Deletion of flavoredoxin gene in Desulfovibrio gigas reveals its participation in thiosulfate reduction

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Abstract The gene encoding Desulfovibrio gigas flavoredoxin was deleted to elucidate its physiological role in the sulfate metabolism. Disruption of flr gene strongly inhibited the reduction of thiosulfate and exhibited a reduced growth in the presence of sulfate with lactate as electron donor. The growth with sulfate was not however affected by the lack of this protein. Addition of sulfite with lactate as electron donor. The growth with sulfate metabolism. Disruption of Desulfovibrio gigas metabolizes broadly distributed in nature. Keywords: Flavoredoxin; Mutant; Thiosulfate reduction; Sulfite reduction; Desulfovibrio gigas

1. Introduction

Desulfovibrio (D) are sulfate-reducing bacteria, strictly anaerobic organisms broadly distributed in nature. Desulfovibrio are able to reduce sulfur compounds, such as sulfate, sulfite or thiosulfate as terminal electron acceptors. Although several studies have been performed with the aim of characterizing the dissipatory sulfate reduction pathway, it remains poorly understood. The sulfate reduction involves three main steps: (i) sulfate activation to APS; (ii) APS reduction to sulfite and (iii) sulfite reduction to sulfide [1]. The last step is a matter of controversy with two mechanisms proposed. The first one, described by Chambers and Trudinger [2] involves the reduction of sulfite to sulfide occurring in a single step through the transfer of six-electrons. The second one proposes the formation of trithionate \((\text{S}_2\text{O}_3^-)\) and thiosulfate \((\text{S}_2\text{O}_4^-)\) as intermediates in the sulfite reduction [3]. This mechanism was further substantiated by several studies [4–8] and requires three different enzymes: sulfite, trithionate and thiosulfate reductases [5].

D. gigas flavoredoxin (Flr) is an 40-kDa homodimer, containing one FMN molecule per monomer and was proposed to be the redox partner of desulfoviridin in the sulfite reduction from molecular hydrogen [9]. Flr appears to belong to the flavin reductase family considering the significant homologies with FMN:NADH:FAD:NADH oxidoreductases [10]. These enzymes are able to reduce free flavins by reduced pyrimidine nucleotides, supplying free reduced flavins to the cellular metabolism [11]. Like in D. vulgaris and D. desulfuricans, D. gigas Flr is encoded by a monocistronic unit and the analysis of the genetic organization around flr gene does not give any additional information on the metabolic pathway in which Flr might be involved [12].

With the aim of elucidating the functional role of Flr in the metabolism of D. gigas, the flr mutant was generated, being the first mutant of this anaerobic bacterium. The phenotypic characterization of this mutant revealed that thiosulfate is an intermediate of sulfite-reduction and that its reduction is dependent on the presence of Flr.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli XL1Blue (Stratagene) and TG2 [13] were used to clone the constructions made in the vectors pZer0-1\textsuperscript{TM} (Invitrogen) and pJRD215 [14], respectively. D. gigas ATCC 19364 was grown anaerobically at 37 °C as previously described [15]. D. gigas wild-type and flr mutant were also grown at 37 °C, in basal medium [16] supplemented with one electron acceptor: 40 mM sulfate; 20 mM sulfite; 40 mM thiosulfate and one electron donor: 40 mM lactate; 40 mM pyruvate, inoculated with 10% (vol/vol) of early stationary phase cultures grown in the same specific medium. Growth was followed measuring OD\textsubscript{kom} in a Smart Spect 3000 (BioRad), using 1 cm pathlength cells. The experiments were reproduced at least twice.

2.2. Plasmid construction

A 4378-bp ApaI–BamHI DNA fragment from the D. gigas genome containing the flr gene was inserted in the cloning vector pZer0-1\textsuperscript{TM}. The oligonucleotides \(\text{flrXbaI-1}/\text{flrXbaI-2}\) and \(\text{Kn}\text{R}^\text{XbaI-1}/\text{Kn}\text{R}^\text{XbaI-2}\) (Table 1) were used to amplify this DNA fragment [94 °C, 2 min; 25 cycles (94 °C, 30 s; 66 °C, 30 s; 68 °C, 12 min); 68 °C, 7 min] and the \(\text{Kn}\text{R}\) gene from the plasmid pJRD215 [14] [94 °C, 2 min; 25 cycles (94 °C, 30 s; 66 °C, 30 s; 68 °C, 12 min); 68 °C, 7 min], respectively. The PCR products were ligated with T4 DNA Ligase (Fermentas) and this construct was transformed in E. coli XL-1Blue. Kanamycin-resistant colonies were selected and their plasmids analyzed. This construct was used in the electrottransformation experiments.

