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Cloning and Expression of Dissimilatory Sulfite Reductase (*dsr*AB) of *Desulfotomaculum geothermicum* Isolated from Indian Petroleum Refinery Cooling Towers

B. Anandkumar[1]*, E.S. Challaraj Emmanuel[2] and S. Maruthamuthu[3]

[1]Department of Biotechnology, [2]Department of Microbiology,
Sourashtra College, Madurai - 625 004, India
[3]Corrosion Protection Division, Central Electro-Chemical Research Institute,
Karaikudi - 630 001, India.

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A thermophilic sulfate reducing bacterium was isolated from corrosion product of cooling tower of an Indian petroleum refinery and characterized as *Desulfotomaculum geothermicum* with morphological and biochemical characters. Gene coding dissimilatory sulfite reductase (*dsr*AB), a key enzyme in the sulfate reduction was sequenced and cloned in an expression vector and over expressed with rDNA technology. Purified protein was identified as dissimilatory sulfite reductase having the molecular size of 97.6 kDa with MALDI - MS and discussed.

Key words: Sulfate reducing bacteria, biocorrosion, dissimilatory sulfite reductase.

Biocorrosion or microbially influenced corrosion (MIC) is the damage caused or accelerated by the presence of bacteria and other microorganisms and their metabolic activities. Beech and Sunner (2004) reviewed the main types of bacteria associated with metals in terrestrial and aquatic habitats are sulfate-, iron- and CO_2- reducing bacteria, sulfur-, iron- and manganese-oxidizing bacteria. In the petroleum industry, engineers have faced problems caused by microorganisms, since the beginning of commercial oil production. Bastin, (1926) reported the presence of SRB in oil environments and was rapidly recognized as responsible for the production of hydrogen sulfide, which is a toxic and corrosive gas responsible for a variety of environmental and economic problems including reservoir souring, contamination of natural gas and oil, corrosion of metal surfaces, and the plugging of reservoirs due to the precipitation of metal sulfides and the consequent reduction in oil recovery [Magot *et al*., 2000; Davidova *et al*., 2001].

Sulfate-reducing bacteria generate a proton motive force by the reduction of oxidized sulfur compounds, such as sulfate, sulfite or thiosulfate [Badziong and Thauer, 1980; Fitz and Cypionka, 1989]. Many redox proteins of these bacteria have been intensively studied and led to the discovery of novel prosthetic groups, such as the putative [6Fe- 6S] prismane cluster [Pierik *et al*., 1992b].

Pereira *et al*., (1996) carried out the purification and preliminary characterization of several proteins involved in the dissimilatory sulfate reduction in *Desulfovibrio desulfuricans* New Jersey strain. They isolated proteins such as hydrogenase catalyzing the reversible oxidation of molecular hydrogen and playing a central role in the energy yielding mechanisms of SRB, cytochrome c_3 having tetraheme playing a role in electron transport, adenylyl sulfate reductase catalyzing the activation of sulfate and reduction

* To whom all correspondence should be addressed.
E-mail.: aklifescience@gmail.com

of adenylyl sulfate forming AMP and sulfite, sulfite reductases (two forms: low spin having low molecular mass due to single polypeptide chain and high spin with a large molecular mass having four different types named desulfoviridin, desulforubidin, P_{582} and desulfofuscsidin) catalyzing the six electron reduction of sulfite to sulfide. Dissimilatory sulfite reductases (SiR) are classified according to their ultraviolet and visible absorption spectra: desulforubidin [Lee *et al*., 1973; Moura *et al*., 1988; Arendsen *et al*., 1993], desulfofuscidin [LeGall and Fauque, 1988], P-582 [Trudinger, 1970] and desulfoviridin [Lee and Peck, 1971; Pierik and Hagen, 1991; Wolfe *et al*., 1994]. Czaja *et al*., (1995) expressed desulforedoxin (*dsr* gene) of *Desulfovibrio gigas* in *E.coli* and characterized the physical and spectroscopic properties of the recombinant protein.

