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Biodegradation of 2,4,6-Trinitrophenol (Picric Acid) and Identification of Metabolic Genes

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Abstract: Biodegradation of pollutants using microbes has become an emerging technology in cleaning contaminated environments. In this work, a molecular characterisation of the *orfB* from the *n*itro*p*henol *d*egradation (*npd*) gene cluster was achieved. The gene was 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.

Key words: biodegradation, microbes, 2,4,6-trinitrophenol, picric acid, *npd* gene cluster.

Introduction

Microbial biodegradation is the process used by living organisms to break down organic substances. 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. Tabak *et al.* (1964) 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 by *Wyman et al.* (1979) 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 as HL PM-1, can induce the enzymes of 2,4-DNP catabolism in the presence of picric acid. Later Heiss *et al.* (2002) 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).

Rajan *et al.* (1996) 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). 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).

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 allowing 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 the assembly of a long contiguous sequence encoding 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). This paper illustrates cloning, expression and purification of the *orfB* contained in the *npd* gene cluster.

Materials and Methods

Bacterial strains, culture growth conditions and plasmids. 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.

Table 1. *Escherichia coli* strains used for DNA manipulations, overexpression and transformations

Bacterial strains	Relevant characteristics	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
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

Table 2. Plasmids constructed in this work

Plasmids	Relevant features	Source
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

Cloning, 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).

Molecular techniques. Standard protocols were used for manipulation of DNA according to Ausubel *et al.* (2001), 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).

Results and Disscusion

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 (Fig. 1.) 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. After an SDS-PAGE gel comparison, more than half of the protein resided in the cells as inclusion bodies (Fig. 2.). 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.

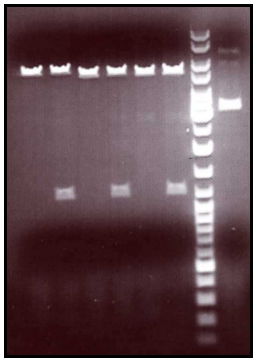

Figure 1. 0.8% agarose gel electrophoresis (1x TAE buffer, 100 V) results showing *Nde*I and *Xho*I digestions of vectors and their respective recombinant plasmids. Lane 2: pARS3, lane 4: pARS4, lane 6: pARS5, lane 7: GeneRuler™ DNA Ladder Mix, lane 8: uncut control.

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.

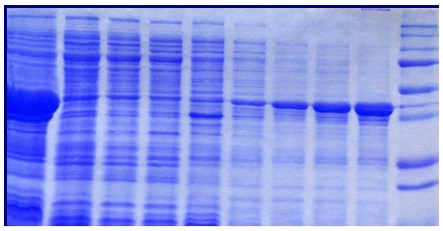

Figure 2. 12% SDS-PAGE (1x SDS running buffer, 100 V) presenting solubilized *orfB* proteins from inclusion bodies using urea. Lane 1: P = pellet sample; lanes 2-9: 0.5 – 4.0 M urea concentrations; lane 10: Precision Plus Protein™ standards.

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 polypeptides. In the present study, OrfB-His recombinant protein also aggregated into inclusion bodies (Fig. 2.). 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). A different protein expression system (the pET system) from Novagen was used in this work and the possibility that OrfB had a toxic effect on the heterologous *E. coli* host can be certainly excluded.

Conclusion

Following the analysis of the data presented in this research, some conclusions could be drawn related 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. 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. In addition, 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 (DNCH) if the non-enzymatic reaction is too slow under physiological conditions. Future work should include chemical synthesis of the 2,4-DNCH and enzyme-substrate studies.

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BIODEGRADACIJA 2,4,6-TRINITROFENOLA (PIKRINSKE KISELINE) I IDENTIFIKACIJA METABOLIČKIH GENA

- originalni naučni rad -

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Rezime

Biodegradacija zagađivača korišćenjem mikroba postala je vodeća tehnologija u prečišćavanju kontaminiranih sredina. U ovom radu, ostvarena je molekularna karakterizacija *orfB* iz nitrofenol degradacijske (*npd*) grupe gena. Gen je kloniran kao His tag spojen rekombinantni protein i ekspresovan u *E. coli*. Teorijsko pI / Mw predviđanje za sekvencu proteina bilo je 5.47 / 42536.23 Da. SDS-PAGE analiza prikazala je dimenziju čistog spojenog proteina, koja je izračunata i dostigla 42.5 kDa. Protein je prečišćen primenjujući Ni-NTA hromatografiju.