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Isolation and Identification of a Sulfide/Sulfoxide Monooxygenase Gene from a Newly Isolated *Rhodococcus* Sp. Strain FMF

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Abstract

Rhodococcus FMF is a gram-positive bacterium isolated for the first time from soil samples of Tabriz refinery in Iran. This microorganism is able to catabolize dibenzothiophene to 2-hydroxybiphenyl and inorganic sulfur without the destruction of carbon-carbon bonds. Three structural genes, dszA, dszB, and dszC have been characterized and shown to be responsible for this phenotype. In this work, dszC gene from *Rhodococcus* FMF was identified and then isolated by PCR technique using the nucleotide sequence of dszC gene from *Rhodococcus erythropolis* IGTS8 to design required primers. After amplification, the purified PCR product was cloned into plasmid vector pTZ57R/T. Further analysis by mapping and sequencing showed the identity of 689 nucleotides from this gene compared to the dszC gene from *Rhodococcus* sp. Strain IGTS8 as a key microorganism in biodesulfurization field.

Keywords: The dszC gene from *Rhodococcus* FMF; Biodesulfurization

Introduction

Sulfur-containing organic molecules found in fossil fuels have been recognized as a major source of environmental pollution in a form of acid rain during combustion [7]. Although physical, chemical, or biological means may accomplish the removal of inorganic sulfur from these fuels, organically bound sulfur is difficult to remove [2]. Nearly 70% of the sulfur in fossil fuels is in the form of heterocyclic organic compounds, such as benzothiophene, dibenzothiophene (DBT), and more complex thiophenes [6]. It seems that, one possible way for reducing the organic sulfur content is to expose these substrates to microorganisms or enzymes that are capable of breaking carbon-sulfur bonds via a sulfur-specific pathway [7,11,13].

Moreover, DBT has been widely used as a model compound to screen microorganisms which might be employed in desulfurization of fossil fuels [17].

A number of microorganisms [1,3,15,16,19,21], particularly *Rhodococcus*, *Bacillus*, *Corinebacterium* and *Arthrobacter* species [10,14,18] have been previously found to metabolize DBT. More recently, *Rhodococcus* sp. Strain FMF has been isolated and shown to be capable of desulfurizing DBT.

Previously it was shown that *Rhodococcus erythropolis* IGTS8 is able to selectively cleave sulfur

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Table 1. Bacterial strains and plasmids

Strain	Genotype and/or description	Source
1. *Rhodococcus* strains		
1. IGTS8	DBT-desulfurizing environmental isolate ATCC 53968	J.J. Kilbane [14]
2. FMF	Newly isolated DBT-desulfurizing strain	Persian Type Culture Collection
2. *E. coli* strain		
DH5αF'	[F'], endA1. HsdRI7 (rk-mkt), supE44, thi-1, recA1, gyrA(Nal^R), relA1, Δ (lacZYA-argF)u469, (ϕ 80 lacZ Δ M15)	Stratagene
3. Plasmids		
1. pESOX3	Dsz^+ pSOX (13 kb)	E. Diaz [9]
2. pESOX4	Dsz^+ pSOX (10 kb)	E. Diaz [9]
3. pTZ57R/T	pUC18 based vector	Fermentas (Germany)

from DBT to form 2-hydroxybiphenyl (HBP) and inorganic sulfur in an enzymatic pathway [12]. Moreover, three structural genes namely dszA, dszB, and dszC have been isolated, cloned, and shown to be responsible for desulfurization [25]. Similar to *Rhodococcus erythropolis* IGTS8, three genes namely sox/dszABC have been characterized in *Rhodococcus* FMF, which act as an operon and are responsible for encoding desulfurization enzymes in this strain. Biodesulfurization ability was confirmed in the related bacterium employing Gibbs assay by Persian Type Culture Collection (PTCC). Among these three enzymes, one encoded by sox/dszC catalyzes the oxidation of DBT to DBT sulfone ($DBTO_2$), which is subsequently converted into 2-hydroxy-biphenyl and sulfate by the enzymes E_A and E_B encoded by sox/dszAB [17]. Whereas this catabolic pathway does not destroy carbon-carbon bonds, the fuel value is retained [12,15].

