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*Full Length Research Paper*

# Evaluation of the conserve flavin reductase gene from three *Rhodococcus* sp. strains isolated in Iran

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**Previously we isolated three native strains, *Rhodococcus* FRF, IPF and GAF from Tabriz and Tehran refineries soil samples in a search for flavin reductase gene (*dszD*). In this study, these three flavin reductase genes were isolated and identified by PCR using the nucleotide sequence of *dszD* gene from *Rhodococcus* sp. The amplified *dszD* DNA sequences were purified and cloned into the T-vector pTZ57R/T and then introduced to the *E. coli* DH5α. Further sequence analysis revealed that the oxidoreductase genes of *Rhodococcus* FRF, IPF and GAF share high similarity to that of *Rhodococcus erythropolis* IGTS8. There were differences only in a few number of nucleotide. Also oxidoreductase enzymes in all strains consist of 193 amino acids that differ only in one amino acid which is located at stop condon of *Rhodococcus* FRF.**

**Key words:** Biodesulfurization, *Rhodococcus* species, flavin reductase gene (*dszD*).

## INTRODUCTION

Fossil fuels contains various organosulfur compounds, including alkaylated dibenzotiophene (DBT), alkaylated benzothiophene (BT) and other thiophene compounds, which are released as sulfur oxyacids upon combustion and cause environmental problems such as acid rains. Therefore desulfurization of petroleum is necessary (Oshiro and Isumi, 1999). One strategy to reduce organic sulfur content in fossil fuel is to expose these substrates to microorganisms that can specifically break carbon-sulfur bounds and release the sulfur in a water-soluble, inorganic form (Monticello, 2000; Oldfield et al., 1997).

Most desulfurizing bacteria are nocardiaforms such as *Rhodococcus* which are considered feasible for petroleum desulfurization (Chang et al., 2000; Matsui et al., 2000). One of the most studied strains of this group is *Rhodococcus erythropolis* IGTS8 that can selectively remove covalently bound sulfur from DBT to form 2-hydroxybiphenyl (HBP) in an enzymatic way, without breaking carbon-carbon bounds (Denome et al., 1994). Three structural genes namely *dszA*, *dszB* and *dszC* have been isolated from *R. erytropolis* IGTS8 and shown to be responsible for desulfurization (Xi et al., 1997; Lizama et al., 1995). Two monooxigenases, DszA and DszC are involved in DBT desulfurization and involved an NAD(P)H/FMN oxidoreductase, encoded by *dszD* which supplies the monooxigenases with the necessary free-reduced flavin ($FMNH_2$) (Gray et al., 1996; Piddington et al., 1995).

Recently *Rhodococcus* sp. strain FMF has been isolated by Persian Type Collection Culture (PTCC) and it was shown to be capable of desulfurization. We have previously isolated and identified desulfurization operon from *Rhodococcus* FMF and it was shown that *dszABC* operon in key organisms of *Rhodococcus* sp. strain IGTS8 and *Rhodococcus* FMF were similar. In this study we isolated and identified the oxidoreductase genes (*dszD)* from three native strains including *Rhodococcus* FRF, IPF and GAF. A comparative sequence analysis provided an evidence of the conserved nature of the *dszD* genes in these three strains.

## MATERIALS AND METHODS

### Bacteria and plasmids

Strains and plasmids used in this study are listed in Tables 1 and 2.

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**Table 1.** Strains used in this study.

| Bacterial Strains | Phenotype | Source |
|---|---|---|
| E.coli DH5α | | NRCGEB |
| Rhodococcus IGTS8 | Biodesulfurization | Shahid Beheshti University |

**Table 2.** The plasmid used in this study.

| Plasmid and Vectors | Information |
|---|---|
| PTZ57R | InsT/A clone ™ PCR Product cloning |

### Chemicals

All restriction enzymes and InsT/A clone™ PCR product cloning kit were from Fermentase (Germany). Molecular weight marker, high pure plasmid purification kit, high pure PCR product purification kit and agarose gel DNA extraction kit were bought from Roche (Germany). All other chemicals were from Merck (Germany).

