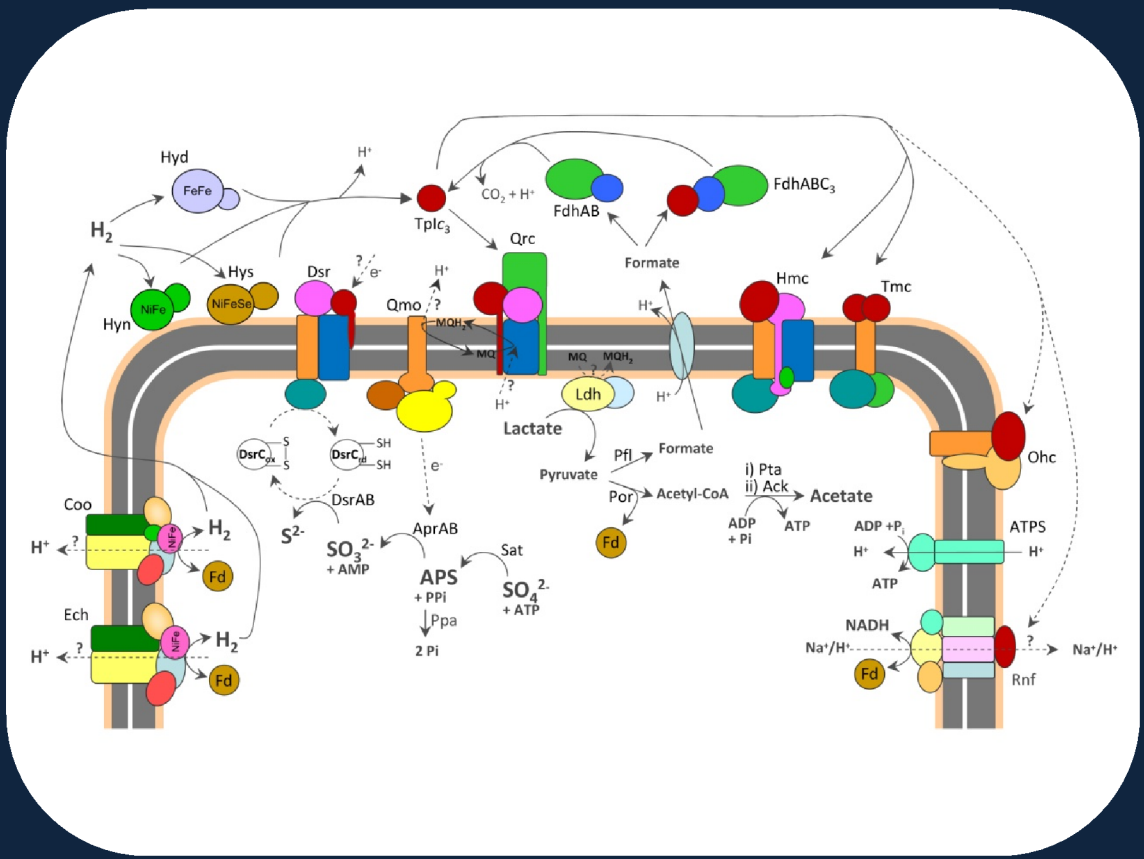
# *Electron transfer chains in sulfate reducing bacteria*

*Sofia Cristina dos Santos Venceslau*




*Dissertation presented to obtain the Ph.D degree in Biochemistry*

*Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa*

*Oeiras, December, 2011*

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA /UNL

Knowledge Creation

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**Supervisor:** Dr. Inês A. Cardoso Pereira

**Co-supervisor:** Dr. Lígia M. Saraiva

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From left to right: Arnaldo Videira, Solange Oliveira, José Moura, Sofia Venceslau, Ben Berks, Inês Pereira, Claudina Rodrigues-Pousada and Lígia Saraiva

December 15, 2011

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# Thesis Outline

The work described in this thesis concerns the study of electron transfer chains in sulfate reducing bacteria, focusing on the link between the menaquinone pool and terminal reductases located in the cytoplasm.

