHWRPRHVGPVTLDPLLARKHRAHHADPRAIPLVFIPWQVELWLFP



 gi|91765513|ref|ZP_01267470.1| Fatty acid hydroxylase [Mycobacterium sp.
MCS]
 gi|91707592|gb|EAS83304.1| Fatty acid hydroxylase [Mycobacterium sp. MCS]

 Length=224
 Score = 105 bits (263), Expect = 4e-22
 Identities = 44/67 (65%), Positives = 53/67 (79%), Gaps = 0/67 (0%)

NEE LAYVGLVAILPVVEWVVHVFILHWKPRRLGPLTLDPLLARKHRAHHADPRAIPLVFIPWQAQLWLAP
FAH L V +VA+ P VEWVVHV ILHW+PRR+G L +DPLLARKHR HH DPR + L+FIPWQA +W+ P
FAH LVPVAMVAVFPFVEWVVHVCILHWRPRRIGRLRVDPLLARKHREHHVDPRVVSLIFIPWQALMWVLP
```

3.2.4. *Nocardioides simplex* FJ2-1A phage library

The genomic library was made with *N. simplex* total DNA using λBlueSTAR™ Vector System (Novagen®). *N. simplex* genomic DNA was partially digested with *Bam*HI and ligated to λBlueSTAR arms. Furthermore, the ligations were packaged using PhageMaker® extracts and plated on the *E. coli* ER1647. Blue/clear screening was achieved by the presence of the complete *E. coli lacZ* gene and control region within the 13 kb stuffer fragment. Non-recombinants were identified as blue plaques on plates containing X-gal, whereas phage containing genomic DNA inserts produced clear plaques (Figure 24). Plasmid sub-clones were obtained by plating λBlueSTAR phage on the *E. coli* BM25.8 strain expressing Cre Recombinase in the presence of ampicillin (50 µg/ml). The colonies obtained carried the genomic insert in a plasmid excised from the lambda vector (Figure 25). The number of clones required to ensure a 99% probability of a given DNA sequence of *N. simplex* being contained within a phage library composed of 10 kb inserts was 2305.

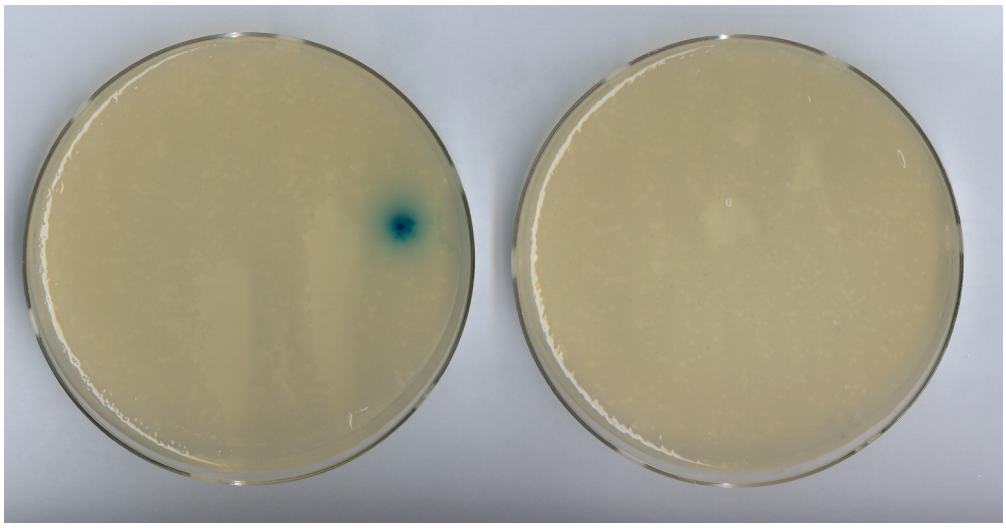


Figure 24. *N. simplex* genomic library in *E. coli* ER1647 using the λBlueSTAR replacement vector. Plates indicate a non-recombinant as the blue plaque, whereas phage containing genomic DNA inserts produced clear plaques.

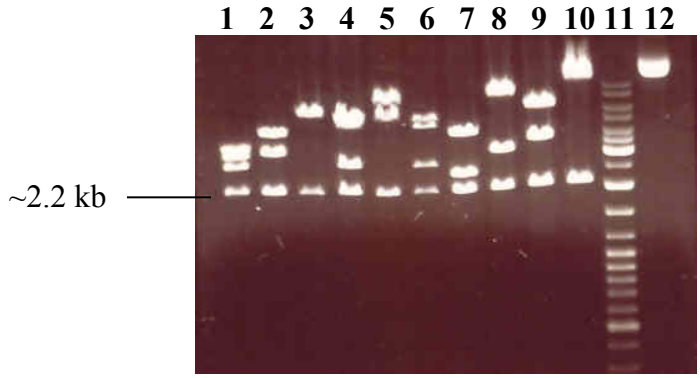


Figure 25. Lanes 1-10: *Bam*HI digested clones from selected recombinants from *E. coli* DH5α resolved on 0.8% agarose in 0.5x TBE, 100 V. GeneRuler™ DNA Ladder Mix (lane 11) and uncut control (lane 12).

N. simplex library screening was achieved using a colony PCR. New set of primers were designed based on the above partial gene sequence (Table 11). The forward primer sequence is 5'-atg aag aac ctg gag ctg gcc tac gtc ggc ctg gtc-3'

and the reverse one is 5'-ccc cac ggt agc acc ccg agc cgg tac gcg tcc ac-3'. The resulting 252 bp PCR product was inserted into the pBluescript II SK (+) cloning vector. New recombinant was designated as pARS6.

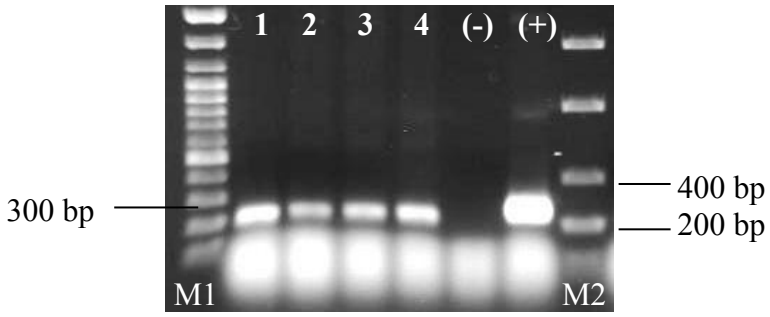


Figure 26. 0.8% agarose gel in 0.5x TBE, 100 V showing positive PCR clones. Negative control (no template) and positive control (pARS6 template) included. M1 = GeneRuler™ DNA Ladder Mix and M2 = FastRuler™ DNA Ladder, Low Range.

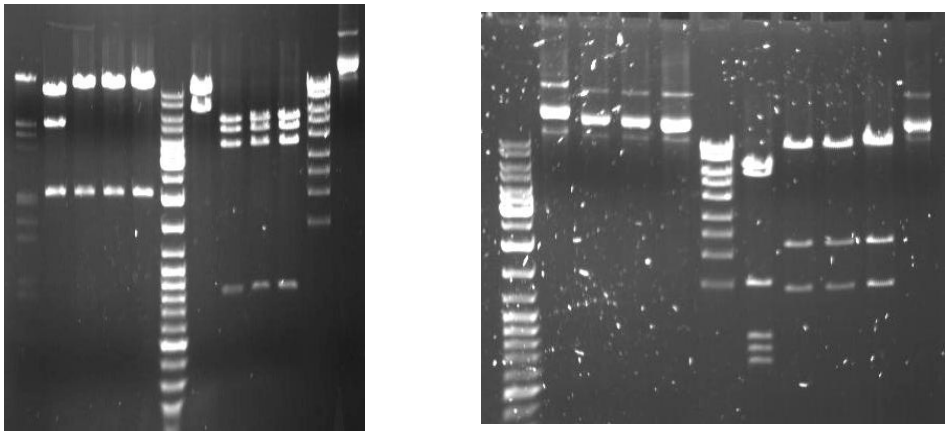


Figure 27. 0.8% agarose gel in 0.5x TBE, 100 V showing single digestions of the clones. Standards used was GeneRuler™ DNA Ladder Mix and MassRuler™ DNA Ladder, High Range.

After restriction analysis of the positive PCR clones, it is evident that three clones show identical digestion pattern implying the presence of the same fragment

within the λBlueSTAR™ vector. Therefore the first positive clone was designated as pARJ1 and the second one as pARJ2. Forward and reverse end DNA sequences from each clones were obtained using T3 and T7 primer sequences found on the vector. pARJ1 – T3 shows similarity to the 442 aa conserved hypothetical protein [*Nocardioides* sp. JS614]. pARJ1 – T7 shows similarity to the 188 aa transferase hexapeptide repeat [*Nocardioides* sp. JS614]. pARJ2-T3 is similar to the 399 aa phosphoglycerate kinase [*Nocardioides* sp. JS614] and pARJ2-T7 is similar to the 576 aa amidohydrolase-like [*Novosphingobium aromaticivorans* DSM 12444]. The DNA sequences and their similarities are shown in the following figures.

Table 13. Forward DNA sequence of pARJ1 clone using the T3 primer.

GCACGTCGCCCAGGCTGCTGCTGTTGTCGTAGACCAGCTCGAGCCAGA
CGAACGCGAACAGCCCCAGGGCCGCCGGCCAGTAGCCGAGCCGGGCC
GGGTAGGTGTAAATCCCGTGCTCCCGGTCGCCACCGGTGGCCCGGCTC
AAGAGGGCGTGGATCGTGCGGAGGGGGCTGATGGATCGCCAGACCGG
CCCGAAGAGCAGCGAGGCGAAGACGAGCCCGACCCACCACCAGACG
TACACGATGCCGAAGAACGGATTGGTGAGACTGTCCTGCCCGAACAG
CGCCACGATGACGGTGTAGGCGAAGAGCATCATGCCGGCGGTCTGCA
ACGTCCGTCGCCACGCGACCGAGAGCAGCACGCGAGCAATGCGGCTG
GAGATCGGTCGCCCTTGAGGCGCGGCTTCGCCGTCGTAGCGGGTCGAT
CGCCACGCGATCGTCATCACCACGAACGACAGGACCAGCGCGACCCC
GGCACCGCCGACCGCGAGCGAGAGCGGGATCGGCAGATCAGCCTGTC
CGCCGATGCCATGCGCCTCGATCATTCCTCACCCTCCGCTCCCACTTGT
CGGGCCTGGAGGCACGGTCCAGATTGGTCGGTCGGTGTGGCCTGCCG
GGTATTCAGAGCCGCGGCGAGTGCGGCCGGTCGCCGTGAGCTGACCA
GCCAGTACGGCGCCGGGTCGGCTGGATCGTCGAT

gi|71368975|ref|ZP_00659450.1| conserved hypothetical protein [*Nocardioides* sp. JS614]
gi|71155296|gb|EAO05748.1| conserved hypothetical protein [*Nocardioides* sp. JS614]

Length=442

