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Short communication

Identification of a new operon involved in desulfurization of dibenzothiophenes using a metagenomic study and cloning and functional analysis of the genes

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ABSTRACT

The presence of sulphur-substituted hydrocarbons in fossil fuels are one of main reasons for the release of sulfur oxides into the environment. Dibenzothiophenes (DBT) are organic sulfur-containing molecules in crude oil, which have the potential for biological oxidation, with the sulphur being removed through an enzymatic cleavage of the C—S bonds. Therefore, finding new strains that can desulfurize this compound has recently become a point of interest. In this study, three new genes involved in the bacterial desulfurization of Dibenzothiophene, which were sequenced in the course of a metagenomic study, were isolated by PCR amplification in the laboratory. The activities of these genes were then analysed following insertion into an expression vector and cloning in *Escherichia coli* DH5α cells. Based on the results, all three genes were actively expressed and their products could act on their corresponding substrates.

1. Introduction

Fossil fuels, including crude oil and coal, are both the main source of energy and the main feed stock for the petrochemical industries, and therefore their usage in industries constantly increases [1]. However, these fossil fuels contain high levels of nitrogen and sulphur-substituted hydrocarbons [2]. The level of sulfur content in crude oils varies between 0.03%–7.89% (w/w) [3]. The combustion of these types of hydrocarbons leads to the production and release of nitrogen/sulfur oxides, which are considered as the main source for the formation of acid deposition [4]. Although there are many methods, such as post-combustion desulfurization techniques, for the removal of these elements from these fossil fuels, they are expensive and inconvenient [5]. Furthermore, while inorganic sulfur compounds, including elemental sulfur, sulphide, sulphite, sulfate and thiosulfate, are easily removed from crude oil and coal using physical and chemical treatments [6], removal of most of organic sulfur containing compounds, such as dibenzothiophene (DBT), benzothiophene and their derivatives is not easily achieved [5,7]. These concerns and increased considerations regarding the acid deposition issues, has increased the interest in the use of microbial sulfur transformation reactions.

Dibenzothiophene and its derivatives, are considered as the prototype for the organic sulfur-containing molecules in crude oil, and much effort has already been put into the development of a biological oxidative approach to the removal of sulfur from these compounds using the enzymatic cleavage of the C—S bonds [7]. The majority of DBT desulfurizing microorganisms and their DBT desulfurizing operons have been found using traditional culture and molecular based approaches [8–15]. However, these systems are not able to identify the majority of these microorganisms in natural sources [16]. Metagenomic approaches; however, are theoretically able to show the whole genome contents of the microbial community present in an environment [17]. In a metagenomic study on crude oil, we obtained a list of microorganisms and the genes involved in DBT desulfurization (unpublished data). Although many of these genes have already been identified and can be found in public data bases, a few were quite different. In this study, we focussed

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Table 1
List of primers designed specifically for amplification of the new dibenzothiophene desulfurization operon.

Genes	Forward	Reverse
DszA	Sul1F: GTCTGGAAAACGCACGTCTG	Sulf1R: CCTGTTTGCCGCTCAGAATG
DszB	Sul2F: TTGCACATGTTCACGATGGC	Sul2R: GCGGGCGATTTCAATGGAAT
DszC	Sul3F: GCGCCCGAGTTCTTGAATAAC	Sul3R: CCAGGTCTTTGCGTCAGACT

on one of these operons in order to obtain its full sequence and to study the desulfurizing activity of the enzymes produced.

2. Material and methods

2.1. Gene sequence and primer design

The original gene fragment obtained from the metagenomic study was expanded using Geneious software and reiterative mapping of short reads from the whole metagenomic data in order to obtain the full length operon sequence. This operon sequence was analysed using Geneious software to find suitable primers for amplification through PCR reactions.

2.2. Gene cloning and enzyme assay

DNA extractions was conducted on 100 ml crude oil using the power water DNA kit (MO BIO), according to the manufacturer's standard instructions. This volume of crude oil was filtered using a disposable filter (0.22 μm) to isolate and concentrate the microbial cells. The microorganisms on the filters were dissolved into 1 ml of solution PW1 (provided by the manufacture), and further DNA extraction processes were performed according to their instructions. PCR amplification reactions for each gene were performed separately using 1 μM of the primers designed by our team (Table 1); The PCR conditions are given in Table 1. The PCR products were purified by electrophoresis on agarose gels and the DNA from the purified bands was ligated with the expression vector pGEM-T Easy (Promega Madison, USA) vector according to the manufacture's instruction. The ligated plasmids were transformed into DH5α cells (provided from Bioline) using standard techniques and plated on plates allowing blue/white selection of recombinant plasmids. The white colonies obtained from growth of bacteria on Luria-Bertani (LB) medium supplemented with ampicillin (100 mg^{-1}) and X-gal were isolated and the inserts sequenced with the universal M13 primers (M13F: CGCCAGGGTTTTCCCAGTCACGAC and M13R: TCACACAGGAAACAGCTATGAC) [18] (using the SA Pathology sequencing facility at Flinders University, Adelaide, Australia).