In this work it has been shown that one of the desulfurization genes in the key organism of *Rhodococcus erythropolis* IGTS8 and *Rhodococcus* FMF (called so by PTCC) are similar to each other. DszC gene from *Rhodococcus* FMF was isolated by PCR technique and cloned into plasmid pTZ57R/T. A comparative map and nucleotide sequence analysis provided an evidence of the conserved nature of the dszC gene in these two strains.

Materials and Methods

Enzymes and Reagents

All restriction endonucleases and InsT/A clone™ PCR product cloning kit were purchased from Fermentas (Germany). Molecular weight markers, High Pure Plasmid Purification kit, High Pure PCR Product Purification kit, and Agarose Gel DNA Extraction kit were from Roche. All the chemicals were purchased from Merck Company.

Bacterial Strains and Plasmids

Strains and plasmids used in this study were described in Table 1.

Bacterial Growth Media

Bacterial strains were routinely grown in Lauria Bertani broth or on Lauria Bertani agar.

Small Scales Genomic DNA Purification of Rhodococcus FMF

A commercially available Wizard™ Genomic DNA Purification Kit (Promega) was used to extract the genomic DNA from *Rhodococcus* FMF. In this method, 1 ml of overnight culture was used and the manufacturer's instructions were followed.

Plasmid DNA Isolation

Large scale preparation of plasmid DNA using a caesium chloride gradient was employed. 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 the alkaline lysis method [24].

Mini/Midi/Maxi Plasmid Purification

A quick and highly purified preparation of plasmid DNA was performed using a commercially available kit

supplied by Boehringer Mannheim Company. These methods were used as the manufacturer's instructions.

Preparation of DNA Fragments from Agarose Gel

The commercially available QIA quick Gel Extraction Kit supplied by QIAGEN was used for the extraction of DNA from agarose gel. The method was carried out as the manufacturer's instruction.

Polymerase Chain Reaction Technique

To isolate dszC gene from *Rhodococcus* FMF, PCR technique was used employing two primers. Amplification was carried out using High Fidelity PCR Master Kit (from Roche) and a Perkin-Elmer (U.S.A) DNA thermal cycler.

Purification of PCR Product

This procedure was performed using High Pure PCR Product Purification Kit manufactured by Roche Company.

Ligation, Transformation, and Cloning Procedures

Purified PCR product was then cloned into a plasmid vector using InsT/Aclone^{TM} PCR Product Cloning Kit # K1213 (Fermentas): An approximately 1300 bp purified PCR fragment was ligated into the plasmid pTZ57R/T (Scheme 1). LacZ Δ M15 mutation (which is related to the partial deletion of β-galactosidase gene) is necessary for identification of transformed bacteria because it is required for blue/white selection. For this reason, competent cells of *E. coli* Strain DH5α (compatible with this kit) were employed to perform transformation.

Sequencing Protocol

The newly constructed plasmid pTZC57K was used to sequence one part of the dszC gene from *Rhodococcus* FMF. Nucleotide sequencing was performed by MWG DNA biotech Company by means of ABI 3700 sequencer (Germany) automatically. For this purpose, one pair of primers consisting of a forward and reverse one was employed.

