

BIODESULFURIZATION GENE EXPRESSION BY PROMOTER REPLACEMENT IN *RHODOCOCCLUS*

Peter C.K. Lau, Claude Denis-Larose,
Tanya MacPherson, Charles W. Greer, Jalal Hawari
Biotechnology Research Institute
National Research Council Canada
Montreal, Quebec, Canada H4P 2R2

Matthew J. Grossman
Corporate Research
Exxon Research and Engineering Company
Annandale, New Jersey, 08801

Bruce M. Sankey
Imperial Oil Resources Ltd, Calgary, Alberta, Canada T2L 2K8

INTRODUCTION

Biodesulfurization is a pathway-driven and enzymatic process, best known in select strains of *Rhodococcus*, that removes sulfur specifically from sulfur heterocycles such as dibenzothiophene (DBT) present in fossil fuels (1-6). The ability of several rhodococcal strains to desulfurize is thus far a plasmid-encoded phenotype involving three genes (*soxABC* [also known as *dszABC*]) and at least a chromosomal gene product which provides the reduced flavin and oxidized NADH required for the two initial oxygenase activities (Fig. 1). The end products of this sulfur-specific removal scheme are liberation of inorganic sulfur as sulfate or sulfite and production of 2-hydroxybiphenyl (2-HBP) without disruption of the original aromatic rings. The practical advantages of this biodesulfurization process include unnecessary loss of calorific value of the fuel upon combustion and a reduced emission of harmful sulfur dioxide into the atmosphere (1, 4, 5, 11-13).

We are interested in developing an efficient desulfurization biocatalyst based on a native *Rhodococcus* strain. This strategy, while not compromising the possible advantages offered by the *Rhodococcus* background, necessitates a resolution of the repression problem faced by the sulfur oxidation genes when the native cells are grown in the presence of sulfur-containing compounds (1, 3, 7, 14, 15). Here we report a promoter replacement strategy and the results of a preliminary study showing the feasibility of *sox* gene expression in a rich and sulfate-proficient background.

EXPERIMENTAL AND DISCUSSION

Bacterial strains and shuttle expression vector

The rhodococcal host used in this study is a plasmid-free derivative of the desulfurizing strain *Rhodococcus* sp. X309 (ATCC 55309), designated *Rhodococcus* sp. strain X309-10-2 (henceforth strain 10-2; ref. 3). The *sox* phenotype was scored by growth on DBT as a sole sulfur source and the fluorescence formed around patches of bacteria on a DBT plate spray assay (3, 7). Bacterial growth media including minimal salt medium (MSM) and general recombinant DNA techniques were as previously described (3, 15, 16).

Plasmid pKSA6-1 (Fig. 2) is capable of replication in both *Escherichia coli* and *Rhodococcus* by virtue of the origin of replication from the *E. coli* pBluescript II KS- vector and the replication region of the pSOX desulfurization plasmid from strain X309 (16). For selection the gene markers are ampicillin (Ap; 50 µg/ml) resistance and chloramphenicol (Cm; 30 µg/ml) resistance, respectively. The pBluescript KS vector component also provides the blue-white color selection when the cells are plated on indicator plates containing IPTG and X-gal. Previously, we found that the levansucrase-encoding gene (*sacB*) from *Bacillus subtilis* (17) could be expressed in *Rhodococcus* and served as a counterselectable marker in the shuttle expression plasmid designated pKSA6-1*sac* (16). Importantly, the *sacB* promoter is not known to be repressed by sulfate, a feature that is explored in the following construct.