MATERIAL AND METHODS

Isolation and characterization of *Desulfotomaculum geothermicum* from the corrosion sample

Corrosion sample collected from an Indian petroleum refinery cooling towers was transported to Sourashtra College Biotechnology laboratory in sterile condition. The sample was enriched with Postgate medium B and enriched sample was inoculated in Modified Baar's medium with acetate as the carbon source and incubated in anaerobic gas pack chamber (Himedia, Mumbai) at 55°C for 3 days. The isolate was characterized for their morphology, motility, H_2S production, and presence of desulfoviridin.

Cloning and expression of dissimilatory sulfite reductase

Genomic DNA of the isolate was extracted with the protocol described by Wawer and Muyzer (1985). The Genomic DNA was used as the template for *dsr*AB gene amplified with primers *DSR*1F (5'- ACG CAC TGG AAG CAC G -3') and *DSR*4R 5' (GTG TAG CAG TTA CCG CA -3') [Wagner *et al*., 1998] in Thermo cycler (Thermo Hybaid) with the PCR program as 30 amplification cycles containing denaturation at 94°C for 30 sec, primer annealing at 54°C for 1 min and primer extension at 72°C for 2 min and 1 cycle of final extension at 72°C for 10 min. The PCR products were purified by QIAquick PCR purification kit as described by the manufacturer and cloned using QIAGEN PCR cloning plus kit as described by the manufacturer. Clones were selected, plasmids with insert isolated and sequenced with M13 sequencing primers using ABI Biosystems automated sequencer. *dsr*AB genes amplified and purified by the above method were cloned in QIAexpress UA Cloning vector (Qiagen) and transformed in competent *E.coli* M15 (pREP4) cells (Qiagen) as described by the manufacturers. The recombinant clones were selected from Luria-Bertani agar plates containing Ampicillin (100µg/ml), and IPTG (50mM). The clone was screened for the presence of *dsr*AB gene in the plasmid isolated from the recombinant. The screened clone was further inoculated in Luria Bertani broth containing Ampicillin (100 µg/ml) and expression of *dsr* protein was induced with IPTG (1mM). The induced cells were lysed in denaturing condition with lysis buffer (100mM NaH_2PO_4, 10 mM Tris HCl, 8M Urea) and centrifuged at 10,000 rpm for 10 minutes. The expressed proteins in the supernatant were resolved in 12% SDS-PAGE with protein molecular weight marker (MBI Fermentas). The proteins were stained using Coomassie-R250 staining solution, destained and the bands were visualized [Sambrook *et al*., 1998].

The proteins obtained were quantified using Bradford's method [Darbre, 1986]. 0.1ml and 0.5ml of sample were withdrawn and made up to 1ml using 0.15M NaCl and to that 1ml of Bradford's reagent (50mg of Coomassie Brilliant Blue G-250 in 25 ml ethanol) was added, kept at room temperature for 2 minutes and reading was taken at 595 nm (Shimadzu UV1700) against NaCl that was used as blank. Bovine Serum Albumin was used as the standard solution.

The proteins were purified using Ni-NTA spin kit (Qiagen) for His tagged protein [Janknecht *et al*., 1991] as described by the manufacturers.

Identification of dissimilatory sulfite reductase

A Maldi ToF MS (Kratos Analytical) was used for Mass analysis. The matrix 3,5-dimethoxy-4-hydroxycinnamic acid was made up to a saturated solution with 1% trifluoroacetic

acid/50% acetonitrile. 0.5 µl of the sample and 0.5 µl of matrix were applied to a sample plate and vacuum dried. A pulsed UV nitrogen laser was used to desorb ions from the sample. The instrument was operated at an accelerating voltage 24kV.The spectra were generated by 50-60 laser shots. The data were acquired from the instruments operating in the positive reflectron mode.

