UDC: 579.222:662.235.1]:579.873
original scientific paper

Acta Agriculturae Serbica, Vol. XIII, 26 (2008) 97-103

Acta Agriculturae Serbica
FACULTY OF AGRONOMY • ČAČAK •

Rhodococcus opacus and *Nocardioides simplex* Metabolites and Enzymes During Biodegradation of Picric Acid

Ana Rebic
School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, South Africa

Abstract: Nitrophenols and polynitrophenols are introduced into the environment through the anthropogenic activity. As a consequence of their uses over the years there are many places heavily contaminated with these xenobiotics. Picric acid (trinitrophenol) is a toxic yellow odourless xenobiotic biodegradable by many microorganisms. Several metabolites and enzymes have been detected and purified from *Rhodococcus opacus* HL PM-1 and *Nocardioides simplex* FJ2-1A as parts of productive catabolic route. In this paper a comparative analysis of known metabolites and enzymes has been presented. It also suggests a catabolic potential and evolutionary implications of large linear megaplasmids detected in both strains since the bacteria are not evolutionary related.

Key words: xenobiotic, biodegradation, picric acid, megaplasmids.

Introduction

Picric acid (TNP) is a toxic yellow odourless crystalline solid that melts at 122°C, 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 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 having 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 (Morrison and Boyd 1992). In addition, the

electron-withdrawing character of the nitro groups results in a highly deficient π-electron system. As a consequence, catabolic pathways initiated by oxygenation are unknown for these xenobiotics (Lenke *et al*. 2000).

A historical overview of known picric acid biodegraders has been described previously (Rebić 2008). Two bacterial genera, *Nocardioides* and *Rhodococcus*, notably, *N. simplex* FJ2-1A and *R. opacus* HL PM-1 use picric acid in their metabolism as a sole source of nitrogen. In addition, Rieger *et al.* (1999) illustrated that *R. opacus* HL PM-1 can use TNP as a sole carbon, nitrogen and energy source. Both microorganisms are Gram-positive Actinomycete, which degrade TNP by the same initial mechanism. While working with these organisms, intriguing questions regarding genomes and proteomes arose since the *npd* gene cluster identified could not provide all the answers. It was described previously that three aromatic nucleus hydrogenations followed by a hydrolytic ring cleavage are the four stages of the proposed upper picric acid biodegradation pathway (Hofmann *et al*. 2004). Comprehension of the bacterial metabolism, metabolic intermediates and enzymes involved in the biodegradation of any xenobiotic is very important for understanding the way a compound is broken down.

This paper presents a comparative analysis of some metabolites and enzymes detected and purified during the biodegradation of picric acid. It also gives an insight into future research efforts since the pathway has not been confirmed yet.

Materials and Methods

Bacterial strains and growth conditions

Nocardioides simplex FJ2-1A was cultured under identical conditions as explained previously (Rebić 2008).

Rhodococcus opacus HL PM-1 and *R. opacus* AR1 (mutant of HL PM-1, deficient in *orfB*) were cultured in Luria-Bertani medium (LB) 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 for *N. simplex* FJ2-1A]. The medium was 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 2,4-Dinitrophenol (DNP) or 2,4,6-Trinitrophenol (TNP) was added at an optical density at 600 nm of 0.5-1.0 and culturing was continued for 3-4 hours.

Molecular techniques

Standard protocols were used for DNA manipulation and gel electrophoresis (Sambrook *et al*. 1989 and Ausubel *et al*. 2001).

Results and Discussion

Collectivity of data on independent research efforts indicates a few differrences between the two bacterial strains, *N. simplex* and *R. opacus*.