Promoter replacement for expression of *soxABC* genes in *Rhodococcus*

The concept is to replace the sulfate-repressed *sox* promoter (14) by that of *sacB* to drive the expression of *soxABC* genes. Knowledge of the precise sequence of these DNAs was used to design primers for PCR (polymerase chain reaction) amplification and cloning of the specific DNA fragments in the core shuttle plasmid pKSA6-1 by a step-wise procedure. i). The *sacB* promoter fragment was amplified by the following primers using pUM24 plasmid as template (18). Primers SacBpro5' (5'-CGCAGGGCCCATCACATATACCTGC) and SacBpro3' (5'-

GCTGACTAG**FCATCGTTCATGTCCTT**) were designed to contain the *Apal* and *SpeI* restriction sites (boldfaced), respectively. The underlined bases correspond to nucleotides 13-30 and 467-448 of the *sacB* noncoding sequence, respectively (17). Both primers have a calculated melting temperature of 54 °C. The CAT sequence adjacent to the *SpeI* site specifies the complementary sequence of the initiator codon of *sacB*. ii). The 3.7-kb DNA fragment of strain X309 pSOX plasmid containing the *soxABC* genes was amplified based on the sequence derived from the *Rhodococcus* sp. IGTS8 strain (7). The forward oligomer contains the sequence 5'-CCTG**ACTAGTCAACAACGACAAATGCATCT** (*SpeI* site underlined), and the sequence of the reverse oligomer is 5'-GT**CTCTAGATCAGGAGGTGAAGCCGGG** (*XbaI* site underlined). The sequence 3' to the *SpeI* site represents the third to eight codons of the *soxB* sequence. In the reverse oligomer the TCA triplet represents the complement of the *soxC* stop codon.

Digestion of the purified *sacB* promoter-containing fragment by *Apal* and *SpeI* endonucleases allowed specific and directional cloning at the MCS of the pKSA6-1 vector (Fig. 2). After plasmid isolation the *soxABC*-containing fragment was cloned at the *SpeI* and *XbaI* sites of the MCS after generation of the compatible restriction ends (Fig. 2).

As a result of transformation in *E. coli* strain DH10B, recombinant plasmids were isolated and checked for authenticity of the clones. A straightforward double digestion by *SpeI* and *XbaI* would verify the correct size of the insert. But it was necessary to first transform the plasmid in an *E. coli dam*-minus strain since in our primer design we had inadvertently introduced an adenine methylation sequence adjacent to the *XbaI* cleavage site (TCTAG**ATCA**; methylated base underlined). The orientation of the clone was verified by *NotI* restriction, an unique site within the *soxB* gene (Fig. 2). Using SacBpro5'as primer the DNA sequence encompassing the cloned junction was also verified.

Expression of *soxABC* genes in *Rhodococcus* and metabolite analysis

Two independent clones of *soxABC* genes (clone #4B and #7) of the above constructs were transformed into rhodococcal strain 10-2 by electroporation using the conditions and Bio-Rad Gene Pulser apparatus as previously described (16). The recombinants were selected on MSM media plates containing DBT (0.52 mM; Aldrich Chemical Co., Milwaukee, WI) and Cm (30 µg/ml). The *sox* phenotype was first examined under UV for fluorescence around patches of bacteria on a DBT spray plate assay. Neither the plate nor the liquid culture imparted fluorescence. This could be due to low level of expression or no expression at all. To check for these possibilities, metabolites of DBT transformation were analyzed by the solid phase microextraction (SPME) technique coupled to GC-MS (19). The solventless SPME technique is known for its speed and sensitivity, detection in the microgram per liter range. Sample preparation and analysis of metabolites were carried out as previously described (19). As a result, although the peaks are minor (not shown), both 2-HPB (*m/z* 170) and DBT-sulfone (*m/z* 216) were detected as metabolites besides the starting material DBT (*m/z* 184). Extracts of cells grown in the rich Luria Bertani (LB) broth were also analyzed. Similar pattern was observed for the recombinant strain but not the native X309 strain which is known to be repressed by the presence of sulfates. As a positive control, the plasmid-free 10-2 mutant strain did not yield any of the characteristic products.