```
 Score = 194 bits (492), Expect = 3e-48
 Identities = 99/182 (54%), Positives = 128/182 (70%), Gaps = 2/182 (1%)
 Frame = -3

Query 547 GMIEAHGIGGQADLPIPLSlavggagvalvLSFVVMTIAWRSTRYDGEAAPQGRPISSRI 368
          G+ AHGIGG DLPI LA+ GA ALV+SF V+ +AWR+ RYD AA GRP + +
Sbjct 5   GITLAHGIGGAKDLPISPELAIAGASAALVVSFTVLAVAWRTPRYD--AATSGRPAPTLL 62

Query 367 ARVLLSVAWRRTLQTAGMMLFAYTVIVALFGQDSLTNPFFGIVYVWWWVGLVFASLLFGP 188
           R++ S WR + GM++F + + A+FG+D + NP FGI YVWWWVGLV S+L GP
Sbjct 63  GRLVDSTGWRVACRVFGMVVFLWAAMAAVFGKDLVINPIFGIFYVWWWVGLVPLSVLLGP 122

Query 187 VWRSISPLRTIHALLSRATGGDREHGIYTYPARLGYWPAALGLFAFVWLELVYDNSSSLG 8
          VW++ISP RTI+ ++ +G D + G+ TYP RLGYWPAALGLFAFVW+ELVY S+ LG
Sbjct 123 VWKAISPARTINLAFAKVSGSDPDRGLLTYPDRLGYWPAALGLFAFVWMELVYPFSTELG 182

Query 7   DV 2
            V
Sbjct 183 PV 184
```

Table 14. Reverse DNA sequence of pARJ1 clone using the T7 primer.

GAGCGACGGTGCGCTCGACGGCTTCGGGGTCGTAGGTCGGCCCCGTC
GCGAGGTCGTAGAGCGGGGCGATCGAGCGATCGCCGTCGGGAGCGAG
GATGAGCGAGATGTTCTTGGCATGGCCGTCCGGGGCGCCGGCGACCA
GGTTGACGGCGCTGACGCCGGTCGCGGCGGCGGCGCGCATGGTGACG
TGCTCGACGAGGGCCTGGTGGTGCAAGACCTTGATGCCGGGGCAACG
GCCTCGTACGAGGACAGGCGTGGCATGTCCTCGTACGAGGGCGGAGG
TGACATGTCCTCGTACGAGGACGGAAAAAAGGTCAGGACCTGCCGTC
GACCGGTACGACGCCCGCGCGGTGCCGCTGCGCGAGGTCGGCGTAGT
AGGGCGCATTGGTCTCGATCCAGAACTGGGCGCCGGTGCCCTCGATC
GGGCGCTTCGCCTCGGCGGGGGCGCCGGCGGCGAGGACGCCCTCGGG
GATCGCGGTGCCGGGCGTGATGACCGCACCGGCGGCGACCAGCGAGC
CCGCGCCGATGGTGGCGCCGTCGAGGATGGTGCTCGCGTTGCCGAGC
AGCGACTTCTCGCCCATGGCGTCGCCGTGGAAGACGCAGCCGTGGGC
GACGGTGGCGTTCGGGCCGATGTCGACGACCACGTCGGGGGCGCCGT
GGACGACCGAGTTGTCCTGGATGTTCGAGCCCTCGCGCACGATGATCG
TGCAGATGTCGG

gi|71366755|ref|ZP_00657293.1| transferase hexapeptide repeat [*Nocardioides* sp. JS614]
gi|71157566|gb|EAO07955.1| transferase hexapeptide repeat [*Nocardioides* sp. JS614]

Length=188

Score = 168 bits (426), Expect = 2e-40
Identities = 76/130 (58%), Positives = 101/130 (77%), Gaps = 0/130 (0%)
Frame = -3

```
Query 716 DICTIIVREGSNIQDNSVVHGAPDVVVDIGPNATVAHGCVFHGDAMGEKSLLGNASTILD 537
          DICTI+VREG+N+QDNSV+H AP + IG +AT+ HGCV H +GE++L+GN ST+LD
Sbjct 49  DICTIVVREGANVQDNSVLHAAPGETLVIGRDATIGHGCVVHCREVGEQALVGNGSTLLD 108

Query 536 GATIGAGSLVAAGAVITPGTAIPEGVLAAGAPAEAKRPIEGTGAQFWIETNAPYYADLAQ 357
           A +G +LVAAG+V+TPGT +P G++AAG PA +PIEGT ++FW+E N PYY +LAQ
Sbjct 109 RAVVGPRTLVAAGSVVTPGTELPGGMVAAGVPARPTKPIEGTSSEFWVEQNPPYYRELAQ 168