RESULTS

The enriched sample from the corrosion product in Postgate medium B was inoculated in Modified Baar's medium formed black coloration in the tubes indicating the formation of H_2S (Fig.1). The isolate was Gram positive, spore forming, acetate oxidizing, desulfoviridin negative and growth at 55°C in anaerobic condition inferring the presence of *Desulfotomaculum geothermicum*. The isolate was further confirmed with the BLAST (Altschul *et al*., 1997) analysis of amplified 1.9 Kb *dsr* gene (Fig.2) sequenced showing 99% similarity with *dsr* gene of *Desulfotomaculum geothermicum* sequences in the database.

Isolate Control

Fig. 1. H_2S production by the isolate in Modified Barr's medium

The *dsr*AB gene purified was cloned in expression vector and over expressed in *E.coli* cells with the induction by IPTG for different time intervals. The cells were lysed and the proteins in crude form and purified using His tag kit were estimated using Bradford's method (Table.1). Mass of the proteins collected after 3 hrs in crude form and His tag purified were analyzed with MALDI ToF and the peaks in the range between 90 and 100kDa were given in the fig. 3 and 4. A peak having 100% area covered corresponding to 97.6 kDa was found in the His tag purified protein sample analysis inferring the expression of dissimilatory sulfite reductase.

Table 1. Concentration of proteins expressed and His Tag purified

S. No.	Samples	Conc. of Protein µg / ml
1.	Uninduced samples	79
2.	Induced sample after 2 hours	97
3.	Induced sample after 3 hours	132
4.	His-Tag purified sample	16

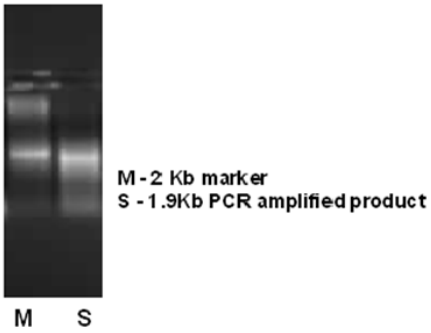

Fig. 2. Electrophoretograph of PCR amplified *dsr* gene product

DISCUSSION

Domestic and commercial heating systems, heat exchangers, and cooling systems favor the development of thermophilic bacteria, which are able to grow under both aerobic and anaerobic conditions [Ford *et al*., 1987]. *Desulfotomaculum geothermicum* was isolated and identified from geothermal water by Daumas *et al*., (1988). They observed that the strain was spore forming, H_2S producing, growing at 56°C, oxidizing acetate incompletely, having no desulfoviridin as their morphological and physiological characters. The high temperature influence the sporulation and incomplete oxidation of acetate transfer electrons and carry out the sulfate reduction to form sulfide. After many works on *Desulfotomaculum geothermicum,*