### Cultivation and genomic DNA extraction

*Rhodococcus* FRF, IPF and GAF were grown in Lauria Bertani broth at 30°C for 48 h, suspensions were centrifuged in 3000 RPM for 5 min and the pellet washed with EDTA (0.5 M, pH 8). Afterward cells were introduced into 10 ml lysis buffer containing lysozyme, RNase and proteinase K and then after incubation at 37°C for 30 min kept at -70°C till the next day. Then proteins were eliminated by two stages of phenol/chloroform and after separation and washing, the genomic DNAs were dissolved in distilled water.

### Plasmid isolation

Large scale preparation of plasmid DNA using a cesium chloride gradient was employed (Sambrook et al., 1989). Using this method, a large amount of high quality plasmid DNA was prepared without using RNase. A small scale preparation of plasmid DNA for identification or screening of recombinant plasmids was carried out using alkaline lysis method (Sambrook et al., 1989).

### Mini/Maxi plasmid purification

A quick and highly purified preparation of plasmid DNA was performed using a commercially available kit (Boehring Company/ Germany) and used according to manufacturer's instruction.

### PCR technique

In order to isolate *dszD* gene from *Rhodococcus* FRF, IPF and GAF, PCR technique was employed using to designed primers. The primers were as follow: 5'-GAA TTC ATG TCT GAC AAG CCG AAT GCC-3' (forward) and 5'-TCT AGA CTA TTG ACC TAA CGG AGT CGG-3' (reverse). Amplification was carried out using High Fidelity PCR Master Kit (Roche) and a Perkin-Elmer (USA) DNA thermal cycler. Amplification conditions were 94°C for 1 min (1 cycle), 94°C for 1 min, 55°C for 1 min, 72°C for 1 min (30 cycles) and a final 5 min at 72°C. The PCR products were purified using High Pure Product Purification kit (Roche).

### Cloning

Purified PCR products were cloned into the plasmid pTZ57R/T using InsT/A clone™ PCR Product Cloning Kit. This plasmid is comprised of ampicillin resistance marker bla site. lacZΔM15 mutation is necessary for blue/white selection. For transformation competent cells of *E. coli* DH5α were employed.

### Sequencing protocol

The new constructed pTZ57R/T plasmids were used for sequencing of the *dszD* gene from *Rhodococcus* FRF, IPF and GAF. Sequencing was done by MGW DNA Biotech Company (Germany) automatically. For this purpose, one pair of primers consisting of a forward: 5'-GAA TTC ATG TCT GAC AAG CCG AAT GCC-3' and reverse 5'-TCT AGA CTA TTG ACC TAA CGG AGT CGG-3' was used.

## RESULTS

### Amplification of dszD gene

Oxidoreductase gene (*dszD*) is necessary for expression of DBT desulfurization activity. We managed to amplify *dszD* gene from *Rhodococcus* FRF, IPF and GAF by means of PCR technique. The nucleic acid sequences of the PCR primers were designed using conserved nucleic acid sequences of the flavin reductase enzyme from *Rhodococcus* sp. strain IGTS8. The oligonucleotide primers were as follows: 5'-GAA TTC ATG TCT GAC AAG CCG AAT GCC-3' (forward) and 5'-TCT AGA CTA TTG ACC TAA CGG AGT CGG-3' (reverse). Digestion sites for EcoRI and HindIII were located at 5' of the forward and reverse primers respectively. After amplification the PCR products were evaluated on agarose gel 1%. 580 bp bands were appeared on agarose gels were the oxidoreductase gene from *Rhodococcus* sp. strain IGTS8 used for cloning.