Results

Amplification and Cloning of dszC Gene from Rhodococcus FMF

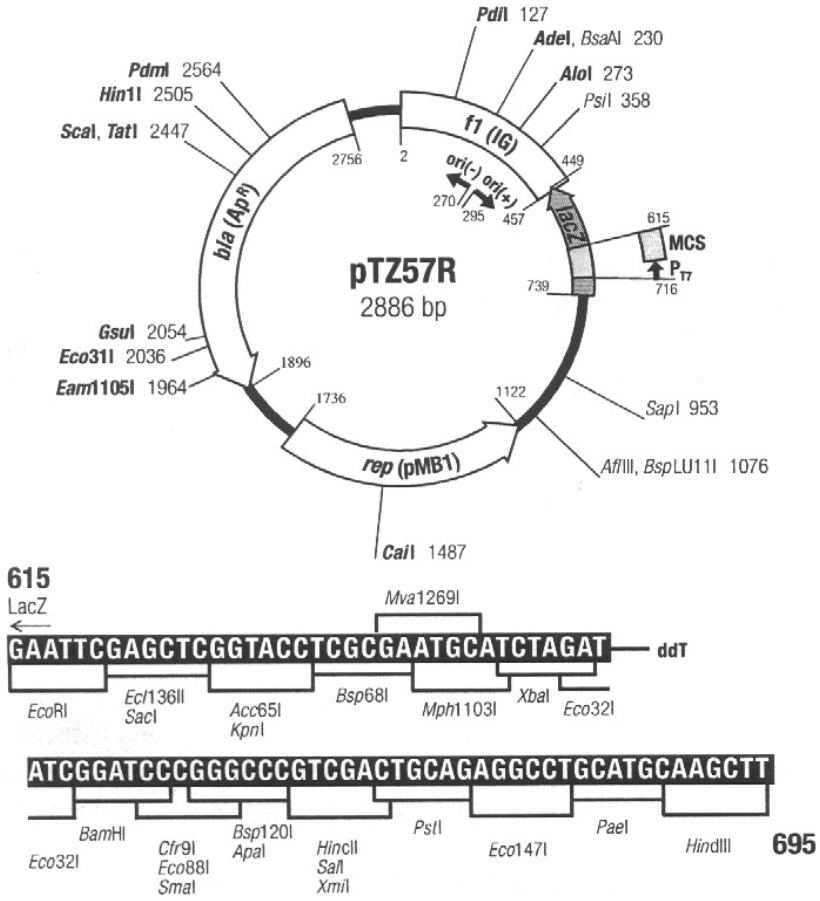
Newly isolated *Rhodococcus* FMF can convert DBT, DBT-Sulfoxide, and DBT-Sulfone to 2HBP via a sulfur-specific pathway similar to 4S pathway that was previously indicated in *Rhodococcus* sp. Strain IGTS8 [11].

Among three genes of desulfurization operon involved in desulfurization of DBT as a model compound, we managed to amplify sox/dszC gene from *Rhodococcus* FMF by means of PCR method. For this purpose, genomic DNA of *Rhodococcus* FMF was employed along with two primers designed using the nucleotide sequence of the dszC gene from *Rhodococcus erythropolis* IGTS8 including forward primer C_1: 5′ GAATTCGCCTCAATGCCCACCGATAC 3′, and reverse primer C_2: 5′ AAGCTTTCAGGAGGTGA AGCCGGGAA 3′. Amplification conditions were 94°C for 5 min (1 cycle), 94°C for 1 min, 70°C for 1 min, 72°C for 2 min repeated for 30 cycles in addition to an extra 72°C for 4 min (Fig. 1).

After amplification, purified PCR product was cloned into plasmid pTZ57R. After plasmid extraction from white colonies (as transformed bacteria), the insertion of relevant PCR fragment into pTZ57R was investigated by single digestion with EcoRI and HindIII. At last, newly constructed plasmid consisting of total PCR product was named pTZC57K. Moreover, in order to confirm the insertion of dszC gene into cloning vector pTZ57R, pTZC57K was separately digested with EcoRI and HindIII. Recognition sites for EcoRI and HindIII had been previously designed at the 5′ ends of forward and reverse primers used in PCR method. In addition, one restriction site for each of these two enzymes exists in MCS of pTZC57K. After digestion, an approximately 1300 bp band including the cloned PCR fragment along with a part of vector (between recognition sites of Eco321 and each of EcoRI and HindIII) was observed in the agarose gel (Fig. 3).