Tab. 1. A comparison of the two picric acid biodegraders

Features	*N. simplex* FJ21-A	*R. opacus* HL PM-1	Reference
Megaplasmid(s)	1 (97 kbp)	3 (190 kbp, 300 kbp and 312.5 kbp)	Dang, unpublished This work
Picric acid pathway	constitutive	Inducible	Rus *et al*. unpublished Dang, unpublished
Gene cluster	not known	*Npd*	Rus *et al*. 2000
Picric acid solid medium	decolourised	red colour	This work
Initial TNP hydrogenation	single Hydride transferase I	Hydride transferase II Hydride transferase I NADPH-dependent F_{420} reductase	Ebert *et al*. 1999 Hofmann *et al*. 2004

kbp = Kilo base pairs

In the *npd* gene cluster *orfB* is localized next to the first intergenic region, which contains a promoter (Hofmann *et al*. 2004, Dang *et al*. 2004). To confirm that 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 1 below shows the growth curves and the expected extremely long lag phase, 48 hours for *R. opacus* HL PM-1 and 96 hours for the mutant AR1. The two curves indicate that the strain AR1 required a longer period of adjustment 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. Results in Figure 1 also confirmed that either chemical could be used as the inducer during biodegradation of the picric acid.

Furthermore, a comparative analysis of some enzymes identified and purified during picric acid metabolism is summarized in Table 2 below. Difficult cell lysis of *R. opacus* changed a course of research to focus on purification of some enzymes from *N. simplex*, although activity for the same enzymes was detected in *R. opacus* (Heiss *et al.* 2002, Hofmann *et al.* 2004). Future work may include purification of the nitrite eliminating enzyme and gene cloning from *R. opacus* since some peptide sequences have been obtained already (Hofmann *et al.* 2004; Rebić 2008). Isolation and DNA sequencing of the large megaplasmids could also facilitate our understanding of the bacterial genomes. It is not excluded that a possible *npd* gene cluster from *N. simplex* could be plasmid borne.