### Cloning of dszD gene

The PCR products were purified using high pure PCR

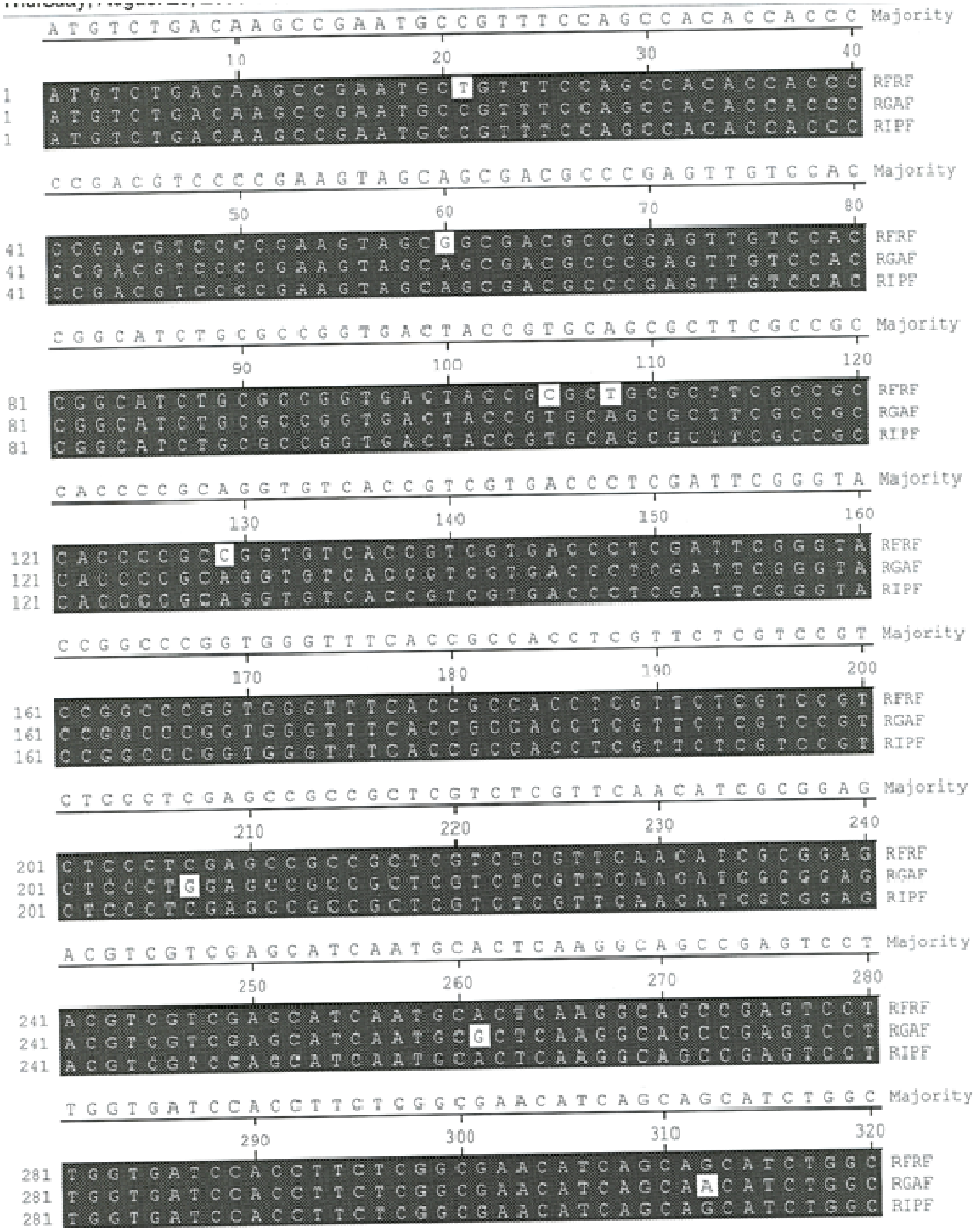


**Figure 1.** Alignment of flavin reductase gene (*dszD*) from *Rhodococcus* sp. FRF, IPF and GAF isolated from Tabriz and Tehran refineries soils.