CONCLUSIONS

This preliminary study showed the feasibility of expression of sulfur oxidation genes in *Rhodococcus* by a heterologous promoter that is not repressed by the presence of sulfate. Undoubtedly, the shuttle expression system provides a good base for further improvement and optimization. Recently, recombinant *Pseudomonas* strains have been constructed to carry out an improved level of biodesulfurization (20).

ACKNOWLEDGMENTS

Funding from Imperial Oil Resources Ltd. is gratefully acknowledged. We thank H. Bergeron for the computer graphics.

REFERENCES

- (1) Kilbane, J. J. *Trends Biotech.* 1989, 7, 97-101.
- (2) Finnerty, W. R. *Annu. Rev. Microbiol.* 1992, 46, 193-218.
- (3) Denome, S. A.; Olson, S. S.; Young, K. D. *Appl. Environ. Microbiol.* 1993, 59, 2837-2843.
- (4) Monticello, D. J. *Environ. Progress.* 1993, 12, 1-4.
- (5) Ohshiro, T.; Suzuki, K.; Izumi, Y. *J. Ferm. Bioeng.* 1996, 81, 121-124.

- (6) Denis-Larose, C.; Labbé, D.; Bergeron, H.; Jones, A. M.; Greer, C. W.; Hawari, J.; Grossman, M. J.; Sankey, B. M.; Lau, P. C. K. *Appl. Environ. Microbiol.* 1997, *63*, 2915-2919.
- (7) Denome, S. A.; Oldfield, C.; Nash, L. J.; Young, K. D. *J. Bacteriol.* 1994, *176*, 6707- 6717.
- (8) Piddington, C. S.; Kovacevich, B. R.; Rambosek, J. *Appl. Environ. Microbiol.* 1995, *61*, 468-475.
- (9) Lei, B.; Tu, S. *J. Bacteriol.* 1996, *178*, 5699-5705.
- (10) Xi, L.; Squires, C. H.; Monticello, D. J.; Childs, J. D. *Biochem. Biophys. Res. Comm.* 1997, *230*, 73-75.
- (11) Gray, K. A.; Pogrebinsky, O. S.; Mrachko, G. T.; Xi, L.; Monticello, D. J.; Squires, C. H. *Nat. Biotech.* 1996, *14*, 1705-1709.
- (12) Finnerty, W. R. *Curr. Opin. Biotech.* 1992, *3*, 277-282.
- (13) Monticello, D. J. *CHEMTECH* 1998, *28*, 38-45.
- (14) Li, M. Z.; Squires, C. H.; Monticello, D. J.; Childs, J. D. *J. Bacteriol.* 1996, *178*, 6409-6418.
- (15) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular cloning: A laboratory manual*, 2nd edition. 1989. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- (16) Denis-Larose, C.; Bergeron, H.; Labbé, D.; Greer, C. W.; Hawari, J.; Grossman, M. J.; Sankey, B. M.; Lau, P. C. K. *Appl. Environ. Microbiol.* 1998, *64*, 4363-4367.
- (17) Steinmetz, M.; Le Coq, D.; Aymerich, S.; Gonzy-Treboul, G.; Gay, P. *Mol. Gen. Genet.* 1985, *200*, 220-228.
- (18) Reid, J. L.; Collmer, A. *Gene*, 1987, *57*, 239-246.
- (19) MacPherson, T.; Greer, C. W.; Zhou, E.; Jones, A. M.; Wisse, G.; Lau, P. C. K.; Sankey, B.; Grossman, M. J.; Hawari, J. *Environ. Sci. Technol.* 1998, *32*, 421-426.
- (20) Gallardo, M. E.; Ferrandez, A.; de Lorenzo, V.; Garcia, J. L.; Diaz, E. *J. Bacteriol.* 1997, *179*, 7156-7160.

