Fuel xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Fuel

journal homepage: www.elsevier.com/locate/fuel

Enhancement of desulfurization activity by enzymes of the *Rhodococcusdsz* operon through coexpression of a high sulfur peptide and directed evolution

Jie Pan, Fan Wu, Jia Wang, Linqing Yu, Naghmeh Hassanzadeh Khayyat, Benjamin C. Stark, John J. Kilbane II *

Biology Division, Department of Biological and Chemical Sciences, Illinois Institute of Technology, Chicago, IL 60616, USA

HIGHLIGHTS

- Demonstrated a novel directed evolution approach to obtain biocatalysts with improved desulfurization specific activity.
- Modified the desulfurization operon by including a gene encoding a sulfur-rich polypeptide to increase the nutritional requirement for sulfur.

ARTICLE INFO

Article history:
Received 31 December 2012
Received in revised form 1 April 2013
Accepted 24 April 2013
Available online xxxx

Keywords:
Biodesulfurization
Biocatalysis
Strain improvement
Directed evolution
dsz

ABSTRACT

Removal of sulfur from petroleum improves the performance of refineries and reduces air pollution from fuel combustion. Biodesulfurization can selectively remove sulfur from petroleum, but improved biocatalysts are needed. This study illustrates a new approach to obtain improved desulfurization biocatalysts. A synthetic gene (Sulpeptide, *S1*) encoding high proportions of the sulfur containing amino acids methionine and cysteine was designed, constructed and cloned, as part of the *Rhodococcus dszABC* (desulfurization) operon, in vector pRESX, under control of the *Rhodococcus kstD* promoter. pRESX*dszABC* and pRESX*dszAS1BC* were electroporated into desulfurization-negative *Rhodococcus opacus* and the strains were transferred through 40 passages in medium with dibenzothiophene (DBT) as the sole sulfur source. Modest increases in desulfurization activity were achieved after 10 passages selecting for rapid growth with DBT. After selection for 40 passages, both the *dszABC* and *dszAS1BC* expressing *R. opacus* strains showed a greater than 20-fold increase in specific desulfurization ability, and exceeded the specific activity of the control, desulfurization positive strain *Rhodococcus erythropolis* IGTS8. This study serves as a proof of concept that engineering with Sulpeptide genes, combined with selective pressure for rapid growth with DBT, can drive evolution of improved desulfurization biocatalysts.

1. Introduction

The demand for clean energy sources, and attention to the environment, is increasing. Currently one aspect of this demand is that for high quality (low sulfur) crude oil. Biodesulfurization is a potentially useful industrial bioprocess for removal of organic sulfur from petroleum, but biocatalysts with improved performance are needed [1,2]. The purpose of this study was to provide a novel strategy for the production of desulfurization genes and microbial strains that have improved performance regarding the biodesulfurization of organosulfur compounds. The biodesulfurization pathway (the 4S pathway) that catalyzes the selective cleavage of carbon–sulfur bonds in molecules such as dibenzothiophene (DBT), and is encoded by the *dsz* operon, has been extensively studied [1,3,4]. Multiple efforts employing genetic engineering of the *dsz* operon have been made to increase biodesulfurization activity, but traditional molecular modification methods, such as increasing the copy number and/or altering the expression of the genes that encode the enzymes of the desulfurization pathway resulted in less than expected levels of improvement [5–7]. Omics methods such as the use of microarrays and metabolic modeling indicate that individual enzymes, much less metabolic pathways, do not operate in isolation. Changing the concentration/activity of an individual enzyme can affect the functioning and/or concentration of other enzymes, often in unexpected ways. Consequently, increasing the

Abbreviations: DBT, dibenzothiophene; *dszABC*, desulfurization operon genes A, B and C; *S1*, gene encoding the first Sulpeptide gene synthesized and cloned; S1, Sulpeptide 1; DCW, dry cell weight; SOE-PCR, sequence overlap extension polymerase chain reaction.

* Corresponding author. Tel.: +1 713 385 1279.
E-mail address: kilbane@iit.edu (J.J. Kilbane II).