This dissertation starts with two introductory chapters. The first focuses on the description of the group of sulfate reducing organisms, with special emphasis on *Desulfovibrio* spp.. The second chapter addresses sulfate respiration, including the dissimilatory sulfate reduction pathway, membrane complexes involved and energy conservation models. Chapters three to five describe the experimental results achieved during this work. Chapter three describes the isolation, characterization and functional studies of a new membrane bound complex from *Desulfovibrio vulgaris*, Quinone-reductase complex, which is involved in sulfate respiration with hydrogen or formate. Chapter four includes studies on the dissimilatory sulfite reduction pathway and proteins involved in that process. Chapter five concerns a genomic comparative survey of 25 genomes of sulfate reducers, focusing on the minimal set of proteins required for sulfate reduction and membrane redox complexes. The last chapter consists of final conclusions and future perspectives of the work.

# List of Publications

## Thesis Publications

- **Sofia S. Venceslau**, Rita R. Lino and Inês A.C. Pereira (**2010**) The Qrc membrane complex, related to the alternative complex III, is a menaquinone reductase involved in sulfate respiration. *Journal of Biological Chemistry*, 285 (30): 22774-22783

- **Sofia S. Venceslau**, Daniela Matos and Inês A.C. Pereira (**2011**) EPR characterization of the new Qrc complex from sulfate reducing bacteria and its ability to form a supercomplex with hydrogenase and Tp$Ic_3$. *FEBS Letters*, 585 (14): 2177-2181

- Inês A.C. Pereira, A. Raquel Ramos, Fabian Grein, Marta C. Marques, Sofia M. da Silva, **Sofia S. Venceslau** (**2011**) A comparative genomic analysis of energy metabolism in sulfate-reducing bacteria and archaea, Review article. *Frontiers in Microbiology*, 2: 69

## Other Publications

The following publications are co-authored by the candidate which, although not a core part of this thesis, are in its majority still related with it:

- Ricardo H. Pires, **Sofia S. Venceslau**, Francisco Morais, Miguel Teixeira, António V. Xavier, and Inês A.C. Pereira (**2006**) Characterization of the *Desulfovibrio desulfuricans* ATCC 27774 DsrMKJOP complex - A membrane-bound redox complex involved in the sulfate respiratory pathway. *Biochemistry*, 45 (1): 249-262

- Filipa M. Valente, Patrícia M. Pereira, **Sofia S. Venceslau**, Manuela Regalla, Ana V. Coelho, Inês A.C. Pereira (**2007**) The [NiFeSe] hydrogenase from *Desulfovibrio vulgaris* Hildenborough is a bacterial lipoprotein lacking a typical lipoprotein signal peptide. *FEBS Letters*, 581 (18): 3341-3344

- Sofia M. da Silva, **Sofia S. Venceslau**, Claúdia L. Fernandes, Filipa M. Valente, Inês A.C. Pereira (**2008**) Hydrogen as an energy source for the human pathogen *Bilophila wadsworthia*. *Antonie Van Leeuwenhoek*, 93 (4): 381-390

- Tânia F. Oliveira, C. Vonrhein, Pedro M. Matias, **Sofia S. Venceslau**, Inês A.C. Pereira, Margarida Archer (**2008**) Purification, crystallization and preliminary crystallographic analysis of a dissimilatory DsrAB sulfite reductase in complex with DsrC. *Journal of Structural Biology*, 164 (2): 236-239

- Tânia F. Oliveira, C. Vonrhein, Pedro M. Matias, **Sofia S. Venceslau**, Inês A.C. Pereira, Margarida Archer (**2008**) The crystal structure of *Desulfovibrio vulgaris* dissimilatory sulfite reductase bound to DsrC provides novel insights into the mechanism of sulfate respiration. *Journal of Biological Chemistry*, 283 (49): 34141-34149

- Fabian Grein, **Sofia S. Venceslau**, Lydia Schneider, Peter Hildebrandt, Smilja Todorovic, Inês A.C. Pereira, Christiane Dahl (**2010**) DsrJ, an essential part of the DsrMKJOP transmembrane complex in the purple sulfur bacterium *Allochromatium vinosum*, is an unusual triheme cytochrome c. *Biochemistry*, 49 (38): 8290-8299

# Dissertation Summary

The dissimilatory reduction of sulfur compounds (*i.e.* sulfate/sulfite reduction and sulfur disproportionation) is considered to have been one of the earliest metabolic processes on Earth able to sustain life. Dissimilatory sulfate reduction, using sulfate as an electron acceptor and organic compounds or hydrogen as electron donors, plays a significant role in the global sulfur and carbon cycles. In anaerobic environments up to 50 % of organic matter mineralization is achieved by sulfate reducers. The molecular mechanisms of sulfate respiration are still poorly understood, as this process is quite different from other respiratory systems and involves novel biological molecules.