Mapping Procedure

In order to compare the map of dszC gene from *Rhodococcus erythropolis* IGTS8 and *Rhodococcus* FMF, several restriction enzymes with reference to the map of dszC gene from *Rhodococcus erythropolis* IGTS8 were selected to digest pTZC57K (Fig. 2). Also SOX4 plasmid extracted from *E. coli* Strain CC118, containing dszC gene from *Rhodococcus* sp. Strain IGTS8 was digested with SalI at 37°C overnight. According to the lack of SalI recognition site in the dszC gene of *Rhodococcus* sp. Strain IGTS8, the intact dszC gene was obtained to use as a control for digestion conditions with different restriction endonucleases during digestion of dszC gene from *Rhodococcus* FMF and mapping analysis (data not shown). By comparing the results of digestion with different restriction



Scheme 1. Restriction map and multiple cloning site of vestor pTZ57R.

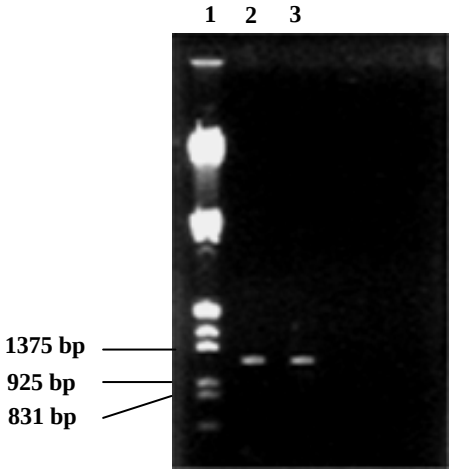


Figure 1. Ethidium bromide-stained agarose gel of PCR product. The position of ~1300bp fragment that contains the dszC gene of Rhodococcus FMF (lanes 2 and 3) is detectable in comparison with DNA molecular weight marker III (lane 1).

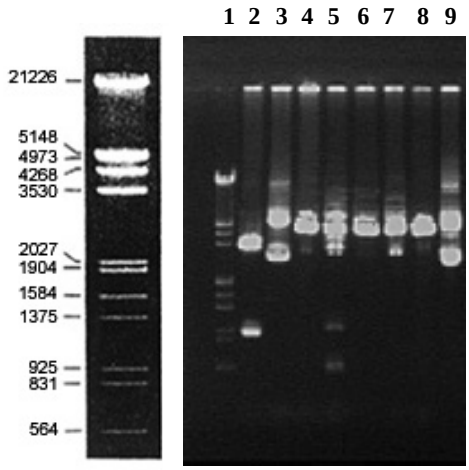


Figure 2. Mapping of dszC gene using several restriction enzymes. PTZC57K was digested with BamHI (lane 2), SnaBI (lane 3), SalI (lane 4), PstI (lane 5), AatII (lane 6), KpnI (lane 7), HincII (lane 8), and non-cut plasmid pTZC57K (lane 9). Lane 1 is molecular weight markerIII.

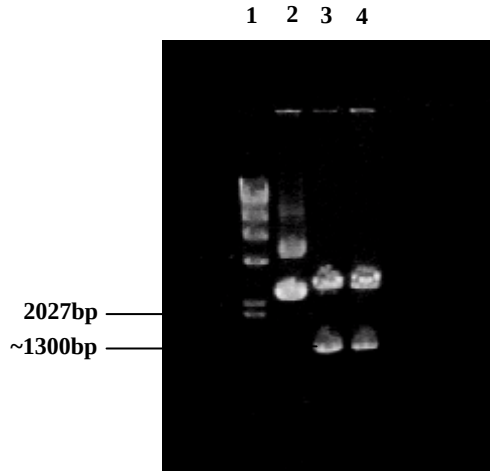


Figure 3. The confirmation of dszC gene cloning into pTZC57K using EcoRI and HindIII restriction enzymes digestion. Lane 1 is molecular weight marker II, lane 2 is non-cut plasmid obtained by minipreparation protocol, lane 3 and 4 are digestions with EcoRI and Hind III, respectively.

enzymes on the agarose gel, it was showed that there is no difference between recognition sites of the restriction enzymes used in mapping procedure for the dszC gene from these two strains.