0016-2361/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fuel.2013.04.065

activity of a pathway, such as biodesulfurization, to levels relevant for an industrial process will likely require multiple steps of metabolic engineering, not just altering the expression of the desulfurization genes [5,6,8].

One such step could be to increase the sulfur demand of desulfurization-competent cultures to force increased desulfurization activity [6], for example by modifying the *dsz* operon so that it encodes a sulfur-rich polypeptide. This is the first report of a study to employ a sulfur-rich polypeptide to improve desulfurization specific activity. If a gene encoding a sulfur-rich polypeptide (Sulpeptide) were inserted into the *dsz* operon, for example between the first two genes (*dszA* and *dszB*) in the operon, and the resulting strain grown with DBT as the sole source of sulfur, the cells would have to express the Sulpeptide gene in order to metabolize DBT. The insertion of the Sulpeptide gene within the *dsz* operon would make it difficult for the microbial culture to delete or inactivate the Sulpeptide gene without also losing desulfurization activity. In the work described here, the Sulpeptide1 gene was constructed. In addition to containing relatively high proportions of cysteine and methionine, S1 also contains a signal sequence intended to facilitate extracellular transport/excretion in order to further increase the demand of cells for sulfur.

A second way to obtain biocatalysts with increased desulfurization activity is to combine the Sulpeptide strategy with directed evolution. The use of directed evolution selection techniques does not require a detailed understanding of cell metabolism, yet it has been used in numerous applications to produce derivative cultures that possess increased levels of enzymatic activity, and/or altered enzymatic activity, while retaining a balanced metabolism [9–12]. Directed evolution could be applied to develop improved desulfurization biocatalysts by using repeated passages in medium with DBT as sole sulfur source, to continually select the fastest growing individuals, to obtain derivative cultures that have increased DBT metabolizing ability.

Here we have combined both strategies by inserting the *S1* gene between *dszA* and *dszB* in the *dsz* operon, transforming this construct into the *dsz*-negative *Rhodococcus opacus,* and subjecting the transformant to repeated passages in medium with DBT as sole sulfur source. The results serve as a proof of concept for this combination of experimental strategies as a way to evolve more efficient DBT metabolizing abilities.

2. Materials and methods

2.1. Bacterial strains and plasmids

Bacterial strains used in this study included *Escherichia coli* DH5α, *Rhodococcus erythropolis* IGTS8 (ATCC 53968), and *R. opacus* (ATCC 17039). pGEM-T-Easy and pGEM-3zf(+) vectors(Promega Corp., Madison, WI) were used for gene/plasmid construction in *E. coli*. For the expression of genes in *Rhodococcus* strains the plasmid pRESX [13], kindly provided by Dr. van der Geize of the University of Groningen, was used. pRESX replicates in both *E. coli* and *Rhodococcus* cultures, contains a gene for kanamycin resistance, and possesses unique restriction endonuclease sites downstream from promoter P*kstD* so that gene fragments can be cloned and subsequently expressed in *Rhodococcus*.

2.2. Growth condition and medium

For growth experiments with *Rhodococcus* cultures a sulfur-free chemically defined medium, designated as CDM medium, was used. CDM medium consists of: K_2HPO_4 (6.96 g L^{-1}), KH_2PO_4 (1.35 g L^{-1}); NH_4Cl (2.7 g L^{-1}); $MgCl_2$ (10.2 g L^{-1}); $CaCl_2$ (2.2 g L^{-1}), adjusted to pH 7.2 with trace elements added at one ml per liter. The composition of the trace elements solution was (pH 6.7): $FeCl_3{\cdot}6H_2O$ (2.04 g L^{-1}); $ZnCl_2$ (70 g^{-3} L^{-1}); $MnCl_2{\cdot}4H_2O$ (100 g^{-3} L^{-1}); $CoCl_2{\cdot}6H_2O$ (200 g^{-3} L^{-1}); $CuCl_2{\cdot}2H_2O$ (20 g^{-3} L^{-1}); $NiCl_2{\cdot}6H_2O$ (20 g^{-3} L^{-1}); $Na_2MoO_4{\cdot}2H_2O$ (40 g^{-3} L^{-1}); H_3BO_4 (20 g^{-3} L^{-1}). Ethanol or glucose, at 2 M^{-3}served as the carbon source, and dibenzothiophene (DBT) was supplied at 0.1 M^{-3}as the sole source of sulfur.