The aim of the work presented in this dissertation was to provide a contribution to the understanding of the electron transfer chain that allows energy conservation in the respiratory chain of sulfate reducers. For this purpose two key points were addressed: i) study of the electron transfer pathway from periplasmic hydrogen and formate oxidation to the membrane quinone pool and from there to the reduction of sulfate in the cytoplasm, and ii) study of the electron transfer chain involved in the last step of sulfate respiration, the dissimilatory reduction of sulfite.

In the first part of the work, a new complex from *Desulfovibrio vulgaris* Hildenborough was isolated and characterized, which reduces the menaquinone pool with electrons transferred from Type I cytochrome $c_3$ (TpI$c_3$). This complex was

named Qrc for Quinone reductase complex. The Qrc complex is composed by four subunits, three periplasmic (QrcABC) and one integral membrane subunit (QrcD). QrcA is an hexaheme cytochrome *c* with a membrane anchor. QrcB is a large protein also anchored to the membrane that belongs to the molybdopterin oxidoreductase family. QrcC is a periplasmic iron-sulfur cluster protein. QrcD is a membrane embedded subunit of the NrfD family. The QrcBCD subunits are closely related with members of the complex iron-sulfur molybdoenzyme family (also known as DMSO reductase family), but Qrc does not contain a molybdenum cofactor, which suggests a change in function from chemical catalysis to electron transfer. The Qrc complex contains six *c*-type low-spin hemes, one $[3Fe\text{-}4S]^{1+/0}$ cluster and at least three $[4Fe\text{-}4S]^{2+/1+}$ clusters. The midpoint redox potentials of the Qrc centers were determined by EPR. The hemes potentials range from +100 to -130 mV, while the $[4Fe\text{-}4S]^{2+/1+}$ clusters are in the interval between -130 to -240 mV, and the $[3Fe\text{-}4S]^{1+/0}$ cluster has a redox potential of +160 mV. These midpoint potentials are in a suitable range for accepting electrons from the physiological partner Tpl$c_3$, whose redox potentials are low (-325 to -170 mV), to the final electron acceptor menaquinone (-70 mV). In addition, the EPR signal of $[3Fe\text{-}4S]^{1+/0}$ cluster is affected by the presence of a menaquinone analogue, suggesting that the location of the menaquinone binding site is close to the periplasmic subunits.

Functional studies with Qrc showed that it acts as a Tpl$c_3$:menaquinone oxidoreductase, with electrons generated from periplasmic hydrogen and formate oxidation. Furthermore, this functional association was also shown at a structural level, since Qrc was observed to form a supercomplex with the [NiFe] hydrogenase and Tpl$c_3$. Qrc is the first respiratory complex in

sulfate reducers shown to act as a quinone reductase. Its physiological importance is related to the fact that, in most sulfate reducers, the periplasmic uptake hydrogenases and formate dehydrogenases lack a membrane subunit for direct quinone reduction. Indeed, Qrc is present in deltaproteobacterial sulfate reducers that have soluble periplasmic hydrogenases and/or formate dehydrogenases, whose electron acceptor is the Tpl$c_3$. In these organisms Qrc is essential for growth in hydrogen or formate. Furthermore, we propose that the Qrc and Qmo complexes may be involved in a redox loop mechanism that sustains the electron transport across the membrane from hydrogen and formate oxidation to the cytoplasmic reduction of sulfate, coupled to proton transfer for ATP synthesis.