product purification kit (Roche) and cloned to the pTZ57R vector at multiple cloning sites. Then the ligation products were transformed to the competent cells of *E. coli* DH5α. Transformed bacteria were differentiated based on α-complementation phenomenon as white colonies. In order to confirm the insertion of *dszD* gene into the pTZ57R, plasmid extraction was done from white colonies and then pTZD57R was digested with EcoRI and Hind III. After digestion, a 580 bp band was observed on agarose gel confirming the cloning of *dszD* gene into the

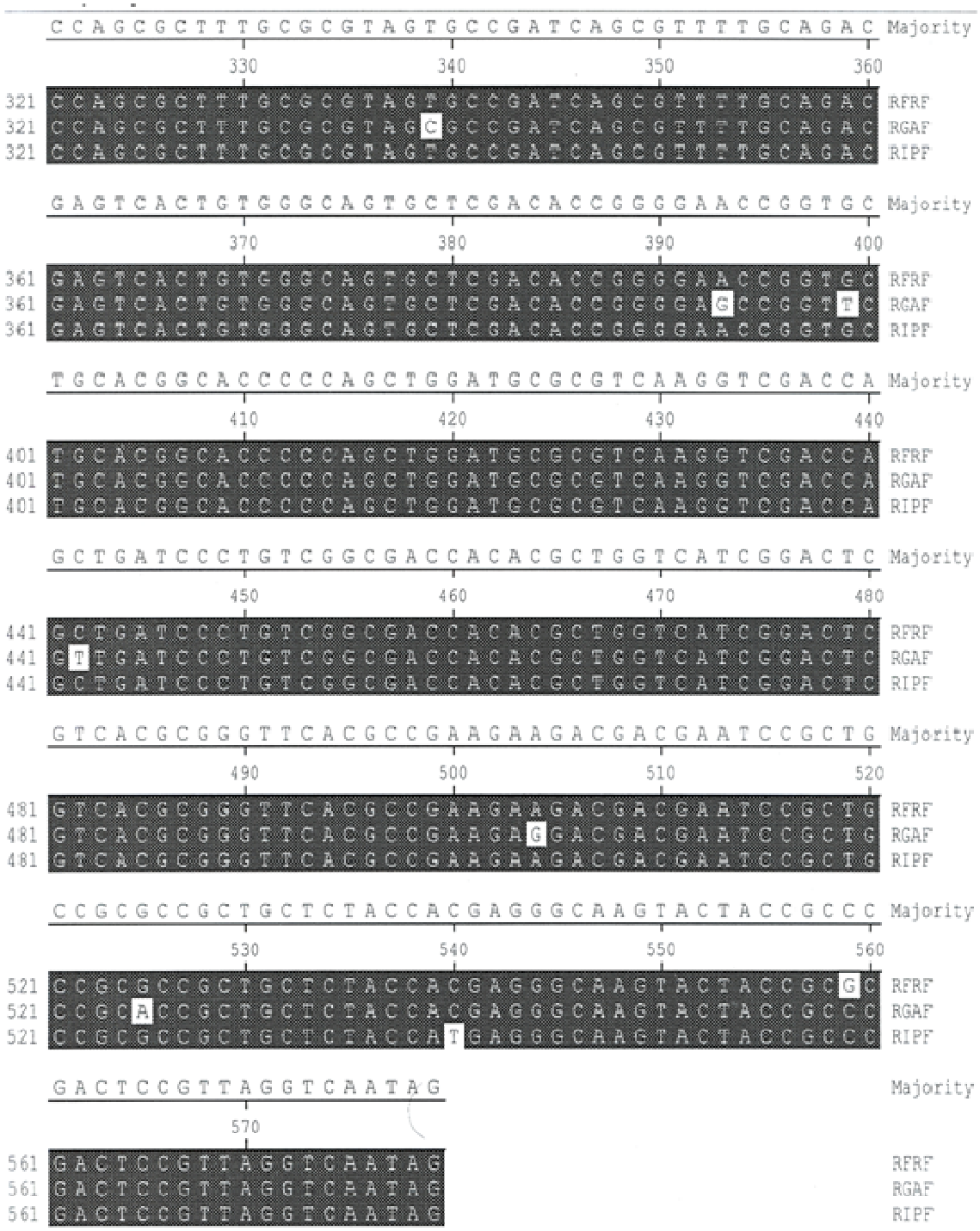


**Figure 1.** Contd.

pTZ57R vector.

## Nucleotide sequencing analysis

Using a pair of specific primers, the *dszD* gene from *Rhodococcus* FRF, IPF and GAF were sequenced using the ABI 3700 sequencer (Germany). The *dszD* gene from these strains was completely aligned with the oxidoreductase gene from *Rhodococcus* sp. strain IGTS8. The existence of only 6 and 9 altered nucleotides for *Rhodococcus* FRF, IPF and no changed nucleotide for *Rhodococcus* GAF confirmed that the oxidoreductase gene in these strains are highly conserved (Figure 1).

## DISCUSSION

*Rhodococcus* FRF, IPF and GAF are native strains which were isolated from Tabriz refinery soil samples. They were able to desulfurize DBT through the 4S pathway. We have already showed that three desulfurization genes (*dszA, dszB* and *dszC*) of *Rhodococcus* FMF constitute an operon similar to that of *Rhodococcus* sp. IGTS8 and each of them shows a considerable homology to the corresponding *dsz* genes (Raheb et al., 2004). However, there is another enzyme involved in DBT desulfurization, flavin reductase (DszD) and two monooxigenases, DszA and DszC, are dependant on $FADH_2$, generated by this NADH-dependant FMN reductase (Gray et al., 1996; Galan et al., 2000). Therefore before the properties of isolated *Rhodococcus* strains can be applied as genetic resources for biodesulfurization, all the biodesulfurization genes must be identified and functionally analyzed.