2.3. Transformation of D. gigas

A pMB1-based suicide vector for D. gigas carrying the mutagenic construction (Fig. 2A) was used to obtain the flr mutant by homologous recombination. Competent cells were prepared based on the

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Abbreviations: Flr, flavoredoxin; D, Desulfovibrio

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method previously described [17] from 340 ml of an early stationary phase culture. 50 l of the cell suspension was mixed with 5.4 l the suicide plasmid. Electroporation was performed with an electro-
porator Jouan GHT 1287B (5500 V/cm during 6 ms). The colonies of flr
mutants were isolated in SOS medium [17] without fructose, supple-
mented with 12% (wt/vol) agar and 50 l kanamycin/ml, using the roll
tube technique. The colonies were picked up after two weeks of growth
and introduced into 2 ml septum-stoppered tubes containing 1 ml of
SOS medium. These tubes were incubated at 37\degree \text{C} in hyperbaric
anaerobic jars (CNRS, Marseille, France) under a N\textsubscript{2}/CO\textsubscript{2} atmosphere (80/20) (2–3 bar pressure) during 10 days. The cultures were then sub-
cultured in 10 ml Hungate tubes. Plating manipulations were
performed in a BS531 anaerobic chamber (Jacomex, Dagneux,
France).

2.4. Southern and Northern blots
The genotypes of kanamycin-resistant mutants were analyzed by
Southern blot. Genomic DNA was purified with "Wizard genomic
DNA purification Kit" (Promega) and digested with Sph\textsubscript{I} (Strata-
genome). Membranes were hybridized with the probes described in
Fig. 2A labeled with [\alpha\textsuperscript{-32P}]dATP as previously described [13]. The
probes 1, 2 and 3 were obtained by PCR, using the oligonucleotides
Fx8R4/Fx88U2, Kn\textsuperscript{R}Xba\textsuperscript{I-1}/Kn\textsuperscript{R}Xba\textsuperscript{I-2} and FlrP10/
flrXba\textsuperscript{I-1}, respec-
tively (Table 1).

Total RNAs were purified from early exponential (OD 600 nm/C25
0.3) D. gigas cultures, using the RNA isolation procedure as de-
scribed by Rodrigues et al. [18]. The RNAs were further fractionated
in 1.2% (wt/vol) agarose gel in MOPS buffer containing 6% (vol/vol)
formaldehyde and transferred onto a positively charged nylon mem-
brane (Amersham Biosciences). The membranes were hybridized with
probe 1 (Fig. 2A) and 16S rRNA probes labeled with [\alpha\textsuperscript{-32P}]dATP as
previously described [19]. The probe 16S rRNA was obtained by PCR
using the oligonucleotides 16SP1 (5\textsuperscript{0} -CCGGGATAACGGTGAA-
CC-3\textsuperscript{0}) and 16SP2 (5\textsuperscript{0} -GTGCAGTTCCCCGGTTGAGCC-
3\textsuperscript{0}) to amplify a region from the 16S ribosomal DNA in the
D. gigas gen-
ome. mRNA levels were quantified (ImageQuant, Molecular Dynam-
ics) and normalized against those of the internal loading control, the
16S rRNA.