The second topic of research concerns the reduction of sulfite to sulfide, where there are still several open questions. These include the electron donor to the final reductase, its coupling to energy conservation, the proteins involved in the process, and finally the mechanism of sulfite reduction itself. The structure of the DsrAB-DsrC complex led to the proposal of a new mechanism for sulfite reduction, which highlights DsrC as a central protein in the process. DsrC is suggested to be directly involved in the mechanism, through release of sulfide via a persulfide intermediate, that leads to an intramolecular disulfide bond, which is then the proposed substrate of the DsrMKJOP complex. The objective of this work was to try to verify the proposed model. First, it was shown that most DsrC in the cell is not strictly associated with DsrAB, and so is not a subunit of DsrAB as previously stated. DsrC contains two conserved redox active cysteines, and one of them is at the penultimate position of a C-terminal flexible arm. Thus DsrC is very prone to form an

intermolecular disulfide-bonded dimer under oxidizing conditions. So it was necessary to develop an oxidation protocol to form specifically the intramolecular disulfide bond in DsrC. A gel-shift assay based on a thiol reactive compound was implemented to study the redox behaviour of the DsrC cysteines. Using this tool, a method was devised to obtain DsrC in reduced and oxidized states. The different redox forms were used to study protein-protein interactions, in which it was observed that the DsrMKJOP complex interacts better with $DsrC_{ox}$ than with $DsrC_{red}$, In addition, DsrC was demonstrated to interact specifically with the DsrK subunit that contains a catalytic $[4Fe\text{-}4S]^{3+}$ cluster, putatively responsible for disulfide reduction, as illustrated by heterodisulfide reductases of methanogens. Electron transfer studies from menadiol to the DsrMKJOP complex were also performed, which indicate that the *b*-type hemes of the membrane bound DsrM subunit and the catalytic $[4Fe\text{-}4S]^{3+}$ cluster of the cytoplasmic DsrK subunit are reduced by menadiol. Overall, the results provide support for the interaction between DsrC and the DsrMKJOP complex, revealing for the first time a link between membrane proteins and cytoplasmic sulfite reduction. Furthermore, they support the involvement of the DsrMK subunits in a two-electron transfer step from the menaquinone pool to the oxidized form of DsrC, which might be associated with proton translocation across the membrane. The essential role of DsrMK in sulfite reduction is also revealed by the fact that the *dsrMK* genes are the only strictly conserved ones among the *dsrMKJOP* genes in all sulfate reducing organisms (SRO).

A comparative study of the 25 available genomes of SRO was carried out to uncover the essential proteins for reduction of sulfate and to analyse membrane redox proteins complexes. The

genes coding for all the proteins already identified as directly involved in sulfate reduction are present in all SRO (*i.e.* sulfate transporters, ATP sulfurylase, APS reductase, pyrofosfatase, DsrAB, DsrC, ATP synthase), together with the two membrane complexes that are considered the electron donors to the terminal reductases, the Qmo and Dsr complexes. The Qmo complex is present in all sulfate reducers analyzed, except in the archaeon *Caldivirga maquilingensis*, but in the Gram-positive organisms the QmoC subunit is absent. These organisms also have a simplified version of the Dsr complex constituted only by DsrMK. The genome analysis also revealed that there are two different groups of SRO, the *Deltaproteobacteria* and *Nitrospira*, characterized by a large number of multiheme cytochromes *c* (and membrane bound redox complexes containing a cytochrome *c* subunit), and a second group, including Archaea and *Clostridia*, that have very few or no cytochromes *c* and proteins associated with periplasmic electron transfer pathways. The genomic analysis also revealed the presence of several putative ion-translocating membrane-bound complexes (*e.g.* complex I homologs, Rnf, Ech/Coo hydrogenases and $H^+$-pyrophosphatases).

Overall, the work in this thesis sheds light on the understanding of how electron transfer chains are linked to energy conservation in sulfate respiration.

# Sumário da Dissertação

A redução dissimilativa de compostos de enxofre (*i.e.* redução de sulfato/sulfito e disproporcionação do enxofre) é considerada um dos processos metabólicos mais antigos na Terra capaz de sustentar vida. A redução dissimilitativa do sulfato, usando o sulfato como aceitador de electrões e compostos orgânicos ou hidrogénio como dadores de electrões, tem um papel significativo nos ciclos globais do enxofre e do carbono. Em ambientes anaeróbios até 50 % da mineralização da matéria orgânica é efectuada por organismos redutores de sulfato. Os mecanismos moleculares da respiração de sulfato são ainda pouco compreendidos, uma vez que este processo é bastante diferente dos outros processos de respiração e envolve novas moleculas biológicas.

O objectivo do trabalho apresentado nesta dissertação foi providenciar uma contribuição para a compreensão da cadeia de transferência de electrões que possibilita a conservação de energia na cadeia respiratória dos redutores de sulfato. Para este efeito dois pontos chave foram abordados: i) estudo da via de transferência de electrões da oxidação periplásmica do hidrogénio e do formato para as quinonas da membrana, e daí para a redução de sulfato no citoplasma, e ii) estudo da cadeia de transferência de electrões envolvidos no último passo da redução do sulfato, a redução dissimilativa do sulfito.