Query 356 RHRAGVVPVD 327
           RH AGV V+
Sbjct 169 RHLAGVAVVE 178
```

Table 15. Forward DNA sequence of pARJ2 clone using the T3 primer.

GCCCGCGTCATCGTCACCGCACACCTGGGCCGGCCGAAGGGCGAGCC
GGACCCGGCGTACTCCCTGGCGCCGGTCGCCGCCCGCCTCGGCGAGCT
GCTCGGCGCGCCGGTCGCCTTCGCGACCGACACCGTCGGCGCCTCGGC
CCaGGAGACGGTGGCCGCGCTGCGGGACGGCCAGGTCGCCCTCTTGG
AGAACGTCAGGTTCAACGCCGGCGAGACCAGCAAGGACGACGTCGAG
CGGGGCGCCTTCGCCGACCGGCTGGCCGGCCTGGCCGACGCGTTCGT
GTCCGACGGCTTCGGCGTCGTGCACCGCAAGCAGGCCAGCGTGTACG
ACGTGGCGCAGCGCCTCCCGCACGCCATGGGCGGCCTGGTCGCCGCC
GAGGTCGCGGTGCTGCGCCGGCTCACCGTCGACCCCGAGCGGCCCTA
TGTCGTCGTCCTCGGCGGCTCGAAGGGCTCCGACAAGCTCGGCGTGAT
CGACAACCTGCTCGGCAAGGCAGACCGGCTGCTCATCGGCGGCGGCA
TGGTGTTCACCTTCCTCAAGGCCGACGGCCATGAGGTCGGCAAGAGC
CTCCTCGAGGAGGACCAGCTCGACACCGTGCGCGCCTA

gi|71368428|ref|ZP_00658927.1| Phosphoglycerate kinase [*Nocardioides* sp. JS614]
gi|71155872|gb|EAO06300.1| Phosphoglycerate kinase [*Nocardioides* sp. JS614]

Length=399

Score = 339 bits (870), Expect = 4e-92
Identities = 176/201 (87%), Positives = 182/201 (90%), Gaps = 0/201 (0%)
Frame = +1

```
Query 1   ARVIVTAHLGRPKGEPDPAYSLAPVAARLGELLGAPVAFATDTVGASAQETVAALRDGQV 180
          ARVIVTAHLGRPKG P+ YSL PV ARLGELLG VAFATDTVG SA+ TV AL+DGQV
Sbjct 51  ARVIVTAHLGRPKGAPEERYSLRPVVARLGELLGQDVAFATDTVGESARATVEALQDGQV 110

Query 181 ALLENVRFNAGETSKDDVERGAFADRLAGLADAFVSDGFGVVHRKQASVYDVAQRLPHAM 360
          A+LENVRFNAGETSKD+ ERGAFADRLA LADAFVSDGFGVVHRKQASVYDVAQRLPHAM
Sbjct 111 AVLENVRFNAGETSKDEAERGAFADRLAALADAFVSDGFGVVHRKQASVYDVAQRLPHAM 170

Query 361 GGLVAAEVAVLRRLTVDPERPYVVVLGGSKGSDKLGVIDNLLGKADRLLIGGGMVFTFLK 540
          G LVA E VLRRLTVDPERPYVVVLGGSK SDKLGVIDNLLGKADRLLIGGGMVFTFLK
Sbjct 171 GALVAKETEVLRRLTVDPERPYVVVLGGSKVSDKLGVIDNLLGKADRLLIGGGMVFTFLK 230

Query 541 ADGHEVGKSLLEEDQLDTVRA 603
          A GHEVGKSLLEEDQLDT R+
Sbjct 231 AQGHEVGKSLLEEDQLDTCRS 251
```

Table 16. Reverse DNA sequence of pARJ2 clone using the T7 primer.

TACCAGGACCTCGGCTTCAAGGTCACCACCAGCGCCGGGTTCGCCTGG
GGCAAGGGCGCGATGTACGGCGAGCGGATCGGCGAGCACGCCTGGCG
CGACCTCGAGCCGCTCCAGCGCCTGCTGGACCAGGGCCTCGAGCTGG
CCGGCGGCTCCGACTGGGGCCCGAAGAACCCCTGGGAGCAGATCGAG
CTCGCCCAGACCCACCGCTTCGCGGGCAGCGACCGGCGCAACGACGG
CCCCGACCAGAGCATCTCGCGGCTCGACGCGCTGCGGATGTGGACGT
CGGCCGCGGCGAGCGTCATCGGCTGGGACGAGGTCGGCTCGCTGACC
CCGGGCAAGCACGCCGACGTCCTCATCGTCGACCGCGACCCGCTGAC
CTGCGACATCGACGACCTCCCCGACACCCGCGTCCTGCGGACCTACCT
GGGCGGCGAGGTCGTCCACGACAGCAAGGAGCTGACGGCATGAGCGC
CGCCACGGAGAAGACCCTCGCCCGCATCGACGACCTGCGCGACGAGA
TGATCGCGACCCTGAGGGAGGCGATCGCGATCCGGAGCGTCAACCCG
ACCTACCCCGACCAGGACTACGACGACCTCGTCGGCGGCGAGACCGA
GGTCTCCCAGCTGCTGGCCGGCCACTACCGGCGGGCCGGCGCGGAGA
CCCAGCTCTTCGGCGACGCACCGGGCCGCGACAACGTGGTCGGC

gi|87199076|ref|YP_496333.1| amidohydrolase-like [*Novosphingobium aromaticivorans* DSM 12444]
gi|87134757|gb|ABD25499.1| amidohydrolase-like [*Novosphingobium aromaticivorans* DSM 12444]

Length=576

Score = 77.8 bits (190), Expect = 4e-13
Identities = 44/131 (33%), Positives = 69/131 (52%), Gaps = 4/131 (3%)
Frame = +1

```
Query 76  IGEHAWRDLEPLQRLLDQGLELAGGSDWG---PKNPWEQIELAQTHRFAGSDRRNDGPDQ 246
          IG+ + P++ L+ G + GSDW NPW IE T + G G Q
Sbjct 439 IGDERMKRWIPIRDALETGALVVAGSDWSVVPSVNPWIAIETMVTRQIPGGSAETLGEGQ 498

Query 247 SISRLDALRMWTSAAASVIGW-DEVGSLTPGKHADVLIVDRDPLTCDIDDLPDTRVLRTY 423
           I+ ALR++T AS +G D+ GS+ G AD ++V+R P ++++ T+VL+T+
Sbjct 499 KITLAQALRIFTENGASFLGQRDQFGSIETGMKADFIVVERSPYKVPVNEIHKTKVLQTF 558