2.5. H\textsubscript{2} consumption rate with thiosulfate as electron acceptor
D. gigas wild-type and flr
mutant were grown in 1.8 l SOS medium [17] without fructose. Cells were collected in early exponential phase (OD600 nm
0.3), washed and resuspended with 2 ml of a degassed buf-
fer (50 mM MOPS buffers, pH 7.2, 5 mM MgCl\textsubscript{2}, 5 mM DTT). All
manipulations were performed under N\textsubscript{2} flux.

\[ \Delta G^\circ = -46.3 \text{ KJ/mol} \]
\[ \Delta G^\circ = -123 \text{ KJ/mol} \]
\[ \Delta G^\circ = -2.1 \text{ KJ/mol} \]

Fig. 1. Proposed mechanisms for sulfite reduction. Single step six-electron reduction of sulfite (a) and trithionate/thiosulfate pathway (b). SO\textsubscript{3}Red, sulfite reductase; S\textsubscript{2}O\textsubscript{3}Red, trithionate reductase and S\textsubscript{2}O\textsubscript{3}Red, thiosulfate reductase.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|}
\hline
\textbf{Oligonucleotides} & \textbf{Sequence} \\
\hline
\textit{K}_{\text{R}}Xba\textsuperscript{1} & 5\textsuperscript{'-}GAAGTTTCTAGA CATGACTGGGCTATCTG-3\textsuperscript{'} \\
\textit{K}_{\text{R}}Xba\textsuperscript{2} & 5\textsuperscript{'-}GAAGTTTCTAGACAGTCCCTGACCCGAGG-3\textsuperscript{'} \\
\textit{FlrP10} & 5\textsuperscript{'-}GAGTTCTAGA CCTCCCTTCCAAAACGG-3\textsuperscript{'} \\
\textit{flrXba\textsuperscript{1}} & 5\textsuperscript{'-}GAGTTCTAGA CAGCTACTGGGCTATCTG-3\textsuperscript{'} \\
\textit{flrXba\textsuperscript{2}} & 5\textsuperscript{'-}GAGTTCTAGA CTGCAGTTCGGGGGCATG-3\textsuperscript{'} \\
\hline
\end{tabular}
\caption{Restriction sites of the endonucleases are underlined.}
\end{table}

Fig. 2. Characterization of flr mutant. (A) Restriction maps of the Apal–BamHI homologous recombination region for the wild-type and mutant strains. A scale of 1 kb is indicated; (B) Southern blot analysis of Sph\textsubscript{I} digested chromosomal DNA from D. gigas wild-type (wt lane) and kanamycin-resistant strains (lanes 1 to 7). Chromosomal DNA was hybridized with probe 1 (flr) (1); probe 2 (\textit{K}_{\text{R}}) (2) and probe 3 (flank) (3). (C) Northern blot analysis of wild-type and mutant strains total RNAs hybridized with probe 1 (flr) and 16S probe as a positive control.
H₂ consumption rate using thiosulfate as electron acceptor was determined by the Warburg manometric technique under a H₂ atmosphere, based on the method described by Akagi et al. [20]. After H₂ atmosphere was established, 300 μl of washed cells were introduced into the main compartment through the side arm. This mixture was equilibrated with H₂ flux during 5 min. To start the reaction, 10 μmol sodium thiosulfate were introduced into the main compartment and the H₂ consumption was measured. To stop the reaction, 100 μl of an 85% (vol/vol) H₃PO₄ solution was added to the reaction mixture and the flask was shaken for an additional 10 min.

2.6. Analytical methods

The protein concentration from the cell suspensions was determined with Bradford assay (BioRad) using bovine serum albumin as the standard. Prior to Bradford assay, cells were broken with 5 cycles of 40 s of sonication.

Trithionate, thiosulfate and sulfide from the cultures supernatants were analyzed by HPLC using a column ASAHIPAK NH₂P-50 (Shodex) [21] under the following conditions: effluent 100 mM Na₂HPO₄, pH 8.5; effluent flow rate, 0.6 ml min⁻¹; sample volume, 20 μl. These sulfur compounds were spectrophotometrically detected at 215 nm (Varian). These measures were carried out immediately after sampling to prevent sulfur compounds from decomposing in the presence of oxygen.