Na primeira parte do trabalho foi isolado e caracterizado um novo complexo de *Desulfovibrio vulgaris* Hildenborough. Este

complexo reduz as menaquinonas com electrões transferidos do citocromo $c_3$ Tipo I (TpI$c_3$). Este complexo foi designado Qrc que significa complexo redutor de quinonas. O complexo Qrc é composto por quatro subunidades, três periplásmicas (QrcABC) e uma subunidade membranar (QrcD). QrcA é um citocromo *c* hexahémico com uma âncora membranar. QrcB é uma proteína grande também ancorada à membrana que pertence à família das molybdopterina oxidoredutases. QrcC é uma proteína periplásmica com centros ferro-enxofre. QrcD é uma subunidade incorporada na membrana que pertence à família das NrfD. As subunidades QrcBCD estão intimamente relacionadas com membros da família do complexo enzimático de ferro-enxofre e molibdénio (também conhecido como a família de enzimas que reduz o DMSO), mas o Qrc não contém o cofactor de molibdénio, o que sugere uma alteração da função de catálise química para a de transferência de electrões. O complexo Qrc contém seis hemos de baixo spin do tipo *c* com coordenação axial do tipo His/His, um centro $[3Fe\text{-}4S]^{1+/0}$, e pelo menos três centros $[4Fe\text{-}4S]^{2+/1+}$. Os potenciais de redução dos centros do Qrc foram determinados por EPR. Os potencias dos hemos variam numa gama de +100 a -130 mV, enquanto os centros $[4Fe\text{-}4S]^{2+/1+}$ estão num intervalo entre -130 a -240 mV, e o centro $[3Fe\text{-}4S]^{1+/0}$ tem um potencial redox de +160 mV. Estes potencias estão numa gama adequada para receber electrões do parceiro fisiológico TpI$c_3$, cujos potencias de redução são baixos (-325 to -170 mV), e para dar electrões à menaquinona (-70 mV), o aceitador final. Adicionalmente, o sinal de EPR para o centro $[3Fe\text{-}4S]^{1+/0}$ é alterado pela presença de um análogo da menaquinona, sugerindo que o local de ligação da menaquinona se encontra perto das subunidades periplásmicas.

Estudos funcionais mostraram que o Qrc funciona como um TpI$c_3$:menaquinona oxidoredutase, com electrões gerados a partir da oxidação periplásmica do hidrogénio e do formato. Além disso, esta associação funcional foi também mostrada a nível estrutural, uma vez que se observou que o Qrc forma um supercomplexo com a hidrogenase [NiFe] e o TpI$c_3$. O Qrc é o primeiro complexo respiratório de organismos redutores de sulfato com uma actividade de redução das quinonas. A sua importância fisiológica está relacionada com o facto de que, na maioria dos redutores de sulfato, a hidrogenase e a formato desidrogenase são periplásmicas e não possuem a subunidade membranar responsável pela redução directa da quinona. De facto, o Qrc está presente em deltaproteobactérias redutoras de sulfato que têm hidrogenases e/ou formato desidrogenases solúveis no periplasma, e cujo aceitador de electrões é o TpI$c_3$. Para além disso, propôs-se que os complexos Qrc e Qmo pudessem estar envolvidos num mecanismo de *redox loop* que permita o transporte de electrões através da membrana desde a oxidação do hidrogénio e formato até à redução do sulfato no citoplasma, e que está acoplado à transferência de protões para a síntese de ATP.