Query 424 LGGEVVHDSKE 456
          + GE V+ S E
Sbjct 559 IDGEQVYLSSE 569
```

4. Discussion and conclusions

4.1. Biodegradation and bioremediation

Microbes have the ability to mediate decomposition of paper, paint, textiles, concrete, hydrocarbons, nitroaromatic compounds and other materials. A general term used to refer to the decomposition process is a biodegradation and several definitions of biodegradability are used in environmental microbiology. It can be defined as a minor change in a molecule or it can be thought as fragmentation, where the original structure of the molecule can be still recognized. Lastly, biodegradation can mean complete mineralization – the complete catabolism of a compound to its inorganic components (Nishino and Spain, 2001; Prescott *et al*., 1999).

A very good example where microorganisms are used to remove pollutants from the environment is called a bioremediation. There are two types of bioremediation: engineered and intrinsic. The first one involved the modifications of the environment (waters and soils) by the addition of oxygen or nutrients in order to stimulate microbial activities. The second one designates a biological process that is happening under natural conditions. Environmental attenuation is seen through the decrease in the levels of contamination via unmanaged physical, chemical and biological processes (Prescott *et al*., 1999). Microorganisms for remediation are usually isolated from the contaminating site itself. Xenobiotic compounds are degraded via a process called co-metabolism because a compound cannot be used as an independent source of energy (Balba, 1993; Prescott *et al*., 1999).

In general, biological treatments are ecologically harmless due to low energy consumption, low emissions and preservation of the biological activity of the soil (Lenke *et al.,* 2000). In the present study, the two nocardioform strains, *Nocardioides simplex* and *Rhodococcus opacus* were exploited. These two

microbes may serve as the potential tools in a development of bioremediation system of nitrophenol-contaminated soils. Both organisms provide a very important set of enzymes involved in the biodegradability of TNP and DNP. During a study that deals with a bioprospecting for industrial enzymes one should consider the steps such as cloning, expression, purification and characterisation of the enzymes to make the top candidates available for large-scale testing (Schäfer and Borchert, 2004).

4.2. Expression of *orfB* gene contained in the *npd* cluster

Cloning a gene is only the first of many steps needed to produce a recombinant protein. Various expression systems have been developed to produce recombinant proteins. Both industrial and academic laboratories work on the development of expression systems an prioritize it as an important research area. The most popular expression systems are the bacteria because they offer simplicity, short generation times and large yields of product with low costs. In addition, the bacterial cells can be induced resulting in a secretion of product into the culture medium allowing successful protein purification. Some drawbacks are also noted with expression in prokaryotic cells. For example, proteins, which are expressed to high levels, may fold incorrectly due to improper formation of disulphide bonds in the reducing environment of the *E. coli* cytoplasm. As a result, a formation of insoluble inclusion bodies is detected and protein extracts from these structures are often inactive (Scopes, 1994).

During expression investigations of *npdC*, *npdG*, *npdH* and *npdI*, all genes were expressed as His-tag fusion proteins. *E. coli* recombinant cells expressing *npdC* or *npdI* were shown to have a substantial amount of inclusion bodies (Heiss *et al.*, 2002). In addition, Dang *et al.* (2004) presented SDS-PAGE analysis of cell extracts from *E. coli* BL21(DE3)/pNGA5 expressing NpdR-His. In this example too, more than half of the protein resided in the cells as inclusion bodies. In the present study, OrfB-His recombinant protein also aggregated into inclusion bodies

(Figure 6). Although a purification procedure using Ni-NTA metal affinity chromatography was successful, further enzyme kinetics study was not possible. Neither biological nor chemical substrate for the protein was available and therefore enzyme-substrate interactions were not tested. Previous attempts to clone and overexpress *orfB* have failed (G. Heiss *et al*., unpublished data). In this work, I used a different protein expression system (the pET system) from Novagen and I could exclude the possibility that OrfB had a toxic effect on the heterologous *E. coli* host. These findings are summarized in the Table 17 below.

Table 17. Cloning of the *orfB* and expression attempts in *E. coli*.

Vector	Vector characteristics	*E. coli* host	Expression	Source
p430.1	Suicide vector	JM109 (*endA*, *recA*)	Not tested	pSTG5
pBW22	Expression vector, constitutive promoter (6His)	BL21(DE3) (Expression of toxic proteins)	none	pSTG1
pJoe2702	Expression vector, Ramnose induction	BL21(DE3)	none	pSTG3
pQE30	Expression vector, IPTG induction	BL21(DE3)	none	pSTG4
pET11a*	Expression vector, no His-tags	Rosetta2(DE3)pLysS	yes	pARS3
pET22b	Expression vector, His-tag (C-term)	Rosetta2(DE3)pLysS	yes	pARS4
pET28a	Expression vector, His-tag (N-term)	Rosetta2(DE3)pLysS	yes	pARS5