Nucleotide Sequencing Analysis

Using a pair of specific primers, one region of the dszC gene from *Rhodococcus* FMF consisting of 689 nucleotides was sequenced. First nucleotide of sequenced part is 27 nucleotide far from the initial nucleotide of dszC gene in this bacterium. Employing DNA star software, the nucleotide sequence of dszC gene from *Rhodococcus erythropolis* IGTS8 and *Rhodococcus* FMF were aligned and compared. It was shown that there is no difference between the nucleotide sequence of this section in these two strains (Scheme 2).

Discussion

We cloned the dszC gene responsible for the conversion of DBT to DBT-sulfone via DBT desulfurization pathway in *Rhodococcus* FMF.

```
    G T T C G A C C A C G C G A C G C C G C C G A C A A C G A T  Majority
    ------------------+------------------+------------------+-
                      10                 20                 30
    ------------------+------------------+------------------+-
  1 G T T C G A C C A C G C G A C G C C G C C G A C A A C G A T  cfmf
  1 G T T C G A C C A C G C G A C G C C G C C G A C A A C G A T  cigts8

    C C C G T C G C G G T T G C C C G T G G G C T A G C C G A A  Majority
    ------------------+------------------+------------------+-
                      40                 50                 60
    ------------------+------------------+------------------+-
 31 C C C G T C G C G G T T G C C C G T G G G C T A G C C G A A  cfmf
 31 C C C G T C G C G G T T G C C C G T G G G C T A G C C G A A  cigts8

    A A G T G G C G A G C C A C C G C C G T C G A G C G T G A T  Majority
    ------------------+------------------+------------------+-
                      70                 80                 90
    ------------------+------------------+------------------+-
 61 A A G T G G C G A G C C A C C G C C G T C G A G C G T G A T  cfmf
 61 A A G T G G C G A G C C A C C G C C G T C G A G C G T G A T  cigts8

    C G C G C C G G G G G T T C G G C A A C A G C C G A G C G C  Majority
    ------------------+------------------+------------------+-
                      100                110                120
    ------------------+------------------+------------------+-
 91 C G C G C C G G G G G T T C G G C A A C A G C C G A G C G C  cfmf
 91 C G C G C C G G G G G T T C G G C A A C A G C C G A G C G C  cigts8

    G A A G A C C T G C G C G C G A G C G G C C T G C T G T C G  Majority
    ------------------+------------------+------------------+-
                      130                140                150
    ------------------+------------------+------------------+-
121 G A A G A C C T G C G C G C G A G C G G C C T G C T G T C G  cfmf
121 G A A G A C C T G C G C G C G A G C G G C C T G C T G T C G  cigts8

    C T C C T C G T C C C G C G C G A A T A C G G C G G C T G G  Majority
    ------------------+------------------+------------------+-
                      160                170                180
    ------------------+------------------+------------------+-
151 C T C C T C G T C C C G C G C G A A T A C G G C G G C T G G  cfmf
151 C T C C T C G T C C C G C G C G A A T A C G G C G G C T G G  cigts8
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Scheme 2. Comparison of nucleotide sequence of a part of dszC gene from *Rhodococcus erythropolis* IGTS8 and *Rhodococcus* FMF.

```
     G G C G C A G A C T G G C C C A C C G C C A T C G A G G T C  Majority
     ----------------+-------------------+-------------------+-
                     190                 200                 210
     ----------------+-------------------+-------------------+-
181  G G C G C A G A C T G G C C C A C C G C C A T C G A G G T C  cfmf
181  G G C G C A G A C T G G C C C A C C G C C A T C G A G G T C  cigts8

     G T C C G C G A A A T C G C G G C A G C C G A T G G A T C T  Majority
     ----------------+-------------------+-------------------+-
                     220                 230                 240
     ----------------+-------------------+-------------------+-
211  G T C C G C G A A A T C G C G G C A G C C G A T G G A T C T  cfmf
211  G T C C G C G A A A T C G C G G C A G C C G A T G G A T C T  cigts8

