UDC: 579.222:577.214
original scientific paper

Acta Agriculturae Serbica, Vol. XIII, 25 (2008) 69-75

Acta Agriculturae Serbica FACULTY OF AGRONOMY • ČAČAK •

Bioremediation and Cloning of a DNA Fragment That Encodes the Nitrite-Eliminating Enzyme Activity

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

Abstract: Bioremediation tends to be an effective technology used to remove pollutants and involving relatively little capital. This study 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. A genomic library using *Nocardioides simplex* FJ2-1A total DNA was constructed. The number of clones required to ensure a 99% probability of a given DNA sequence being contained within a library composed of 10 kb inserts was 2305. The similarity with fatty acid hydroxylase from *Nocardioides* sp. JS6614 was detected.

Key words: bioremediation, nitrite-eliminating enzyme, picric acid.

Introduction

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 means 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 are 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 the behaviour of a single bacterial genus in the terrestrial environment and a pathway by which the organism degrades a xenobiotic is a prerequisite for a successful bioremediation strategy.

The nitrite elimination reaction from the *aci*-nitro form of dihydride Meisenheimer complex of TNP ($2H^-$-TNP) during the picric acid biodegradation pathway was of 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 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 the 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. This paper illustrates cloning of a DNA fragment encoding nitrite-eliminating enzyme activity using the reverse genetics approach. Also it suggests a bioremediation system since the pathway is proposed and not confirmed.

Materials and Methods

Bacterial strains, culture conditions, vectors and their 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 an appropriate antibiotic.

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 $MgSO_4 \times 7H_2O$, 50 mg/L of $CaCl_2 \times 2H_2O$ and 1 ml of SL6 trace element solution (100 mg/L $ZnSO_4 \times 7H_2O$, 30 mg/L $MnCl_2 \times 4H_2O$, 300 mg/L H_3BO_3, 200 mg/L $CoCl_2 \times 6H_2O$, 10 mg/L $CuCl_2 \times 2H_2O$, 20 mg/L $NiCl_2 \times 6H_2O$, 30 mg/L $Na_2MoO_4 \times 2H_2O$). 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.

Table 1. Plasmids and vectors used in this study

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
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

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).The genomic library was made with *N. simplex* FJ2-1A total DNA using λBlueSTAR™ Vector System (Novagen®). Internal protein sequencing was performed by TopLab (Martinsried).

Results and Discussion

FPLC enrichment of the NEE and peptide sequencing. The nitrite-eliminating enzyme was purified from *N. simplex* FJ2-1A by Hofmann *et al.* (2004). An FPLC enrichment was performed 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. Upon detection of the prominent band of the NEE, the protein was excised from the gel 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 digestion, the resulting peptides were separated on a 320 μm x 150 mm capillary HPLC column. 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.

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 designed using the highest frequencies from the codon table and the reverse primer was designed 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). It was necessary to perform 2 step PCR due to different annealing temperatures of the primers. The PCR product was cloned and sequenced showing respective amino acid sequence (Tab. 2.).

Partial gene sequence encoding the putative nitrite-eliminating enzyme was further analysed. Homology searches were performed with BLASTN, BLASTP and BLASTX Altschul *et al.* (1990). The similarity with fatty acid hydroxylase (246 amino acid residues) from *Nocardioides* sp. JS614 was detected (Fig. 1.).

Table 2. 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

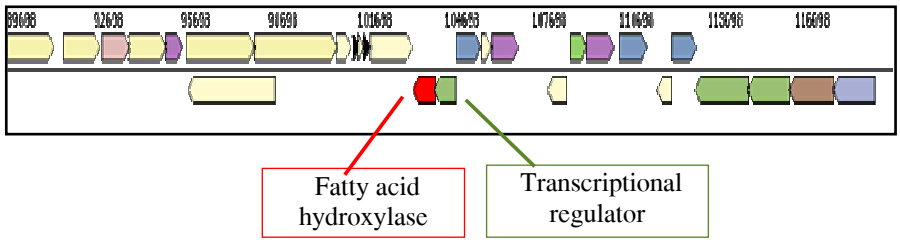

Figure 1. Chromosomal view of the *Nocardioides* sp. JS614 indicating a location of the putative NEE gene.