O segundo tópico de investigação está relacionado com o processo de redução do sulfito a sulfureto, aonde ainda existem várias questões em aberto. Estas incluêm o dador de electrões à última reductase, o seu acoplamento ao processo de conservação de energia, as proteínas envolvidas no processo, e finalmente o mecanismo para a redução de sulfito. A estrutura do complexo DsrAB-DsrC levou a que se propusesse um novo mecanismo para a redução de sulfito, no qual sobressai o papel central da DsrC no processo. Foi sugerido que a DsrC esteja directamente

envolvida no mecanismo, sendo reponsável pela libertação de sulfureto tendo por intermediário um persulfureto, que leva à formação de uma ligação de dissulfureto intramolecular, que será o substrato do complexo DsrMKJOP. O objectivo deste trabalho foi tentar verificar o modelo proposto. Primeiro, mostrou-se que a maioria da DsrC na célula não está associada à DsrAB, e como tal não é uma subunidade desta como afirmado anteriormente. A DsrC contém duas cisteínas redox activas, e uma delas é o penúltimo resíduo do C-terminal que é um braço flexível. Assim a DsrC é muito propensa a formar dímero por uma ligação dissulfureto intermolecular em condições oxidantes. Pelo que foi necessário desenvolver um protocolo de oxidação específico para a formação da ligação dissulfureto intramolecular na DsrC. Foram implementados ensaios de *gel-shift* baseados num composto reactivo a tióis para estudar o comportamento redox das cisteínas da DsrC. Utilizando esta ferramenta, foi desenvolvido um método para obter a DsrC nos estados reduzido e oxidado. As diferentes formas redox foram usadas para estudar interacções proteína-proteína, nas quais se observou que o complexo DsrMKJOP interage melhor com a $DsrC_{ox}$ do que com a $DsrC_{red}$. Também se demonstrou que a DsrC interage especificamente com a subunidade DsrK, subunidade esta que contém o centro catalítico $[4Fe\text{-}4S]^{3+}$, hipoteticamente responsável pela redução de dissulfuretos, como descrito para as heterodissulfureto redutases de metanogénicos. Estudos da transferências de electrões do menadiol para o complexo DsrMKJOP também foram efectuados, e observou-se que o menadiol reduz os hemos *b* da subunidade membranar DsrM e o centro catalítico $[4Fe\text{-}4S]^{3+}$ da subunidade citoplásmica DsrK. Em conjunto, os resultados demonstram a interacção entre DsrC e o complexo DsrMKJOP, revelando pela

primeira vez um elo de ligação entre proteínas membranares e a redução citoplásmica do sulfito. Estes resultados também suportam o envolvimento das subunidades DsrMK no passo de transferência de dois electrões da menaquinona para a forma oxidada da DsrC, mecanismo este que pode estar associado à translocação de protões através da membrana. O papel essencial da DsrMK na redução do sulfito também está patente no facto de que os genes *dsrMK* são os únicos estritamente conservados em todos os organismos redutores de sulfato (SRO) de entre os genes *dsrMKJOP.*

Um estudo comparativo a 25 genomas disponíveis de SRO foi realizado para perceber quais as proteínas essenciais à redução de sulfato e para analisar complexos membranares redox. Os genes que codificam para todas as proteínas que já haviam sido identificadas como estando directamente envolvidas na redução de sulfato estão presentes em todos os SRO (*i.e.* transportadores de sulfato, ATP sulfurilase, APS redutase, pirofosfatase, DsrAB, DsrC, ATP sintetase), juntamente com os dois complexos membranares que são considerados os dadores de electrões para as redutases terminais, complexos Qmo e Dsr. O complexo Qmo está presente em todos os SRO analisados, excepto no arquaia *Caldivirga maquilingensis*, mas em organismos Gram-positivos a subunidade QmoC está ausente. Estes organismos também têm uma versão simplificada do complexo Dsr estando constítuidos apenas por DsrMK. A análise dos genomas também revelou que existem dois grupos diferentes de SRO, os *Deltaproteobacteria* e *Nitrospira*, caracterizados por terem um grande número de citocromos *c* multihémicos (e complexos membranares redox contendo uma subunidade citocromo *c*), e um segundo grupo, que inclui Arquaia e *Clostridia*, que têm poucos ou nenhuns

citocromos *c* e proteínas associadas à via periplásmica de transferência de electrões. A análise genómica também revelou a presença de vários complexos membranares possivelmente associados à translocação de iões (*e.g.* homólogos do complexo I, Rnf, Ech/Coo hidrogenases and $H^+$-pirofosfatases).

No seu conjunto, os resultados dos trabalhos reportados nesta tese abrem novas prespectivas sobre a compreensão de como as cadeias de transferência de electrões estão ligadas à conservação de energia na respiração de sulfato.