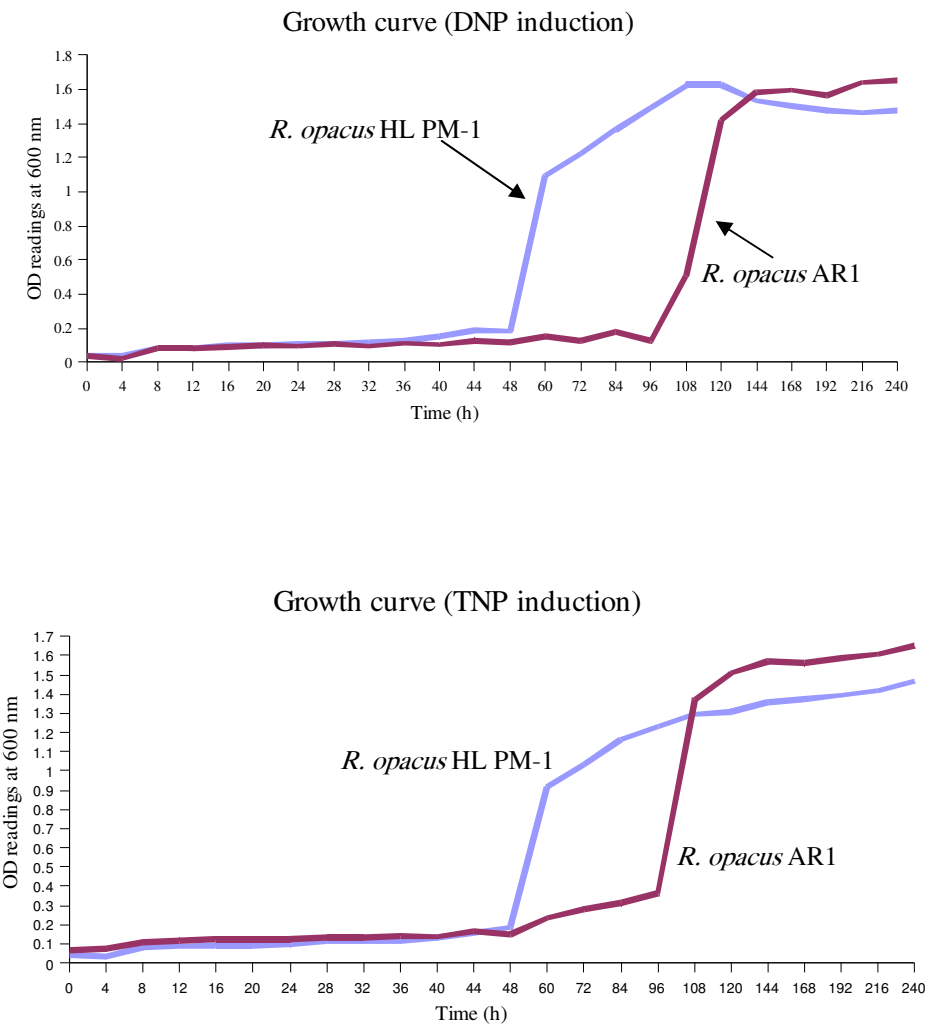

Fig. 1. Growth curves of *R. opacus* strains using different inducing agents. The inducing agent was added at t=0. Growth was achieved with 0.5 mM picric acid as a sole nitrogen source. Increase in cell density was determined photometrically at 600 nm.

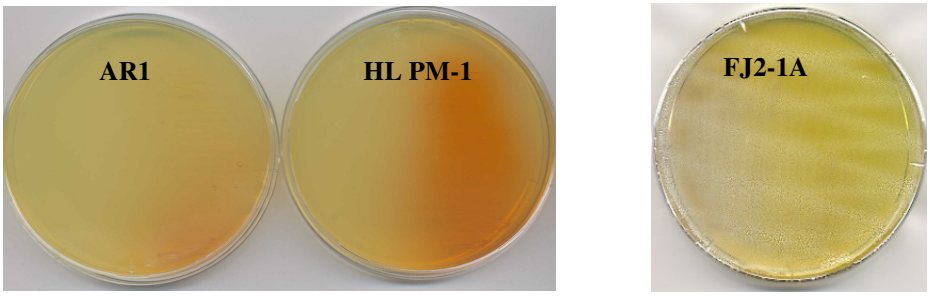

Fig. 2. Hydride-Meisenheimer complex released into the environment by *R. opacus* AR1 and HL PM-1 during picric acid biodegradation (left). Decolourisation of the TNP medium by *N. simplex* FJ2-1A (right).

Tab. 2. Enzymes detected and purified during picric acid

Enzyme	***N. simplex* FJ21-A**	***R. opacus* HL PM-1**
NADPH-dependent F_{420} reductase	His-tag fusion protein	His-tag fusion protein
Hydride transferase II	His-tag fusion protein	His-tag fusion protein
Hydride transferase I	Overexpressed in *E. coli* BL21	His-tag fusion protein
Tautomerase	His-tag fusion protein	His-tag fusion protein
Nitrite-eliminating enzyme	Purified by FPLC	Activity in crude extracts
Hydrolase	Purified by FPLC	Activity in crude extract

Conclusion

Although some enzymes have been identified and characterized during TNP metabolism, the pathway was proposed, but this was certainly insufficient to confirm the actual biodegradation. Nevertheless, elucidation of the nitrophenol catabolic genes from *N. simplex* is an imperative to deepen our knowledge regarding TNP degradation. There is always a possibility that *N. simplex* carries another hydride transferase in its genome since the sequence similarity is not that high. In addition, a detailed study of the regulation regions in *N. simplex* is also necessary.

References

Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A., Struhl K. (2001): *Current protocols in molecular biology*. John Wiley and Sons, Inc., New York, N.Y.

Dang P.N., Altenbuchner J., Heiss G.H. (2004) NpdR, a repressor involved in 2,4,6-trinitrophenol degradation in *Rhodococcus opacus* HL PM-1. *J. Bacteriol.,* **186(1)**: 98-103.

Ebert S., Rieger P.-G., Knackmuss H.-J. (1999) Function of coenzyme F420 in aerobic catabolism of 2,4,6-trinitrophenol and 2,4-dinitrophenol by *Nocardioides simplex* FJ2-1A. *J. Bacteriol.,* **181(9)**: 2669-2674.