After analysis of the data presented in this work, some conclusions could be drawn relating to the function of *orfB* contained within the *npd* gene cluster. An explicit function to *orfB* cannot be assigned due to lack of the enzyme substrate. The mutation in *orfB* had no effect on the biodegradation of picric acid, and both strains, HL PM-1 and AR1, utilized the entire picric acid molecule. This result

was conclusive after detection of metabolites using HPLC and upper pathway intermediates. There remains the possibility of involvement of the gene product in the lower TNP biodegradation steps or even some other cellular function.

4.3. Sucrose sensitivity as a positive selection marker in *R. opacus*

Gene expression of the secreted enzyme levansucrase, encoded by *sacB* of *Bacillus subtilis* (Gay *et al*., 1983) is lethal in the presence of sucrose in many bacteria. The enzyme catalyzes hydrolysis of sucrose and synthesis of levans, high-molecular weight fructose polymers. The basis for toxicity of levansucrase action on sucrose is unknown. Pelicic *et al.* (1996) suggested that the toxicity is probable due to an accumulation of levans, which restrain the periplasm because of their high-molecular weight. Alternatively, fructose residues could be transferred to improper acceptor molecules resulting in a toxic effect on the bacterial cell. The use of *sacB* as a positive selection marker during the isolation of insertion sequence elements from various bacterial strains, including *Rhodococcus fascians* has been reported by Jager *et al.* (1995). Later on, *sacB* was used in *Rhodococcus erythropolis* SQ1 during unmarked gene deletion mutagenesis as counter-selectable marker, allowing screening for the rare second recombination event, which resulted in the actual gene replacement (van der Geize *et al*., 2001). Here and work from Dang *et al.* (2004) provide information which suggests that a sucrose sensitivity is very useful selection marker in *Rhodococcus opacus*. The effect of 10% sucrose on the growth of *R. opacus* resulted in a detectable phenotype. Therefore, the *sacB* selection could be further tested using different rhodococci giving a possibility of developing a successful mutagenesis system in this genus. Some members of the genus carry megaplasmids, which may interfere during mutagenesis experiments. Large numbers of insertion sequence elements are associated with catabolic genes and various catabolic genes and gene clusters often resides on mobile genetic elements. A list and brief description of catabolic plasmids from genus *Rhodococcus* are presented in Table 18 (Nojiri *et al.*, 2004). It has been also

reported that not all Gram-positive bacteria, for example *Streptomyces lividans*, are sensitive to sucrose implying a different sucrose sensing pathway (Jager *et al.*, 1992).

Table 18. Examples of linear catabolic plasmids found in genus *Rhodococcus*.

Plasmid	Substrates	Strain	Size (kb)	Catabolic gene
pBD2	Isopropylbenzene	*R. erythropolis* BD2	210	*ipbA1A2A3A4C*
pLP6	Biphenyl/PCBs	*R. globerulus* P6	650	*bphC2*
pNC30	Propene	*R. corallinus* B-276	185	*amoABC*
pNUO1	Biphenyl/PCBs	*R. opacus* M213	750	*edoD*
pRHL1	Biphenyl/PCBs	*Rhodococcus* sp. RHA1	1 100	*bphA1A2A3A4CB, etbD1*
pRHL2	Biphenyl/PCBs, ethylbenzene	*Rhodococcus* sp. RHA1	450	*bphB2, etbA1A2C, bphDEF, ebdA1A2A3, etbD2, bphC2, bphC4*

4.4. *Rhodococcus opacus* mobile genetic elements

Even with a rapid development of modern biotechnology, not much is known about the linear plasmids involved in the degradation of xenobiotics. In 1998, Kalkus *et al.* studied the terminal structures of linear plasmids from *R. opacus*. Using a gel retardation assay they showed presence of proteins bound to the ends of linear plasmids. The wild-type strains MR11 and MR22 each carry three linear plasmids. In the genome of MR11, the linear genetic elements pHG201, pHG202 and pHG203 were detected and sized 270 kbp, 400 kbp and 420 kbp respectively. The strain MR22 contains pHG204 (190 kbp), pHG205 (280 kbp)and pHG206 (500 kbp). Coincidently, Figure 14 shows also *R. opacus* HL PM-1 strain harbouring three megaplasmids. There is not much information on the respective genetic elements. According to their sizes, 190 kbp, 300 kbp and 312.5 kbp, one

could speculate about their catabolic potential. In the past it has been shown that some of the TNP catabolic genes, *npdG*, *npdH*, *npdI* and *npdR*, from *R. opacus* CB24-1, exist as a double copy within the genome (G. Heiss *et al*., unpublished data). One was situated on the chromosomal DNA and the other on a plasmid. Detailed investigations into the catabolic capacity of these plasmids could involve separate isolation and sequencing of the DNA. It would be also interesting to identify a replication region of each plasmid and collect information that would deepen our knowledge related to a horizontal gene transfer. Megaplasmids certainly offer future prospects as a tool in bioremediation. Presence of xenobiotic catabolic genes on the plasmids that mediate horizontal gene transfer, could be further useful if the plasmid DNA is introduced into the indigenous bacterial population in the contaminated soils (Nojiri *et al.*, 2004).

4.5. *Nocardioides simplex* proteome analysis