Escherichia coli DH5α cells were employed as the host for all three genes. The cells were grown in LB broth supplemented with ampicillin (100 mg/ml) under 37 °C, 200 rpm for an overnight. When the cell absorbance reached 0.5, gene expression was induced by addition of IPTG for 2 h. These cell suspensions were centrifuged and the pellets were washed twice with 0.1 M potassium phosphate buffer (PBS) (pH 7.0). The pellets were then re-suspended into the same PBS buffer. These cell suspensions were lysed using sonication, and the supernatants were separated from cell debris using centrifugation (10,000 × *g*, 4 °C) for 5 min. Following addition of 9 mM NADH and 0.01 mM FMN to the supernatants, the concentration of cell-free lysates was adjusted to 2.0 mg/ml. All three substrates, dibenzothiophene (DBT), dibenzothiophene sulfone ($DBTO_2$) and 2-(2-hydroxyphenyl)-benzene sulfonate (HBPS), were separately dissolved as dimethylformamide solutions to a volume of 100 mM, and were mixed with an adequate of cell-free lysate mixtures to a final concentration of 1 mM. The enzyme activity of the DszA and DszC cloned genes were assayed using absorbance of NADH at 340 nm for 2 h (using UV.360-Shimadzu Spectrophotometry) in separate suspensions containing DszA and DszC recombinant *E. coli* DH5α and the corresponding substrates, and their comparison with this value in the suspensions containing the non-recombinant *E. coli* DH5α and the corresponding substrate for each enzyme. The enzyme activity of DszB was determined based on pH changes in the suspension containing DszB recombinant *E. coli* DH5α and substrate and comparison with this value in the non-recombinant *E. coli* DH5α.

2.3. Accession number

The nucleotide sequence data for these three desulfurization genes, were submitted to the GenBank databases under the accession numbers KT630579, KT630580 and KT630581 for genes ADRO1, ADRO2 and ADRO3 respectively.

3. Results and discussion

The dibenzothiophene desulfurizing bacteria harbour an operon containing three genes (DszA, DszB and DszC), which perform the desulfurization process in four steps though the 4S desulfurization pathway [19,20] (Fig. 1). The first two reactions, performed by dibenzothiophene monooxidase (DszC) convert DBT first to dibenzothiophene-sulfoxide and then to dibenzothiophene sulfone ($DBTO_2$) [21,22]. This intermediate product is later catalysed by $DBTO_2$ monooxygenase (DszA) to produce 2-(2-hydroxyphenyl)-benzene sulfonate (HBPS) [21,22]. The product of DszB gene (HBPS-desulfinase) releases sulfur from the compound and leaves 2-Hydroxybiphenyl (HBP). The first three steps are O_2-dependent oxidative reactions and require an electron and hydrogen transportation system, composed of $FMNH_2$ and NAD(P)H as a reductant [21,22]. Several gene fragments responsible for DBT desulfurization were found in a metagenomic study on crude oil and crude oil field soils (unpublished data). Although many of these fragments showed high similarity with the genes deposited in gene databases, several new sequences were also discovered in these datasets. Gene walking expansion of these fragments using Geneious software to reiteratively map short reads from the metagenomic data produced whole gene sequences from these fragments. Several of these gene sequences showed quite low degrees of similarities to the genes present in public databases. Further expansion of one of the gene sequences in this study produced a whole operon potentially encoding the genes responsible for DBT desulfurization. This operon carried three DBT desulfurizing genes, referred to as *dszA*, *dszB* and *dszC*, which showed low similarities to genes deposited in databases; *dszA* with 78% similarity to dibenzothiophene desulfurization enzyme A of *Gordonia* sp. RIPI, *dszB* with 73% similarity to 2′-hydroxybiphenyl-2-sulfinate desulfinase of *Gordonia* sp. SYKS2 and *dszC* with 77% similarity to dibenzothiophene desulfurizing enzyme C of *Gordonia* sp. RIPI [23]. These new DBT desulfurizing genes contained 1167 bp (*dszA*), 1116 bp (*dszB*) and 1242 bp (*dszC*) including initiation and stop codons and were nominated as ADRO1, ADRO2 and ADRO3 in the gene bank. The big differences in the similarity of sequences and the nucleotide numbers of these three genes in comparison to other corresponding dsz genes present in gene data banks indicated the existence of new sets of dsz genes belonging to uncultured microorganisms. The presence