Heiss G., Hofmann K.W., Trachtmann N., Walters D.M., Rouviere P., Knackmuss H.-J. (2002): *npd* gene functions of *Rhodococcus (opacus) erythropolis* HL PM-1 in the initial steps of 2,4,6-trinitrophenol degradation. *Microbiology,* **148**: 799-806.

Hofmann K.W., Knackmuss H.-J., Heiss G. (2004): Nitrite elimination and hydrolytic ring cleavage in 2,4,6-trinitrophenol (picric acid) degradation. *Appl. Environ. Microbiol.,* **70(5)**: 2854-2860.

Lenke H., Achtnich C., Knackmuss H.-J. (2000): Perspectives of bioelimi-nation of polynitroaromatic compounds. *In* Spain J.C., Hughes J.B., Knackmuss H.-J. (eds.), *Biodegradation of Nitroaromatic Compounds and Explosives*. CRC Press, Boca Raton, FL., 91-126 pp.

Morrison T.R., Boyd R.N. (1992): *Organic Chemistry*, 6th edition. Prentice Hall International, Inc., Englewood Cliffs, New Jersey. Pg. 493-545, 889-912.

Rebić A. (2008): Bioremediation and Cloning of a DNA Fragment That Encodes the Nitrite- Eliminating Enzyme Activity. *Acta Agriculturae Serbica,* **25**: 69-75.

Rebić A. (2008) Biodegradation of 2,4,6-Trinitrophenol (Picric Acid) and Identification of Metabolic Genes. *Acta Agriculturae Serbica*, **25**: 87-93.

Rieger P.G., Sinnwell V., Preuss A., Francke W., Knackmuss H. -J. (1999): Hydride-Meisenheimer complex formation and protonation as key reactions of 2,4,6-trinitrophenol biodegradation by Rhodococcus erythropolis. *J. Bacteriol.,* **181**: 1189-1195.

Rieger P.-G., Meier H.-M., Gerle M., Vogt U., Groth T., Knackmuss H.-J. (2002): Xenobiotics in the environment: present and future strategies to obviate the problem of biological persistence. *J. Biotechnol.,* **94**: 101-123.

Russ R., Walters D.M., Knackmuss H.-J., Rouviere P.E. (2000): Identifi-cation of genes involved in picric acid and 2,4-dinitrophenol degradation by mRNA differential display. *In* Spain J. C., Hughes J.B., Knackmuss H.-J. (eds.), *Biodegradation of Nitroaromatic Compounds and Explosives*. CRC Press, Boca Raton, FL., 127-143 pp.

Sambrook J., Fritsch E.F., Maniatis T. (1989): *Molecular Cloning: A Laboratory Manual*. 2nd edition. Cold Spring Harbor Laboratory Press, USA.

Rhodococcus opacus i *Nocardioides simplex* METABOLITI I ENZIMI ZA VREME BIODEGRADACIJE PIKRINSKE KISELINE

- originalni naučni rad -

Ana Rebić
Witwatersrand Univerzitet, Johanesburg, Južna Afrika

Rezime

Nitrofenoli i polinitrofenoli su uvedeni u čovekovu životnu sredinu kroz antropogene delatnosti. Kao ishod njihovih upotreba tokom godina, mnoga mesta su jako kontaminirana sa ovim ksenobioticima. Pikrinska kiselina (trinitrofenol) je toksičan, zuti, bezmirisni ksenobiotic, biodegradativan mnogim mokroorganizmima. Nekoliko metabolita i enzima su detektovani i purifikovani iz *Rhodococcus opacus* HL PM-1 i *Nocardioides simplex* FJ2-1A kao delovi produktivne katabolicke putanje. U ovom radu, komparativna analiza do sada poznatih metabolita i enzima je prezentovana. Ovaj rad nudi sugestije o katabolickom potencijalu i evolutivnim implikacijama velikih linearnih megaplazmida detektovanih u oba soja bakterija budući da nisu evolutivno srodne.