3. Results

3.1. Generation of flr mutant

DNA from seven isolated kanamycin-resistant colonies was analyzed by Southern blot for replacement of the flr gene by the KnR cassette. Hybridization with probe 1 (flr gene) resulted in a positive band only in the lane corresponding to the wild-type strain genomic DNA (Fig. 2B.1), indicating that the genomic DNA from the kanamycin-resistant strains was deleted for flr gene. Hybridization with probe 2 (KnR gene) resulted in two positive bands, 1731 and 606 bp, in lanes 1 to 7, indicating that the kanamycin cassette was integrated in the genomic DNA of the aforementioned kanamycin-resistant colonies. As expected, no hybridization occurred with this probe in the wild-type lane (Fig. 2B.2). Hybridization with probe 3 (flanking region) resulted in one positive band in all lanes, a 1928 bp band in the wild-type lane and a 1731 bp in lanes from 1 to 7, which is consistent with the expected size of the fragments in the deleted strain (Fig. 2B.3). The genomic organization analysis of the mutants confirmed the planned deletion strategy (Fig. 2A). None of the tested recombinants exhibited a single cross over, indicating that D. gigas has a high recombination efficiency, as previously reported in D. fructosovorans [22].

Total RNAs of D. gigas wild-type and mutant strains were analyzed by Northern blot. Hybridization with probe 1 (flr) revealed that the mRNA was detected only in the wild-type strain (Fig. 2C), which confirmed that in the mutant strain the flr gene was indeed disrupted.

3.2. Characterization of the flr mutant

The ability of the D. gigas wild-type and flr deleted strains to metabolize different substrates revealed that the mutant was not able to use thiosulfate as the final electron acceptor (Fig. 3A). Comparison of the growth curves revealed a dramatic decrease in the growth of the mutant strain when thiosulfate was the terminal electron acceptor using lactate or pyruvate as electron donors (Fig. 3A), corresponding to 86% and 70% decrease of the growth rates, respectively. Growth was also followed using sulfate or sulfite as the electron acceptor (Fig. 3B and C). No significant growth differences were detected between the wild-type and mutant strains, except for the 35% decrease of the mutant growth rate in sulfite–lactate. The efficient reduction of sulfite when pyruvate is the electron donor might be explained by the very low electron potential of the pyruvate-acetate-couple (E⁰pyruvate-/acetate=−670 mV) that can reduce thiosulfate by diverted pathway.

In order to verify whether thiosulfate accumulated during growth, the sulfur-compounds that appeared in the medium were analyzed using a new HPLC technique. No intermediates were detected, in both wild-type and mutant strains, when sulfite was used. Indeed, sulfate concentration decreased and sulfide concentration increased at the same rate (data not shown). In contrast, when sulfite was used, trithionate was formed in the medium, giving rise to thiosulfate, which disappeared along with sulfide formation. In the mutant strain growing with lactate and sulfite, thiosulfate accumulated at a higher extent in the medium and sulfide was formed at a much lower level when compared with wild-type strain (Fig. 4).

The flr mRNA expression was analyzed under the growth conditions, lactate/sulfate, pyruvate/sulfate, lactate/sulfite, pyruvate/sulfite, lactate/thiosulfate and pyruvate/thiosulfate (Fig. 5). The level of flr expression is significantly increased when D. gigas is grown with sulfite (5.7 and 3.7 times higher for lactate and pyruvate) and thiosulfate (4.3 and 2.7 times higher for lactate and pyruvate) at 37 °C. However, such increase is not significant when grown with both thiosulfate and lactate or pyruvate.

![Fig. 3](image-url) Growth curves under different growth conditions. Growth curves of D. gigas wild-type (——) and mutant (---) strains using different electron acceptors [40 mM thiosulfate (A), 20 mM sulfite (B) and 40 mM sulfate (C)] and different electron donors [40 mM lactate (x) and 40 mM pyruvate (●)]. These cultures were inoculated with 10% of early stationary phase cultures grown in the same specific medium and incubated at 37 °C. The values correspond to the average of duplicate cultures.
higher for lactate and pyruvate), when compared with growth in the presence of sulfate.

Our aforementioned data indicated the involvement of Flr in the thiosulfate reduction. Therefore, we determined the H$_2$ consumption rates of wild-type and flr mutant strains cell suspensions, as indirect indication of thiosulfate reductase activity. Wild-type and flr mutant strains revealed a H$_2$ consumption rate of 1.04 ± 0.02 and 0.58 ± 0.07 mol H$_2$/min/mg protein, corresponding to a decrease of about 50%.

