

NITROREDUCTASES FROM THERMOPHILIC BACILLUS SPECIES

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Abstract: Nitroreductases (NTR) are members of the NAD(P)H/FMN oxidoreductase that exists widely in bacteria. NTRs have raised a great interest due to their potential in biomedicine, especially in prodrug activation for chemotherapeutic cancer treatments, in bioremediation and enzyme-based biosensors for nitro-sensitive compounds. Thermophilic *Bacillus* species isolated from hot water springs in Turkey were identified by 16S rRNA gene sequence analysis. The previously identified species, *Bacillus paralicheniformis* (5NK) and *Bacillus paralicheniformis* (FMB2) with sequence similarity of 97% and 99.65, respectively, were used. They were all found to possess the putative NADPH nitroreductase genes. Genomic DNAs for isolation of nitroreductase genes were isolated by conventional phenol-chloroform extraction method. The nitroreductase genes from thermophilic *Bacillus* species were amplified by PCR, using the designed three primer pairs. The corresponding DNA fragments were fractionated by agarose gel electrophoresis and PCR products were purified and concentrated for cloning and DNA sequencing.

Keywords: Nitroreductases, Thermophilic *Bacillus*, PCR

Introduction

Most nitroaromatic compounds are toxic for living organisms, which are released to the environment during manufacturing and handling, through filtration and losses of the storage tanks and during transport and intensive military activities, especially TNT and other explosives. There have been human health risk with regard to nitrosubstituted compounds due to the metabolites causing genotoxic and mutagenic effects and the generation of reactive nitrogen oxide species, which readily react with biological macromolecules (Cortial et al. 2010).

It has been found that various microorganisms have developed reductive pathways to degrade or transform these compounds. Bacterial nitroreductases including *Bacillus* species are flavoenzymes that catalyze the NAD(P)H-dependent reduction of the nitro groups on nitroaromatic and nitroheterocyclic compounds. Therefore, the nitroreductases are used for several biotechnological applications for bioremediation, as biosensors and for clinical uses (Gwenin et al. 2015, Roldán et al. 2008, Chaignon et al. 2006).

Particularly, prodrug activation gene therapy is a promising approach to cancer treatment, whereby prodrug-activating enzymes are expressed in tumour cells. Following administration of a non-toxic prodrug, the nitroreductase enzyme expressed in tumour cells converts it to cytotoxic metabolites which directly kill the cancer cells. The enzyme nitroreductase, isolated from *Escherichia coli* B, converts CB1954 ((5-aziridin-1-yl)-2,4-dinitro-benzamide) into a potent DNA-crosslinking cytotoxic agent that kills tumor cells (Drabek et al. 1997).

The use of any prodrug activating enzyme is, however, limited by factors such as its stability of which is needed when for example conjugated to an antibody. From this point of view, enzymes from thermophilic organisms have considerable potential because they are resistant and remain fully active under denatured conditions compared to most enzymes from mesophilics. On the other hand, thermophilic stable enzymes still need to have sufficient catalytic activity at 37 °C if they are utilised in prodrug therapy (Emptage 2009).

Turkey has lots of different ecological areas, which possesses a broad microbial diversity. Turkey is well known for its geothermal activity and there are so many thermal springs all over the country. Therefore, there should be a great deal of opportunities for newly isolated microorganisms from extreme environments, including thermophilic ones with numerous biotechnological applications. For many decades, the *Bacillaceae* family members have been good sources in biotechnological processes concerning whole cells or enzymes. In Turkey, the isolated and identified thermophilic members of the *Bacillaceae* family include *Anoxybacillus*, *Geobacillus*, *Bacillus*, *Brevibacillus*, *Aeribacillus*. Members of *Bacillus* genus are well known to be widespread all over the world in various extreme and geographical areas including hot springs of Turkey (Güven et al 2018).

In the present study, we report on the identification and characterization of nitroreductase genes from the Gram-positive thermophilic *Bacillus* species isolated from hot springs in Turkey.

Material and Methods

Bacteria, Plasmids and Media

Bacillus paralicheniformis 5NK, *Bacillus paralicheniformis* FMB2 and *E. coli* DH5 α strains used for transferring the nitroreductase gene in to pET28a + vector.

Bacterial strains and *E. coli* DH5 α maintained at -20 ° C in medium containing 30% v / v glycerol. Bacterial strains were produced in Luria Bertani Broth (LB) medium (Bacto Trypton 10 g / L, Bacto-Maya Extract 5 g / L, NaCl 10 g / L, pH 7.0), 40-55 ° C and aerobic conditions. *E. coli* DH5 α strains were produced at 37 ° and aerobic conditions in LB broth. 1.5% w / v agar was added to breeding media for solid medium.

Isolation of chromosomal DNA from *Bacillus* strains

Bacteria were produced in aerobic conditions in LB medium and chromosomal DNA was isolated from the produced bacteria using the Thermo Fischer Scientific DNA isolation kit. The amount and purity of DNA in the spectrophotometer was measured and prepared for the PCR reaction.