2.3. Construction of S1 and recombinant plasmids

Sulpeptide 1 is a synthetic gene of 267 base pairs (bp). It was designed to have a leader sequence of 89 bp, and a ribosome binding site. The protein coding region of the Sulpeptide 1 gene contains an ATG start codon and 3 consecutive stop codons. The entire translated region is 50 amino acids long, 25 of which are a bacterial signal sequence that includes a bacterial secretion signal sequence [14] (to facilitate the excretion of the Sulpeptide 1 protein from the bacterial cell) while of the remaining 25 amino acids in the mature sequence, 7 are cysteines and 6 are methionines (sulfur-containing amino acids). A DNA sequence corresponding to the entire amino acid sequence and optimized for codon usage was synthesized by Eurofins MWG Operon (Ebersberg, Germany) and supplied as plasmid pUC57. The portion of the native *dsz* operon from *R. erythropolis* IGTS8 from the intiation codon of the first gene in the operon (*dszA*) through the termination codon of the third gene in the operon (*dszC*) had previously been cloned into pGEM T-easy (Promega) [15]. It was PCR amplified from this construct and cloned into pGEM-3Zf(+) (Promega), producing pGEM3Zf(+)*dszABC* to use as starting material for all subsequent modifications.

DszA was PCR amplified from pGEM-3Zf(+)*dszABC* using a 5′ primer incorporating an EcoRI site and a 3′ primer designed for overlap extension PCR (SOE-PCR) with S1 that was PCR amplified from pUC57 using a 3′ primer incorporating a BamHI site and a 3′ primer designed for SOE-PCR with the *dszA* amplicon. Following this SOE-PCR step, the *dszA-S1* amplicon was cleaved with EcoRI and BamHI, gel purified, and cloned into the EcoRI and BamHI sites of pGEM3Zf(+) to form pGEM*dszAS1*.

The *dszBC* fragment from pGEM-3Zf(+)*dszABC*was PCR amplified using a 5′ primer incorporating an XbaI site and a 3′ primer incorporating a HindIII site. After cleavage of this amplicon as well as pGEM*dszAS1* with XbaI and HindIII, both DNAs were gel purified and ligated together. As the XbaI and HindIII sites are located, in that order, downstream of the BamHI site in pGEM-3Zf(+), this produced plasmid pGEM*dszAS1BC* containing an insert with the following gene sequence: *dszA-S1-dszBC*.

The *dszA-S1-dszBC* insert was then PCR amplified using a 5′ primer incorporating an NdeI site and 3′ primer incorporating a BglII site. After cleavage of this amplicon with NdeI and BglII it was cloned into NdeI–BglII double digested vector pRESX [13], placing the *dszA-S1-dszBC* sequence under the control of the *kstD* promoter in plasmid pRESX*dszAS1BC*. Plasmid pRESX*dszAS1BC* was then electroporated into *R. opacus* as described in the following section. At the same time *dszABC* was PCR amplified from pGEM-3Zf(+)*dszABC* using the same primer set, and also cloned in the same way into pRESX downstream and under control of the *kstD* promoter.

2.4. Electroporation into R. opacus

Electroporation of *R. opacus* employed cells grown in NZ broth at 30 °C and harvested at OD_{600nm} of 0.6 [13]. Cells were collected by centrifugation at 4 °C and washed 4 times with 1.0 ml ice cold 0.3 M sucrose; they were then resuspended in one fiftieth volume of 0.5 M sucrose.100 L^{-6} (microliters) of cells were added to ice cold 0.2 cm electroporation cuvettes, 2 L^{-6} plasmidDNA was added, and then electroporation was performed at 25 μF, 2.4 kV, 800 Ω. Cells were diluted immediately in 5.0 ml NZ broth and

Please cite this article in press as: Pan J et al. Enhancement of desulfurization activity by enzymes of the *Rhodococcusdsz* operon through coexpression of a high sulfur peptide and directed evolution. Fuel (2013), http://dx.doi.org/10.1016/j.fuel.2013.04.065