4. Discussion

In order to investigate the role of Flr in the metabolism of D. gigas, we have constructed the flr disrupted strain and evaluated its ability to grow under different conditions. flr deletion abolishes growth in the presence of thiosulfate (Fig. 3A), revealing that Flr is involved in the thiosulfate reduction. A lower H$_2$ consumption rate using thiosulfate as electron acceptor is also observed with flr mutant when compared with wild-type cells, which reinforces the involvement of Flr in the thiosulfate reduction. The 50% H$_2$ consumption rate detected in the mutant strain (see Section 3) can be justified by the presence of other electron carriers that might replace Flr, although with much less efficiency. Previous results obtained by Hatchikian [23] using in vitro assays in the absence of Flr, already suggested the existence of another natural redox protein in the coupling of molecular hydrogen oxidation with thiosulfate reduction. Although the system here described may differ from the in vitro mechanism, our study points towards Flr being the missing component of the system that couples hydrogen oxidation with thiosulfate reduction.

As the flavoredoxin deletion specifically impairs thiosulfate reduction, alterations in the sulfate and sulfite reduction were not expected in the mutant strain (Fig. 3A). However, in the presence of sulfite, a slower growth rate was observed when lactate was the electron donor. This intriguing observation revives the old debate on the possible presence of intermediates during sulfite reduction [3–6], as our results indicate that under these specific conditions thiosulfate reduction might be involved. In order to investigate whether thiosulfate is an intermediate of sulfite reduction, the sulfur compounds formed during growth in lactate-sulfite medium were analyzed (Fig. 4). Our results clearly show that in both wild-type and mutant strains, sulfite reduction occurs with the transient formation of trithionate and thiosulfate, suggesting that sulfite, trithionate and thiosulfate would be sequentially at play (Fig. 4A and B, respectively). In the mutant, the process stalled upon thiosulfate formation due to its inability to further reduce it into sulfide, thus thiosulfate accumulates to a much higher extent (Fig. 4B). Although, the mutant is affected in its ability to reduce thiosulfate, growth is still possible in sulfite because enough energy is driven from the first two reaction steps (see Fig. 1). The analysis of the kinetics presented in Fig. 4 shows that in both wild-type and mutant strains, sulfite reduction occurs with the transient formation of trithionate and thiosulfate, suggesting that sulfite, trithionate and thiosulfate would be sequentially at play (Fig. 4A and B, respectively). In the mutant, the process stalled upon thiosulfate formation due to its inability to further reduce it into sulfide, thus thiosulfate accumulates to a much higher extent (Fig. 4B). Although, the mutant is affected in its ability to reduce thiosulfate, growth is still possible in sulfite because enough energy is driven from the first two reaction steps (see Fig. 1). The analysis of the kinetics presented in Fig. 4 shows that in both wild-type and mutant strains, sulfite reduction occurs with the transient formation of trithionate and thiosulfate, suggesting that sulfite, trithionate and thiosulfate would be sequentially at play (Fig. 4A and B, respectively). In the mutant, the process stalled upon thiosulfate formation due to its inability to further reduce it into sulfide, thus thiosulfate accumulates to a much higher extent (Fig. 4B). Although, the mutant is affected in its ability to reduce thiosulfate, growth is still possible in sulfite because enough energy is driven from the first two reaction steps (see Fig. 1).
and Cypionka [5] and explains why the production of intermediates is still controversial. The flr gene expression in the presence of thiosulfate and sulfate is highly induced (Fig. 5), suggesting that Flr is indeed required for the reduction of thiosulfate. In the presence of sulfate, the induction observed reflects the formation of thiosulfate, showing a metabolic relationship between these compounds. With sulfate there is no thiosulfate accumulation and only a flr basal expression is observed. When sulfate is the electron acceptor, it can be postulated that sulfite is produced at a very low rate through the reduction of APS, as APS synthesis requires ATP consumption. Therefore, at this stage two hypotheses might explain the absence of intermediates: (1) the cellular concentration of sulfite is low enough to avoid the inhibition of trithionate reduction; (2) the intermediates are not produced. Although it is still not possible to discriminate between them, we favor the latter, as it is the only one that is consistent with the normal growth of the mutant in the presence of sulfate. Overall, our results suggest that the presence of intermediates depends on sulfite concentration and clearly demonstrate that Flr is crucial for \textit{D. gigas} thiosulfate reduction.

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