Agarose Gel Electrophoresis

For the volume required, SeaKem® GTG® pure agarose was weighed and added to the volume of 1X TAE buffer in a mulled beer and kept on the flame until dissolved. After cooling to the tangible temperature (45-50 ° C), the EtBr solution was added to give a final concentration of 0.5 μ g / mL. The gel was poured carefully into the container and gel tray was placed and waited for about 40 minutes to freeze. 1X TAE buffer was added to the gel electrophoresis tank until it was covered. DNA samples (3 μ L BFB and 10 μ L DNA) were inserted into the wells formed by removing the comb. One DNA standard (4 μ L) was loaded from the wells to calculate the DNA size. After the progress of the molecules in the electric field, the gel was kept in the pure water for 10 minutes and the gel was observed by translucinator.

PCR

A nucleotide BLAST search of the *Bacillus* genome was performed using the *nfnB* gene sequence of *E. coli*. *nfnB* gene was identified as BC_1619a: putative oxygen-insensitive NADPH nitroreductase, Primers were obtained from Sentegen (Ankara/Turkey). Primers for amplification of the *nfnB* gene were ATAGGATCCATGACTAACTCAGTAAAGAC (5' primer) and ATCAAGCTTTTATTTCCATTCAGCAAC (3' primer), *Bam*HI and *Hind*III sites are underlined. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN Ltd., UK) according to the manufacturer's instructions. The purity and approximate size of the PCR products were confirmed using agarose gel electrophoresis.

Cloning

The purified PCR products obtained from the bacteria and the vector DNA to be used for cloning were partially cleaved by *Bam*HI and *Hind*III restriction enzymes. 500 ng of chromosomal DNA and 50 ng of plasmid DNA digested with *Bam*HI and *Hind*III were ligated. Prior to transformation, the ligation mixture was subjected to ethanol precipitation and ligase samples were inactivated by incubation at 65°C for 15 min prior to transformation. The inactivated self ligation and ligation samples were transformed with the prepared competent bacteria. All of the liquids in the transformation tube were first separately produced at 37 ° C for 1 hour, poured into S.O.C liquid media. With this application, the bacteria that have become sensitized by the competing processes are returned to their normal state, and the adaptation of the bacteria to the antibiotic fattening, which will be inoculated after one hour, is provided. At the end of one hour, the bacterium was removed and plated on a LB/Kanamycin petri dishes, and the plates were dried for 5-10 min, then inverted and left at 37 ° C for 1 night. The next day, transformants were transformed with recombinant (expanded) and non-recombinant (widely closed) plasmid DNAs. Recombinant colonies were selected by means of a sterile toothpick and transferred to tubes containing 10 mL of LB/Amp100 nutrient and produced at 37 ° C with shaking for 1 night and plasmid isolation was performed as described below with the aid of QIAGEN mini-prep kit. The amount of plasmid DNA was measured and then the purity was tested by agarose gel. Sequence analysis was carried out by Sentegen (Ankara/Turkey). Nitroreductase gene sequences were examined in the Blast program at the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/BLAST/>), CLC Workbench v 4.0 Software (CLC bio, Aarhus, and other *Bacillus* species, and their homology affinities were determined.

Results and Discussion

Thermophilic *Bacillus* species isolated from hot water springs in Turkey were identified by 16S rRNA gene sequence analysis. The previously identified species, *Bacillus paralicheniformis* (5NK), *Bacillus paralicheniformis* (FMB2), with sequence similarity of 97% and 99.65% respectively, were used. Genomic DNAs of *Bacillus* species were isolated and shown in Figure 1.



Figure 1: Genomic DNAs of *Bacillus* species, **B2:** Genomic DNA of *Bacillus paralicheniformis* (FMB2), **5NK:** Genomic DNA of *Bacillus paralicheniformis* (5NK), **L:** DNA marker (New England Biolabs)

One gene from *Bacillus* species were successfully amplified during PCR using the primer BC_1619a (putative oxygen-insensitive NADPH nitroreductase) among three primers designed (Figure 2). The primers were designed on the basis of *Bacillus cereus* nitroreductase genes which have been found to metabolise prodrug CB1954 and its metabolites as superior cell killing ability, which were a promising candidate for enzyme prodrug therapy (Gwenin et al. 2015).

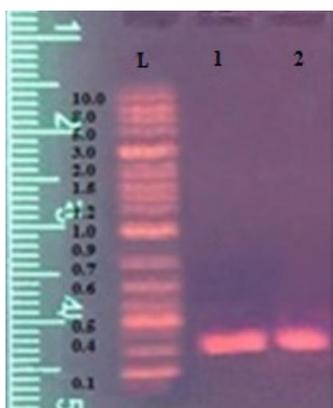


Figure 2: PCR products, **L:** DNA marker (New England Biolabs), **1:** PCR products of *Bacillus paralicheniformis* (FMB2), **2:** PCR products of *Bacillus paralicheniformis* (5NK)

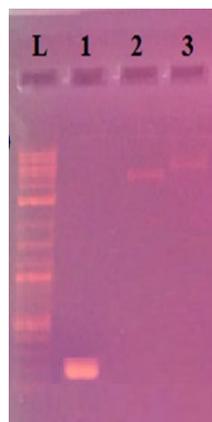


Figure 3: **L:** DNA marker (New England Biolabs), **1:** PCR products of *Bacillus paralicheniformis* (5NK) after ligation, **2:** pET28a+, **3:** Ligation (double digested DNA of strain 5NK and double digested pET28a+)

These PCR products were successfully inserted into pET28a+ expression vector, which consists N-terminal His-tag for ease of protein purification (Figure 3).

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