     T T G G G A C A C C T G T T C G G A T A C C A C C T C A C C  Majority
     ----------------+-------------------+-------------------+-
                     250                 260                 270
     ----------------+-------------------+-------------------+-
241  T T G G G A C A C C T G T T C G G A T A C C A C C T C A C C  cfmf
241  T T G G G A C A C C T G T T C G G A T A C C A C C T C A C C  cigts8

     A A C G C C C C G A T G A T C G A A C T G A T C G G C T C G  Majority
     ----------------+-------------------+-------------------+-
                     280                 290                 300
     ----------------+-------------------+-------------------+-
271  A A C G C C C C G A T G A T C G A A C T G A T C G G C T C G  cfmf
271  A A C G C C C C G A T G A T C G A A C T G A T C G G C T C G  cigts8

     C A G G A A C A A G A A G A A C A C C T G T A C A C C C A G  Majority
      ----------------+-------------------+-------------------+-
                     310                 320                 330
     ----------------+-------------------+-------------------+-
301  C A G G A A C A A G A A G A A C A C C T G T A C A C C C A G  cfmf
301  C A G G A A C A A G A A G A A C A C C T G T A C A C C C A G  cigts8

     A T C G C G C A G A A C A A C T G G T G G A C C G G A A A T  Majority
     ----------------+-------------------+-------------------+-
                     340                 350                 360
     ----------------+-------------------+-------------------+-
331  A T C G C G C A G A A C A A C T G G T G G A C C G G A A A T  cfmf
331  A T C G C G C A G A A C A A C T G G T G G A C C G G A A A T  cigts8

     G C C T C C A G C G A G A A C A A C A G C C A C G T G C T G  Majority
     ----------------+-------------------+-------------------+-
                     370                 380                 390
     ----------------+-------------------+-------------------+-
361  G C C T C C A G C G A G A A C A A C A G C C A C G T G C T G  cfmf
361  G C C T C C A G C G A G A A C A A C A G C C A C G T G C T G  cigts8

     G A C T G G A A G G T C A G C G C C A C C C C G A C C G A A  Majority
     ----------------+-------------------+-------------------+-
                     400                 410                 420
     ----------------+-------------------+-------------------+-
391  G A C T G G A A G G T C A G C G C C A C C C C G A C C G A A  cfmf
391  G A C T G G A A G G T C A G C G C C A C C C C G A C C G A A  cigts8

     G A C G G C G G C T A C G T G C T C A A T G G C A C G A A G  Majority
     ----------------+-------------------+-------------------+-
                     430                 440                 450
     ----------------+-------------------+-------------------+-
421  G A C G G C G G C T A C G T G C T C A A T G G C A C G A A G  cfmf
421  G A C G G C G G C T A C G T G C T C A A T G G C A C G A A G  cigts8

     C A C T T C T G C A G C G G C G C C A A G G G G T C G G A C  Majority
     ----------------+-------------------+-------------------+-
                     460                 470                 480
     ----------------+-------------------+-------------------+-
451  C A C T T C T G C A G C G G C G C C A A G G G G T C G G A C  cfmf
451  C A C T T C T G C A G C G G C G C C A A G G G G T C G G A C  cigts8

     C T G C T G T T C G T G T T C G G C G T C G T C C A G G A T  Majority
     ----------------+-------------------+-------------------+-
                     490                 500                 510
     ----------------+-------------------+-------------------+-
481  C T G C T G T T C G T G T T C G G C G T C G T C C A G G A T  cfmf
481  C T G C T G T T C G T G T T C G G C G T C G T C C A G G A T  cigts8

     G A T T C T C C G C A G C A G G G T G C G A T C A T T G C T  Majority
     ----------------+-------------------+-------------------+-
                     520                 530                 540
     ----------------+-------------------+-------------------+-
511  G A T T C T C C G C A G C A G G G T G C G A T C A T T G C T  cfmf
511  G A T T C T C C G C A G C A G G G T G C G A T C A T T G C T  cigts8
```

Scheme 2. Continued.