# List of Abbreviations

| | |
|---|---|
| ACIII | alternative complex III |
| ATP | adenosine triphosphate nucleotide |
| APS | adenosine-5'-phosphosulfate |
| BN | blue native |
| BRC | bacterial respiratory complex |
| CISM | complex iron-sulfur molybdoenzyme |
| CN | clear native |
| *D.* | *Desulfovibrio* |
| *D. desulfuricans* | *Desulfovibrio desulfuricans* ATCC 27774 |
| *D. vulgaris* | *Desulfovibrio vulgaris* Hildenborough |
| DDM | *n*-dodecyl-β-D-maltoside |
| DMSO | dimethyl sulfoxide |
| Dsr | dissimilatory sulfite reductase |
| DTT | dithiothreitol |
| $e^-$ | electron |
| $E_m$ | midpoint redox potential |
| EDTA | ethylenediamine tetraacetic acid |
| EPR | electron paramagnetic resonance |
| Fd | ferredoxin |
| Fdh | formate dehydrogenase |
| Fe-S | iron-sulfur cluster |
| *g* | EPR *g*-factor |
| Hase | hydrogenase |
| Hdr | heterodisulfide reductase |
| HEPES | *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid |
| Hmc | high molecular mass cytochrome *c* complex |
| HPLC | high performance liquid chromatography |
| HQNO | 2-heptyl-4-hydroxyquinoline-*N*-oxide |
| IPTG | isopropyl-β-D-thiogalactoside |
| MALDI-TOF | matrix-assisted laser desorption ionization/time-of-flight |
| MalPEG | methoxy-polyethylene glycol maleimide |
| MOPS | 3-(*N*-morpholino)propanesulfonic acid |
| MQ | menaquinone |
| $MQH_2$ | menaquinol |
| Nar | nitrate reductase |
| Nrf | nitrite reductase |
| Nhc | nine-heme cytochrome complex |
| Ohc | octaheme cytochrome complex |

| | |
|---|---|
| PAGE | polyacrylamide gel electrophoresis |
| pmf | proton motive force |
| PVDF | polyvinylidene difluoride |
| Qmo | quinone-interacting membrane oxidoreductase complex |
| Qrc | quinone-reductase complex |
| Rnf | *Rhodobacter* nitrogen fixation complex |
| SDS | sodium dodecyl sulfate |
| Sox | sulfur oxidizing enzyme system |
| sp. | species |
| SPR | surface plasmon resonance |
| SRB | sulfate reducing bacteria |
| SRO | sulfate reducing organisms |
| TBHP | *tert*-butyl hydroperoxide |
| Tmc | Transmembrane complex |
| TpI$c_3$ | type I cytochrome $c_3$ |
| Tricine | *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine |
| Tris | tris(hydroxymethyl)-aminomethane |
| UV | ultra-violet |
| wt | wild type |

## Latin abbreviations

| | |
|---|---|
| *c.* | *circa*, around |
| *e.g.* | *exempli gratia*, for example |
| *et al.* | *et alia*, and other people |
| *i.e.* | *id est*, that is to say |
| *vs* | *versus*, in opposition to |

## Amino acids

| | | | | | |
|---|---|---|---|---|---|
| Ala | A | Alanine | Leu | L | Leucine |
| Arg | R | Arginine | Lys | K | Lysine |
| Asn | N | Asparagine | Met | M | Methionine |
| Asp | D | Aspartate | Phe | F | Phenylalanine |
| Cys | C | Cysteine | Pro | P | Proline |
| Gln | Q | Glutamine | Ser | S | Serine |
| Glu | E | Glutamate | Thr | T | Threonine |
| Gly | G | Glycine | Trp | W | Tryptophan |
| His | H | Histidine | Tyr | Y | Tyrosine |
| Ile | I | Isoleucine | Val | V | Valine |

# CHAPTER 1

# SULFATE REDUCING ORGANISMS AND *DESULFOVIBRIO*

## 1.1 THE SULFUR CYCLE

Biological and geophysical processes are essential for life. Biogeochemical cycles describe the pathways of a chemical element that is cycled between the living (biotic, "bio") and non-living (abiotic, "geo") compartments through terrestrial, marine and atmospheric environments. These dynamic processes rely heavily in the biotic part, namely on microbes – especially bacteria and fungi – that play essential roles in the global cycles of several elements.