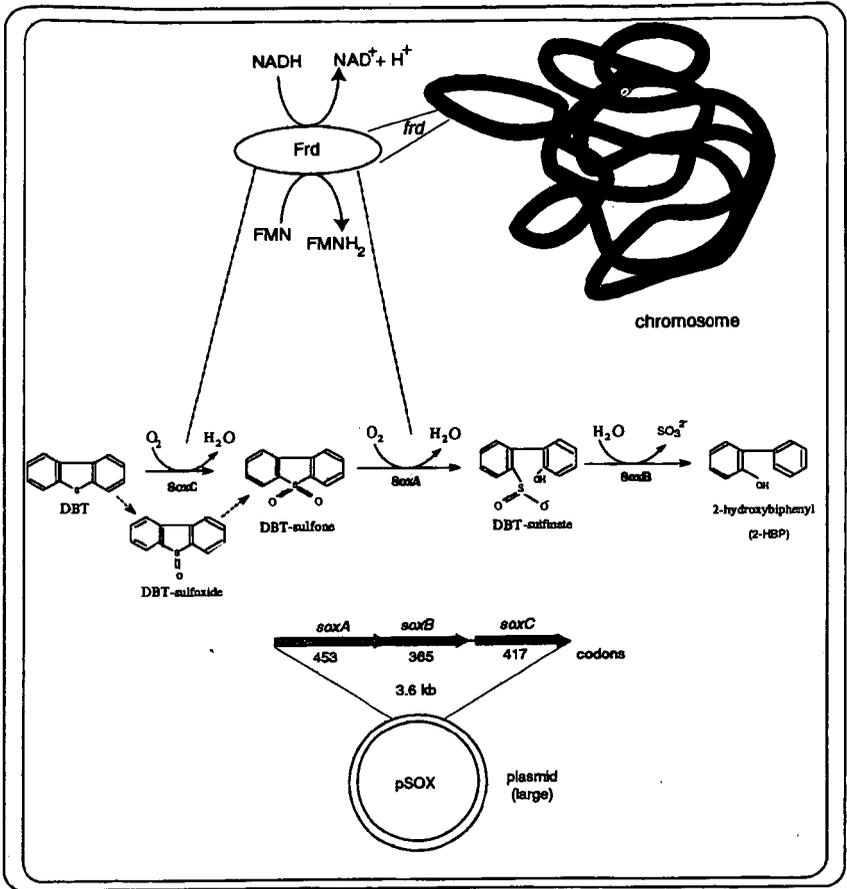


Fig. 1 The sulfur oxidation (*sox*)/desulfurization pathway of dibenzothiophene (DBT) and its genes in *Rhodococcus* spp. SoxA, DBT sulfone monooxygenase; SoxB, 2-(2-hydroxybiphenyl)-benzenesulfinate; SoxC, sulfite/sulfoxide monooxygenase; Frd, flavin reductase (FMN-NADH oxidoreductase). Compiled from references 6-11.

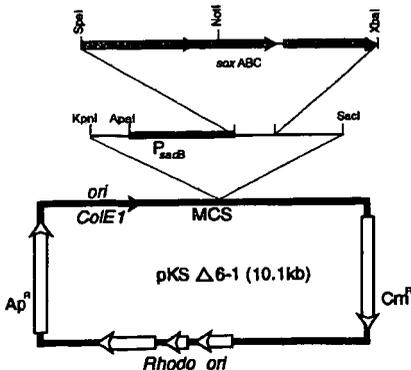


Fig. 2 Step-wise construction of the *sox*-gene expression plasmid under the control of the *Bacillus subtilis sacB* promoter element (*PsacB*). MCS is the multiple-cloning site of the *Rhodococcus-Escherichia coli* shuttle plasmid pKSΔ6-1 as previously described (16). *Cm^R*, chloramphenicol resistance gene; *Ap^R*, ampicillin resistance gene. *Rhodo ori* and *ColE1 ori*, origins of replication of *Rhodococcus* sp. strain X309 and *E. coli*, respectively.