Furthermore, the genomic library was made with *N. simplex* total DNA using λBlueSTAR™ Vector System (Novagen®). 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 screening was achieved using a colony PCR. New set of primers were designed based on the partial gene sequence. The forward primer sequence was 5'-atg aag aac ctg gag ctg gcc tac gtc ggc ctg gtc-3' and the reverse one was 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. The new recombinant was designated as pARS6. The screening of the gene bank additionally identified four positive clones (Fig. 2.).

After restriction analysis of the positive PCR clones, it was evident that three clones showed identical digestion pattern (Fig. 2.) 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. It was very interesting to see that the small DNA fragment (Tab. 2.) showed 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 indicates its own transcriptional regulator. This work certainly hints the possible parallel pathway, which is turned on during the biodegradation of picric acid.

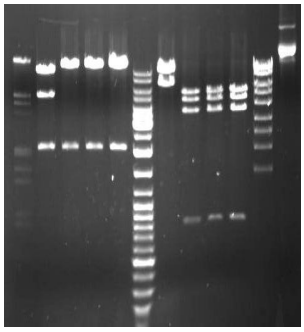

Figure 2. 0.8% agarose gel in 0.5x TBE, 100 V showing single digestions (*Bam*HI and *Bgl*II) of the clones. Standards used were GeneRuler™ DNA Ladder Mix and MassRuler™ DNA Ladder, High Range.

Conclusion

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 the remediation effort (Philp *et al.*, 2005).

An aerobic sequencing batch reactor for picric acid biodegradation seeded with *R. opacus* strain JW01 was reported by Weidhaas *et al.* (2007). In the future, the same could be applied to *N. simplex* and *R. opacus* HL PM-1. 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. Pivotal work on the nitrite-eliminating enzyme in this study has opened many questions. With respect to the current knowledge, one could attempt to answer the following questions:

- Where does the gene come from and is there indeed 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?

References

Altschul S.F., Gish W., Miller W., Myers E.W., Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.*, 215, 403-410.

Atlas R., Unterman R. (1999) Bioremediation. In: Demain A.L., Davies J.E. (eds.), *Manual of Industrial Microbiology and Biotechnology, 2nd edition*, ASM Press, Washington, D.C., USA, 666-681 pp.

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.

Burden D.W., Whitney D.B. (1995: *Biotechnology: Proteins to PCR*, A Course in Strategies and Lab Techniques. Birkhauser, Boston.

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.

Inoue H., Nojima H., Okayama H. (1990) High efficiency transformation of Escherichia coli with plasmids. *Gene*, 96, 23-28.

Philp J.C., Bamforth S.M., Singleton I., Atlas R.M. (2005) Environmental polution and restoration: a role for bioremediation. In: Atlas, R.M., Philp, J.(eds.) *Bioremediation: applied microbial solutions for real-world environmental cleanup*, ASM Press, Washington, D.C., 1-48 pp.

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.

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

Weidhaas J.L., Schroeder E.D., Chang D.P.Y. (2007) An aerobic sequencing batch reactor for 2,4,6-trinitrophenol (picric acid) biodegradation. *Biotechnology and Bioengineering*, 97(6), 1408-1414.

BIOREMEDIJA I KLONIRANJE DNK FRAGMENTA KOJI KODIRA AKTIVNOST NITRO-ELIMINACIONOG ENZIMA

- originalni naučni rad -

Ana Rebić
Witwatersrand Univerzitet, Johanesburg, Južna Afrika

Rezime

Bioremedija teži da postane efektivna tehnologija, upotrebljavana u otklanjanju zagađivača i obuhvata relativno mali kapital. Istraživanjem je identifikovan DNK fragment, koji kodira aktivnost nitro-eliminacionog enzima (NEE). Ovo je ostvareno obogaćivanjem aktivnosti uz pomoc FPLC hromatografije i sekvenciranjem proteina. Dve unutrašnje sekvence su napravljene: L P G D Y T D Q L L R i S G A I V G L G H A Q V D R. One su iskorištene za izradu degenerisanih *primera* sa ciljem da se izvuče klon iz nokardijskog genoma. Konstruisana je genomska biblioteka koristeći *Nocardioides simplex* FJ2-1A celokupnu DNK. Broj klonova potreban da obezbedi 99% verovatnocu da se data DNK sekvenca sadrzi u biblioteci, sačinjen od 10 kb inserta, bio je 2305. Otkrivena je sličnost sa hidroksilazom masnih kiselina od *Nocardioides* sp. JS6614.