The peculiarity of the dependence of DszA and DszC on $FADH_2$ has been noted; compared with the enzymes which utilize a built-in FAD cofactor, this mode of operation is apparently relatively rare in nature (Oldfield et al., 1997). Although destroying the *dszD* gene by insertional inactivation causes of losing DBT-desulfurization completely, it has no effect on the viability of this strain. It seems therefore that the sole function of DszD is to provide $FADH_2$ for DszA and DszC (Lei and Tu 1996; Li et al., 1996).

Sequence analysis showed that there was significant sequence identity between *dszD* genes of the three isolated strains and that of *Rhodococcus* sp. IGTS8. The oxidoreductase gene of *Rhodococcus* FRF, IPF and GAF share 99, 98.5 and 100% similarity, respectively, to that of *Rhodococcus* sp. IGTS8 and There were differences only in 6 and 9 nucleotides for *Rhodococcus* FRF and IPF respectively (Figure 1). Furthermore ORF studies revealed that oxidoreductase enzyme in all strains consists of 193 amino acids. Alignment of ORFs were carried out by DNASTAR soft ware. The result showed a difference of only one amino acid for *Rhodococcus* FRF which is located at stop condon.

We have already showed that there is a high conservation between chromosomal encoded sox operon in *Rhodococcus* FMF and its plasmid counterpart in *Rhodococcus* sp. strain IGTS8 (Raheb et al., 2004). The results of this study also showed that the dszD gene is highly conserved in *Rhodococcus* FRF, GAF and IPF and that of *Rhodococcus* sp. IGTS8. Therefore it can be concluded that these strains are closely related to the *Rhodococcus* sp. stain IGTS8.

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