Proteome databases, SWISS-2DPAGE, Sub2D, SSI-2DPAGE, based on the proteins resolved by 2-D gel electrophoresis are being developed for a number of model organisms. For example, proteomic databases of *Escherichia coli*, *Bacillus subtilis*, *Haemophilus influenzae*, *Helicobacter pylori*, *Mycobacterium tuberculosis* and *Mycoplasma pneumoniae* are available. Till today, there are no reports on the proteome database from any known nocardioform. Only under different environmental and stress conditions, more of the bacterial proteome could be obtained. This study aimed to give a preliminary overview of the *N. simplex* proteome and it is shown in Figure 28 below.

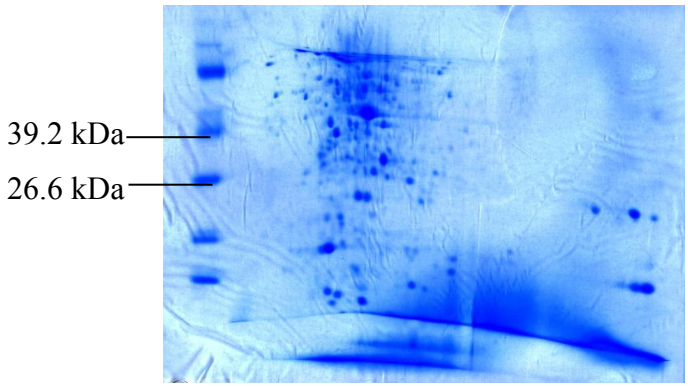


Figure 28. 2-D electrophoresis results using the crude extracts from *Nocardioides simplex* FJ2-1A. The Premixed Protein Molecular Weight Marker, low-range used as a standard.

4.6. Discovery of industrially important enzymes

Chemicals used in many industries after a certain period of time cause detrimental effects on the environment. Various industries have been using biodegradable enzymes instead of chemicals. For example, use of enzymes for detergents, baking, beverage, and dairy, feed and paper. At the moment there are three major suppliers of industrial enzymes: Novozymes A/S (Denmark), Genencor International Inc. (United States) and DSM N.V. (Netherlands). The discovery and development of novel biocatalysts is usually based on several parametres. These include: the performance of the enzyme in the target application, safe and economic production of the protein and patentability of the protein and/or the application to gain product protection. In addition, successful economic production of a biocatalyst starts with a production strain, development of the fermentation processes, product recovery as well as process approval (Schäfer and Borchert, 2004).

In a modern expanding world innovative biotechnology is closely linked with the detection of novel microorganisms, their activities, metabolic products, or genes.

Discoveries are dependent on the recognition of novel organisms, the definition of biotechnological targets as well as invention of search and screening strategies. Biotechnology is seen as the art of exploiting biology. It is possible to make use of biological products obtained directly from genetic resources or via recombinant DNA technology. Also, chemically modified derivatives of natural products or even chemically synthesized compounds are widely used today (Bull, 2004).

Pivotal work on the nitrite-eliminating enzyme and its potential applications in industry has been presented in this work. The approach that was employed did not rely on expression of the cloned gene but it was reliant on functional assays to detect the enzyme activity. It was based on obtaining some amino acid sequence from proteolytic digests of the pure protein (this work) and from the N-terminus (Hofmann *et al*., 2004). Degenerate oligonucleotide primers were designed from the amino acid sequence and used to direct the amplification of a small DNA fragment. Thereafter, the same set of primers was used to screen clones in a library. By this method, numerous genes homologous to the initial gene sequences can be easily identified. This is illustrated in Figure 21 after a few randomly selected clones showed different insert sizes. The limitation of this method was that enzyme variants rather than totally novel enzymes were detected (Schäfer and Borchert, 2004). Also it was very interesting to see that the small DNA fragment shows similarity and 83% sequence identity to the fatty acid hydroxylase (FAH) from *Nocardioides* sp. JS614. There was no experimental evidence under laboratory conditions about the function of the FAH from this bacterium. Hence, one could speculate that the fatty acid hydroxylase is indeed the nitrite-eliminating enzyme, since the chromosomal view (Figure 22) indicates its own transcriptional regulator. This work certainly hints the possible parallel pathway, which is turned on during the biodegradation of picric acid.

Diversity during removal or even productive metabolism of nitro groups has been addressed by Spain (1995). He explained four different strategies involved in these processes. Firstly, some bacteria can reduce the aromatic nucleus of

polynitroaromatic compounds by the addition of a hydride ion to form a hydride-Meisenheimer complex that rearomatizes with the elimination of nitrite. Secondly, monooxygenases can add a single oxygen atom and eliminate the nitro group from nitrophenols. If the two hydroxyl groups are inserted into the aromatic ring by dioxygenases, the spontaneous elimination of the nitro group is accelerated. Finally, reduction of the nitro group to the hydroxylamine is the initial reaction during the productive metabolism of nitrobenzene, 4-nitrotoluene and 4-nitrobenzoate (Philp *et al.*, 2005).

Due to financial and time constrains, the chemical synthesis of the substrate for the nitrite-eliminating enzyme was not possible. It still remains unclear if the enzyme catalyze release of the two remaining nitrite groups from 4,6-DNH since no activity was reported for this reaction or from the aromatic moiety. There is a call for detailed enzyme-substrate investigation that does not have to be dependent on the entire gene sequence. The enzyme could be purified as previously described by Hofmann *et al.* (2004) and using the two possible substrates, kinetic data could be generated and compared.