```
     G C C G C T A T C C C G A C A T C G C G G G C T G G C G T T  Majority
     -----------------+-----------------+-----------------+-
                      550               560               570
     -----------------+-----------------+-----------------+-
541  G C C G C T A T C C C G A C A T C G C G G G C T G G C G T T  cfmf
541  G C C G C T A T C C C G A C A T C G C G G G C T G G C G T T  cigts8

     A C G C C C A A C G A C G A C T G G G C C G C C A T C G G C  Majority
     -----------------+-----------------+-----------------+-
                      580               590               600
     -----------------+-----------------+-----------------+-
571  A C G C C C A A C G A C G A C T G G G C C G C C A T C G G C  cfmf
571  A C G C C C A A C G A C G A C T G G G C C G C C A T C G G C  cigts8

     A T G C G G C A G A C C G A C A G C G G T T C C A C G G A C  Majority
     -----------------+-----------------+-----------------+-
                      610               620               630
     -----------------+-----------------+-----------------+-
601  A T G C G G C A G A C C G A C A G C G G T T C C A C G G A C  cfmf
601  A T G C G G C A G A C C G A C A G C G G T T C C A C G G A C  cigts8

     T T C C A C A A C G T C A A G G T C G A G C C T G A C G A A  Majority
     -----------------+-----------------+-----------------+-
                      640               650               660
     -----------------+-----------------+-----------------+-
631  T T C C A C A A C G T C A A G G T C G A G C C T G A C G A A  cfmf
631  T T C C A C A A C G T C A A G G T C G A G C C T G A C G A A  cigts8

     G T G C T G G G C G C G C C C A A C G C C T T C G T T C T X  Majority
     -----------------+-----------------+-----------------+-
                      670               680               690
     -----------------+-----------------+-----------------+-
661  G T G C T G G G C G C G C C C A A C G C C T T C G T T C T    cfmf
661  G T G C T G G G C G C G C C C A A C G C C T T C G T T C T C  cigts8

     X X X X X X X X X X X X X X X X X X X X X X X X X X X X X  Majority
     -----------------+-----------------+-----------------+-
                      700               710               720
     -----------------+-----------------+-----------------+-
689                                                           cfmf
691  G C C T T C A T A C A A T C C G A G C G C G G C A G C C T C  cigts8
```

Scheme 2. Continued.

Previously, DNA sequence and molecular subclone analysis in *Rhodococcus* sp. Strain IGTS8 revealed that the desulfurization pathway consists of three genes: dszA, dszB and dszC [23]. The organization of these genes initially suggested that they act as an operon. Also the three genes are transcribed in the same direction. The termination codon for dszA and the initiation codon for dszB overlap and there is only a 13-bp gap between dszB and dszC. Previous expression studies revealed that dszA and dszB had to be coexpressed in order to observe the activity of these genes [23].

Rhodococcus FMF desulfurizes DBT by a modification of 4S pathway. The first step in this pathway is catalyzed by a sulfide/sulfoxide monooxygenase encoded by dszC gene. This enzymatic reaction converts DBT to DBT-sulfone directly.

In a previous report, Denome *et al.* [5] had also described the cloning of the *Rhodococcus* sp. Strain IGTS8 desulfurization cluster which codes for three proteins DszA, DszB, and DszC under the control of a single promoter [22].

The size of two large plasmids in *Rhodococcus* sp. Strain IGTS8 was previously estimated to be 150 kb and 90 kb. However, size estimation for the IGTS8 plasmids according to Denome *et al.* were 120 and 50 kb [5]. In order to isolate large plasmids from *Rhodococcus* FMF, we employed the procedure modified by Denis-Larose *et al.* [4]. However, no large plasmid with desulfurization activity was isolated in this strain.

In this work we demonstrated that the dszC desulfurization gene from *Rhodococcus* FMF exhibited significant levels of sequence similarity with the dszC gene from *Rhodococcus* sp. Strain IGTS8.

It was for the first time that we showed the existence of desulfurization cluster on the chromosomal DNA of *Rhodococcus* FMF. The next challenge is to overexpress the cloned gene to construct a system for removing organic sulfur from fossil fuels more efficiently.

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