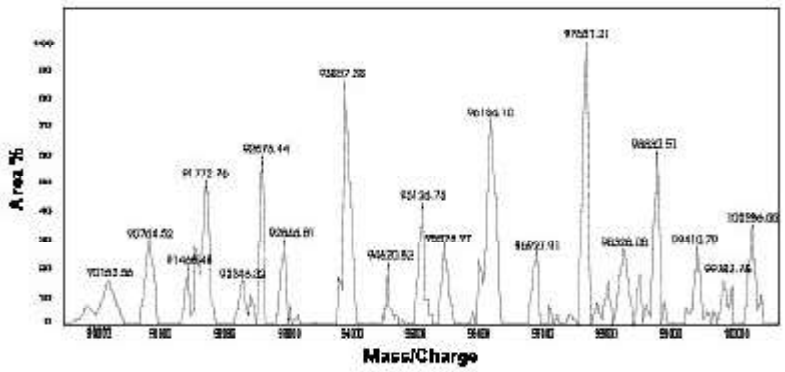

Fig. 3. Maldi MS analysis of unpurified protein extract

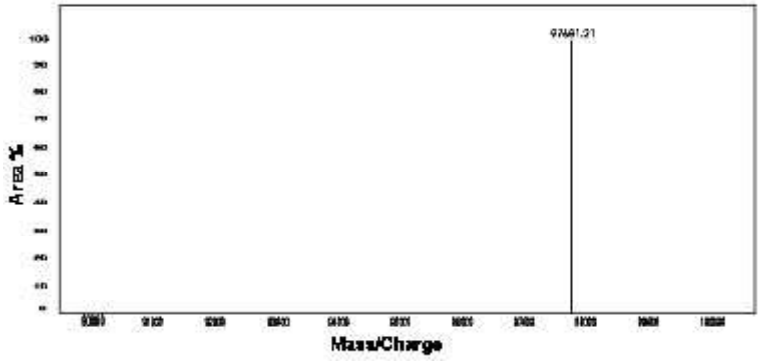

Fig. 4. Maldi MS analysis of purified protein extract

it was confirmed that the vegetative cells respond to Gram's reaction as positive.

The isolate was confirmed after sequencing the gene dissimilatory sulfite reductase amplified from genomic DNA extarcted from the isolate. Wagner *et al*., (1998) and Klein *et al*., (2001) studied on the *dsr*AB genes encoding the dissimilatory sulfate reductase, the key enzyme in dissimilatory sulfate reduction, can be used as a phylogenetic marker for identification of SRBs and these genes have been found in all known sulfate-reducing prokaryotes [Zverlov *et al*., 2005].

Dissimilatory sulfite reductases consist of relatively long polypeptide chains containing both semi-conserved and highly conserved regions, and occur in sulfate- and sulfite-reducing prokaryotes as well as in some sulfur oxidizers [Dahl *et al*., 1993; Karkhoff-Schweizer *et al*., 1995; Hipp *et al*., 1997]. Therefore, the sulfite reductase subunits in principle appeared to be suited to trace the evolution of dissimilatory sulfur metabolism. Lee and Peck (1971) found sulfite reductases of the desulfoviridin type in *Desulfovibrio* species. Lee *et al*., (1973) initially described their subunit structure as $\alpha_2\beta_2$, with a molecular mass of 50 kDa for the α and 40 kDa for the β subunits, but a third subunit, ã (11 kDa), was discovered, and an $\alpha_2\beta_2\gamma_2$ structure was proposed by Pierik *et al*., (1992a) for *Desulfovibrio vulgaris* (Hildenborough), *D. vulgaris oxamicus* (Monticello), *D. gigas*, and *D. desulfuricans* ATCC 27774. The identified protein in the study is similar in their size and subunit structures with that of eubacterial and archael SRB species.

The genes for the α- and β-subunits (*dsr*A and *dsr*B) are contiguous in the order *dsr*A*dsr*B and most probably comprise an operon with the directly following *dsr*G and *dsr*C genes. *dsr*G and *dsr*C encode products which are homologous to eukaryotic glutathione S-transferases and the proposed γ-subunit of *Desulfovibrio vulgaris* sulfite

reductase, respectively. *dsr*A and *dsr*B encode 44.2 kDa and 41.2 kDa peptides which show significant similarity to the two homologous subunits *Dsr*A and *Dsr*B of dissimilatory sulfite reductases [Molitor *et al*., 1998]. They observed significant differences in their subunit composition, especially with regard to the ã subunit between the enzymes of the two species. á and â subunits of Dissimilatory sulfite reductases in hyperthermophilic archaeon *Pyrobaculum islandicum* were expressed and purified by recombinant DNA methods and immunoblotting methods. Steuber *et al*., in 1995 analyzed the expressed sulfite reductase of *Desulfovibrio desulfuricans* with the enzyme of *Desulfovibrio vulgaris* by immunodetection with antibodies raised against α, β and γ-subunits of *D.vulgaris* enzyme. Solution structure of ã-subunit *dsr*C of sulfite reductase in *Pyrobaculum aerophilum* [Cort *et al*., 2001] was determined by NMR spectroscopy. The structures analyzed and discussed by many authors give an idea about the localization of sulfite reductases in the membrane region.

The protein expressed in this study may be used in the development of immobilized enzyme sensor probe to detect the SRB in the environment without cultivation and molecular approach protocols.

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