Nevertheless, according to Cheetham (1998) we need people with many different skills if we want to create a successful biocatalyst. A screening program for industrial enzymes starts with a problem to be solved using enzymes and a business justification. And the task to find the one ideal performing enzyme in the enormous diversity of molecules should not be undertaken lightly. The first steps have been made in this work in searching for the biocatalyst that will selectively target NO2 bonds from all possible substrates. It is important to stress that a discovery of industrial enzymes is a multidisciplinary effort dependent on different technologies. The novel product should be of the highest possible quality of diversity that will lead to the ultimate goal, in this case a removal of nitrite group from nitro compounds (Schäfer and Borchert, 2004).

4.7. Developing a bioremediation strategy

Bioremediation is very much an evolving technology (Philp *et al.*, 2005). There are five major types of bioremediation: above-ground bioreactor, solid phase treatment, composting, land farming and in situ treatments (Balba, 1993). Nitro- and polynitroaromatic compounds are abundantly present in nature mainly from anthropogenic activities. The degradation of these compounds does not only occur in pure culture but also *in situ* – in soil, water and sewage. Complete removal of these xenobiotics from the environment by bacterial mineralization has been characterized extensively (Russ *et al.*, 2000). In the development of bioremediation strategy, a combination of biological and chemical reagents is crucial. For example, biodegradation of DNT will occur under the following conditions: oxygen concentration greater than 1 mg/L, adequate and stable moisture, pH between 6.5 and 8.5, moderate soil / water temperature, adequate macronutrients (phosphate and sulphate) and lastly appropriate bacterial biomass (Nishino and Spain, 2001). The similar combination of external factors could be also taken into consideration when establishing the TNP strategy.

Large-scale culturing of *N. simplex* and *R. opacus* is important because organisms provide a certain arrays of enzymes necessary for biodegradation of TNP. As mentioned earlier, in *N. simplex* these enzymes are constitutively present while in *R. opacus* enzyme induction is necessary (Russ *et al.*, 2000). By introducing both organisms into the contaminated soils, horizontal gene transfer, as well as the time need to break down the xenobiotic, may be facilitated. In addition, many clusters of catabolic genes are located on transposons and novel catabolic capabilities can arise by the rearrangement of DNA sequences (Philp *et al.*, 2005). Also, novel spontaneous mutants can be selected that utilize TNP as sole source of carbon, nitrogen and energy. (Rieger *et al*., 2002). *In situ* bioremediation of nitroaromatic compounds has been considered at sites where aerobic conditions are present or can be engineered, where appropriate organisms are present or can be introduced effectively and lastly if the potential for nitrite or nitrate accumulation is

manageable (Nishino and Spain, 2001).

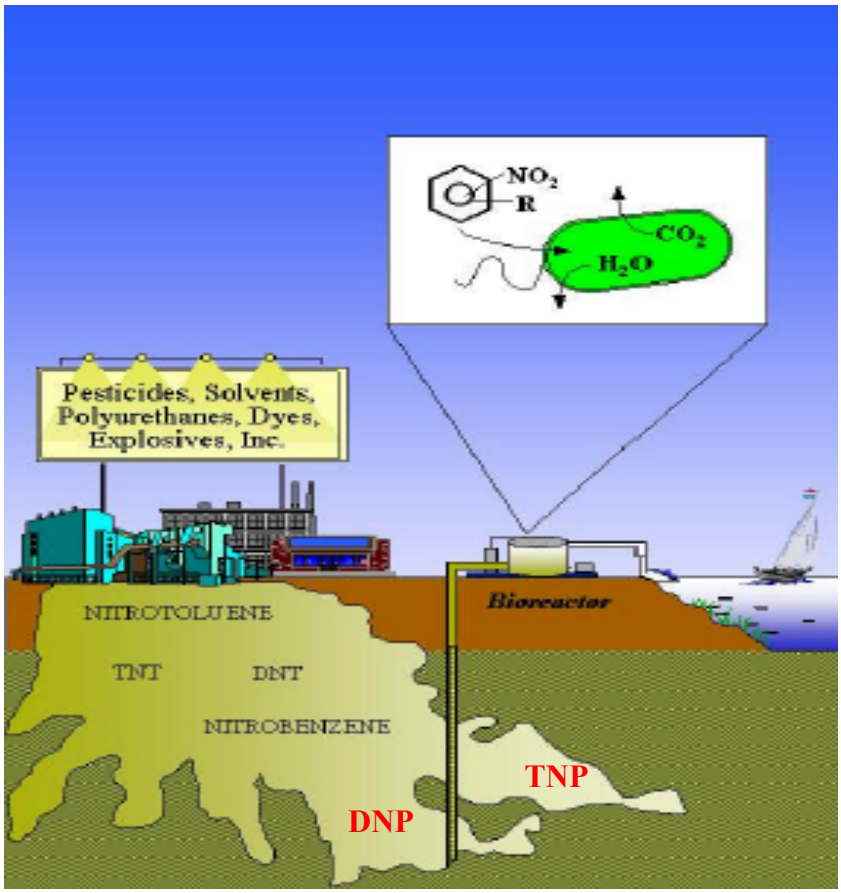


Figure 29. Diagrammatic presentation of the bioremediation technology of nitroaromatic compounds (Adapted from Nishino and Spain, 2001).

4.8. Factors affecting bioremediation process

Degradability of a xenobiotic in soil or water is diminished if the compound is not easily available to the organisms capable of mineralization. In addition, the chemical structure of the xenobiotic and the ability of microbial enzymes to accept these as substrates also influence its degradability. Only at high substrate concentrations a selective advantage is possible in such a way that microbes harbouring complete catabolic pathways complement the genetic pool. Consequently, the evolution of new catabolic pathways for xenobiotics is

enhanced (Rieger *et al*., 2002). Therefore, the most important factors affecting any bioremediation process are: the bioavailability of the substrates to the microorganisms, a viable microbial population able to degrade the contaminant and environmental conditions suitable for microbial biodegradative activities (Balba, 1993; Philp *et al.*, 2005).

In conclusion, like many other technologies, there are limitations to the application of bioremediation. The knowledge transfer from the laboratory to field-scale bioremediation is not a straight line process. Further considerations should include the ambient and seasonal environmental conditions, as well as the composition of the indigenous microbial community. Decontamination of soils is particularly complex due to the heterogeneous soil structure. Also performance monitoring is a critical part of remediation effort (Philp *et al*., 2005). Until recently, most investigations focused on biodegradation were conducted at mesophilic temperature. Scientists have provided the evidence suggesting that the indigenous microbial communities will adapt to the *in situ* temperature. Bej *et al.* (2000) isolated a *Rhodococcus* species from an Antarctic soil. The strain was capable of degrading alkanes at –2ºC but was inhibited at a higher temperature.

Appendices

APPENDIX A: Southern blotting and Southern hybridization solutions

Southern blotting

Acid solution	0.25 M HCl
Denaturation solution	0.5 M NaOH 1.5 M NaCl
Neutralization solution	0.5 M Tris 1.5 M NaCl pH 7.5
20x SSC	3.0 M NaCl 0.2 M Tri-sodium citrate

Autoclave all solutions at 121°C for 20 min.

Southern hybridization

Hybridization buffer	5x SSC 0.1% (w/v) N-lauroylsarcosine 0.02% (w/v) SDS 1% (w/v) Blocking reagent

Do not autoclave, use sterile distilled H_20, microwave at 650 W, cool down and store at −20°C.

Buffer H1	0.10 M Maleic acid 0.15 M NaCl pH 7.5 adjusted with NaOH pellets, autoclaved
Buffer H2	1% Blocking reagent in buffer H1 Microwave at 650 W, cool down and store at −20°C.
Buffer H3	0.1 M Tris-HCl 0.1 M NaCl 0.05 M $MgCl_2$ pH 9.5 adjusted with liquid HCl, autoclaved
Washing buffer	0.3 % (v/v) Tween 20 in buffer H1

Stringency wash solutions

Low stringency wash	2.0x SSC, 0.1% (w/v) SDS
High stringency wash	0.1x SSC, 0.1% (w/v) SDS

APPENDIX B: SDS-PAGE solutions

SDS-PAGE Buffer System

- 30% Acrylamide/Bis-acrylamide solution, 37.5:1 mixture (30%T, 2.67% C) (Bio-Rad)

- 10% (w/v) SDS

Dissolve 10 g SDS in 90 ml H_2O with gentle stirring and bring to 100 ml with deionised H_2O. Store at room temperature.

- 1.5 M Tris-HCl, pH 8.8

27.23 g Tris base (18.15 g/100 ml)
80 ml deionised H_2O

Adjust to pH 8.8 with HCl. Bring total volume to 150 ml with deionised H_2O and store at 4°C.

- 0.5 M Tris-HCl, pH 6.8

6.0 g Tris base
60 ml deionised H_2O

Adjust to pH 6.8 with HCl. Bring total volume to 100 ml with deionised H_2O and store at 4°C.

- Sample Buffer (SDS Reducing Buffer, 2x)

4.8 ml deionised H_2O

1.2 ml 0.5 M Tris-HCl, pH 6.8
2.0 ml 10% (w/v) SDS
1.0 ml glycerol
0.5 ml 0.5% (w/v) bromophenol blue (dissolved in H_2O)

Store at room temperature. Prior to use take 950 µl sample buffer and add 50 µl 2-mercaptoethanol (β-ME). Sample should contain approximately 20 µg of total protein.

- 10x Electrode (Running) Buffer, pH 8.3 (makes 1.0 L)

	(1x Running Buffer, 1.0 L)
30.3 g Tris base	(3.0g)
144.0 g Glycine	(14.4g)
10.0 g SDS	(1.0g)

Dissolve and bring total volume up to 1.0 L with deionised H_2O. No need to adjust pH with acid or base. Store at 4°C but if precipitation occurs, warm to room temperature before use. Dilute 50 ml of 10x Tris/Glycine/SDS stock with 450 ml deionised H_2O for each electrophoresis run. Mix thoroughly before use.

- 10% (w/v) Ammonium persulfate (APS) (fresh daily)

100 mg APS

Dissolve in 1.0 ml of deionised H_2O.

SDS-PAGE staining and destaining

Coomassie Blue R-250 staining solution (for 500 ml)

0.1% (w/v) Coomassie Brilliant Blue R-250	(0.5 g)
40% (v/v) Isopropanol	(200 ml)
10% (v/v) Acetic acid	(50 ml)
50% (v/v) Deionised H_2O	(250 ml)

Dissolve Coomassie Brilliant Blue R-250 in isopropanol first and then add acetic acid and water. Store at room temperature. After staining, pour off remaining staining solution back into a storage container and reuse it.

Destaining solution	(for 500 ml)
20% (v/v) Acetic acid	(100 ml)
80% (v/v) deionised H_2O	(400 ml)

Store at room temperature.

Bradford Assay

Bradford Reagent (for 500 ml)

50 mg Coomassie Brilliant Blue G-250
25 ml 95% Ethanol
50 ml 85% *ortho*-Phosphoric acid
Make up to 500 ml with distilled H_2O

Dissolve the Coomassie Brilliant Blue G-250 in the 95% ethanol in a 100 ml measuring cylinder. Add the *ortho*-phosphoric acid and pour resulting mixture into a 500 ml volumetric flask. Rinse the cylinder until clear with distilled water, adding each rinse to the flask. Make up volume to 500 ml with water. Filter the reagent through Whatman N°1 filter paper and store at 4°C in a bottle wrapped in foil.

Calibration curve

Bovine Serum Albumin (BSA) has been chosen as the standard. It is stored as a stock solution of 1 mg/ml at −20°C. Since protein has a tendency to absorb water during storage, it is necessary to obtain the exact concentration of BSA by measuring its absorbance at 280 nm in a disposable plastic cuvette. A 1 mg/ml

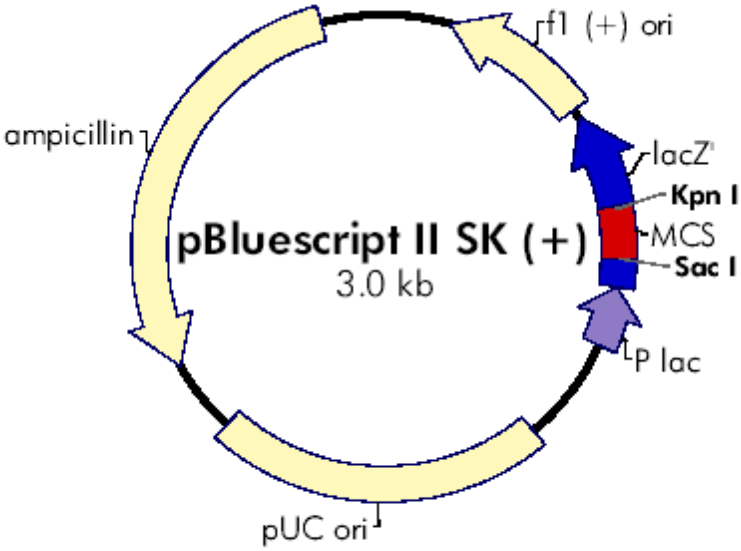
solution of BSA should have an absorbance of 0.66. Prepare duplicate standards as per table below.

µg BSA	µl BSA	µl H_20	µl Bradford Reagent
0	0	100	900
1	1	99	900
2.5	2.5	97.5	900
5	5	95	900
10	10	90	900
15	15	85	900
20	20	800	900

Plot and interpret a standard curve. In theory, the relationship is linear, but if data appear to curve, it is important to fit a curve rather than a straight line. The spectrophotometre is set at 595 nm and is zeroed against the water blank. The absorbance of the other tubes is measured 5 min after addition of reagent at 595 nm. The absorbance of each tube (y-axis) is plotted against the protein mass in µg (x-axis) and a straight line is drawn through the points. The amount of protein in the unknown samples is determined by its absorbance and comparison to the standard protein curve. The concentration of the solution is calculated by the following equation:

Concentration of unknown = mg of protein determined by assay / 0.1 ml

APPENDIX C: Vectors used in this work



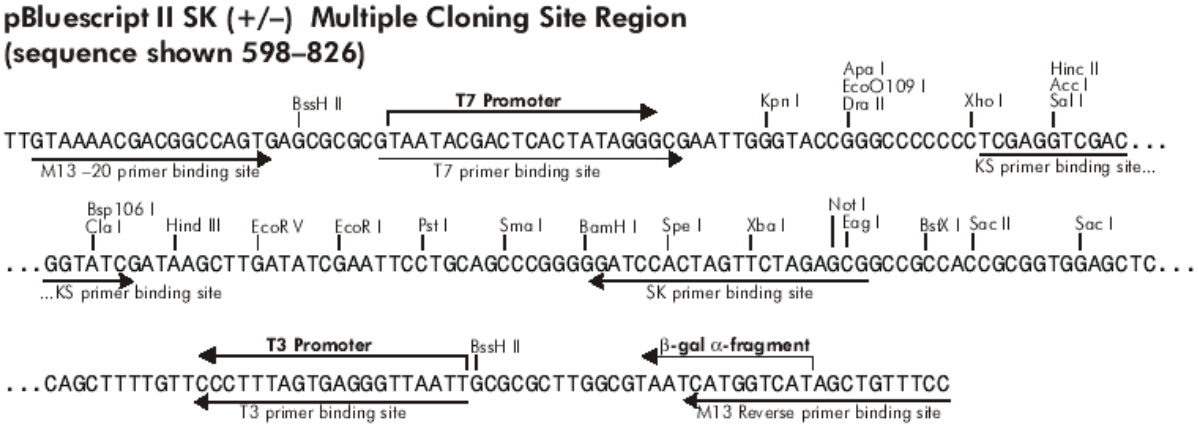


Figure C.1. Restriction map of pBluescript II SK (+) (Adapted from pBluescript® II Phagemid Vectors Instruction Manual, 2004).

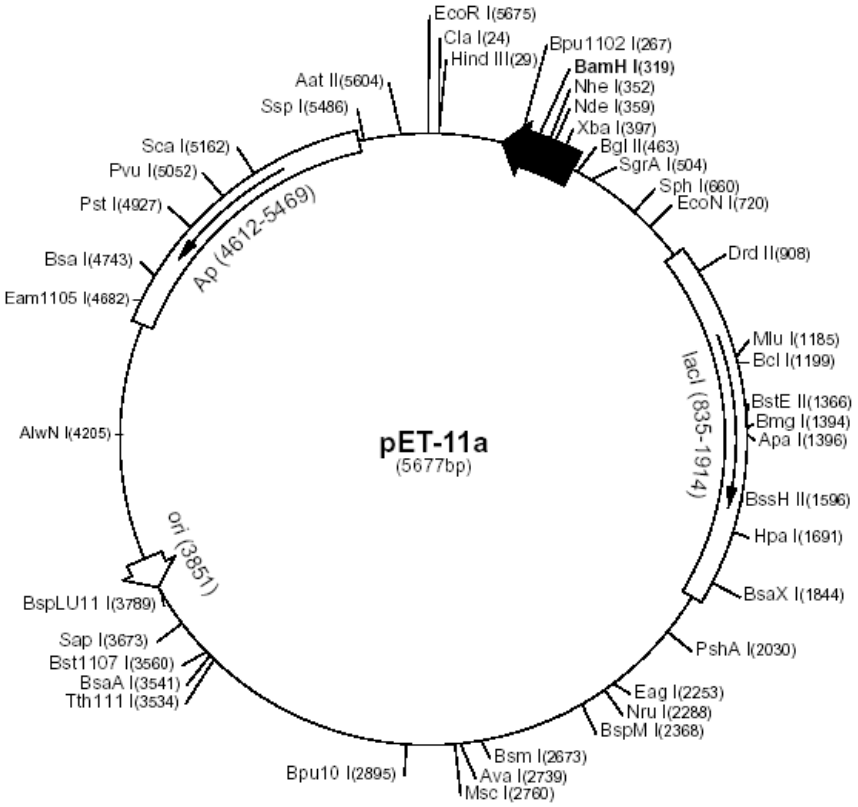


Figure C.2. Restriction map of pET11a (Adapted from Novagen® catalogue, 2004/2005).

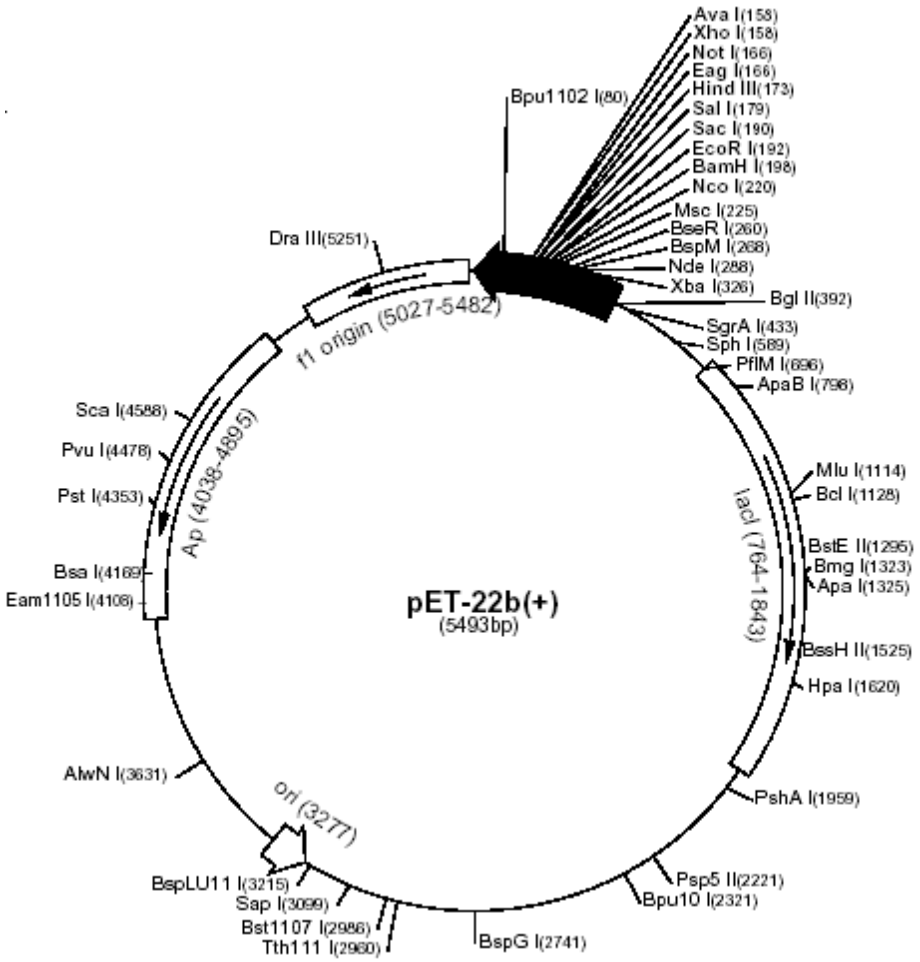


Figure C.3. Restriction map of pET22b (Adapted from Novagen® catalogue, 2004/2005).

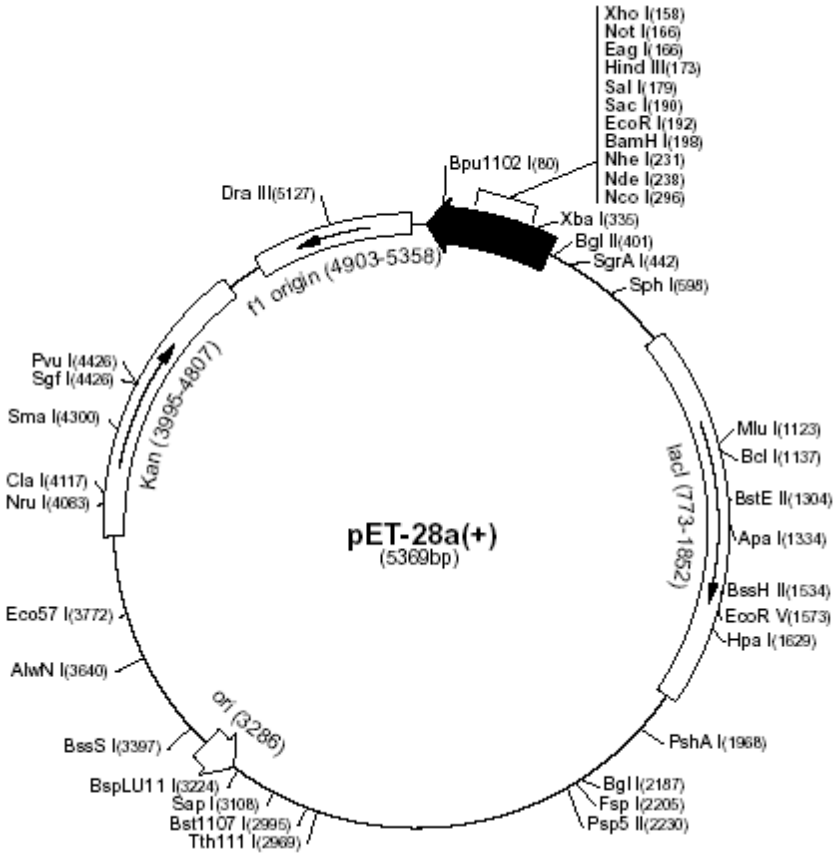


Figure C.4. Restriction map of pET28a (Adapted from Novagen® catalogue, 2004/2005).

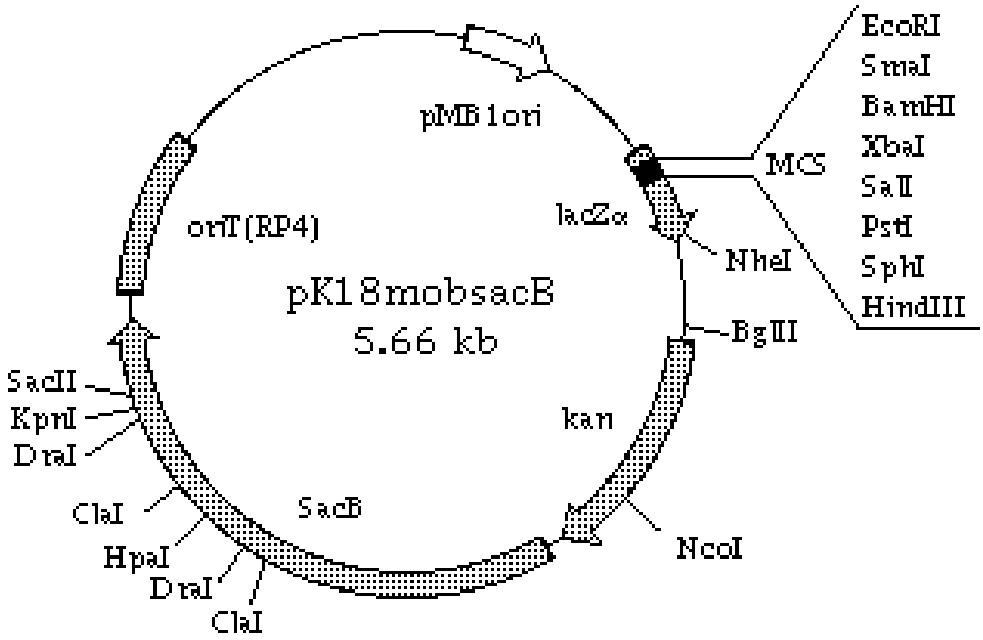


Figure C.5. Restriction map of pK18*mobsacB* (Adapted from Schäfer *et al*., 1994).

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