Sulfur stands out in nature because of its great (bio)chemical versatility, since its oxidation states can range from -2 ($H_2S$) to +6 ($SO_4^{2-}$) (**Table 1.1**). The interconversion between these redox states constitutes the biogeochemical cycle of sulfur, on which biological processes play a major role. The sulfur compounds presented in the table 1.1 are the most biologically significant ones. Sulfide is the most reducing form of S and is present in different forms (*e.g.* $H_2S$, $HS^-$ and $S^{2-}$) depending on the pH. It is naturally found in large amounts in gas reservoirs at the underground level or, due its nucleophilicity, it reacts with iron to form sulfide minerals, particularly pyrite ($FeS_2$), present in minerals and rocks. It is also produced by living organisms, since sulfide is the end-product of sulfate respiration. However, the sulfide produced from sulfate reduction near oxygenated zones is, in its majority, directly or indirectly reoxidized back to sulfate.

**Table 1.1** – Biologically relevant inorganic sulfur compounds and their oxidation states (taken from (Dahl *et al.*, 2008)).

| Compound | Chemical formula | Oxidation state |
|---|---|---|
| Sulfide | $H_2S/HS^-$ | -2 |
| (Poly)sulfides | $S_n^{2-}$ ($n$ >1) | -1 (terminal S)/±0 (inner S) |
| Elemental sulfur | $S_n$ (S rings) or $S_\infty$ (polymeric sulfur) | 0 |
| Sulfite | $HSO_3^-/SO_3^{2-}$ | +4 |
| (Poly)thionates | $-O_3S(S)_nSO_3^-$ | +5 (sulfone S)/±0 (inner S) |
| Thiosulfate | $S_2O_3^{2-}$ | +5 (sulfone S)/-1 (sulfane S) |
| Sulfate | $SO_4^{2-}$ | +6 |

Polysulfides are produced by the reaction of sulfide with elemental sulfur or when sulfide is partially oxidized. Zero-valent sulfur is present in a wide range of allotrophes, and at standard conditions the thermodynamically most stable form of elemental sulfur is the yellow orthorhombic α-sulfur, structured as a ring of $S_8$ (also called cyclo-octasulfur). It can be found near hot springs, volcanic regions and also in deposits. Sulfite is present for example in hot springs, and it can either be reduced to sulfide or oxidized to sulfate by biological pathways. Thiosulfate and polythionates are present in soils and volcanic areas, and are produced by biochemical and chemical processes, but are rapidly converted to other sulfur compounds due to their instability. Sulfate is the most oxidized form of S and it can function as a sulfur source in assimilative pathways, or it can be employed as respiratory acceptor dissimilatory energy generation in the case of prokaryotes. The sulfate ion accumulates in seawater, where it is the second most abundant

anion, next to chloride, with a concentration of ~28 mM (Vairavamurthy *et al.*, 1995), while in freshwaters this is several orders of magnitude lower. Another sulfate reservoir is found in mineral gypsum ($CaSO_4 \times 2H_2O$). In the modern world, sulfate is generated and discharged from many industrial processes including production of edible oil, molasses fermentation, tannery operations, food production, coal burning power plants, and pulp and paper processing (Liamleam and Annachhatre, 2007). The wastewaters generated from these industries may contain high concentrations of other S-species such as sulfide, sulfite, thiosulfate and dithionite (Amend *et al.*, 2004; Hulshoff Pol *et al.*, 1998).

Sulfur is also part of a broad variety of biomolecules, such as amino acids (cysteine and methionine), nucleosides (thionucleosides in transfer RNAs), protein cofactors (iron–sulfur clusters and molybdopterin), co-enzymes (co-enzyme A, lipoic acid), vitamins (thiamine or vitamin $B_1$ and biotin), metabolites, sulfolipids (sulfatides) and hormones (insulin and growth hormone). Besides being part of several biological compounds, sulfur and sulfur compounds can be used biologically by prokaryotes in energy metabolism, so jointly this makes sulfur the sixth most abundant element on Earth (Amend *et al.*, 2004).

There are two biological roles for inorganic sulfur compounds: the biosynthesis of sulfur biomolecules (assimilatory processes), or as electron donors or acceptors for energy production (dissimilatory processes) (**Figure 1.1**). The first is an energy-requiring process performed by many bacteria, archaea,

fungi, plants and animals, while dissimilatory sulfur processes are limited to prokaryotes (archaea and bacteria).

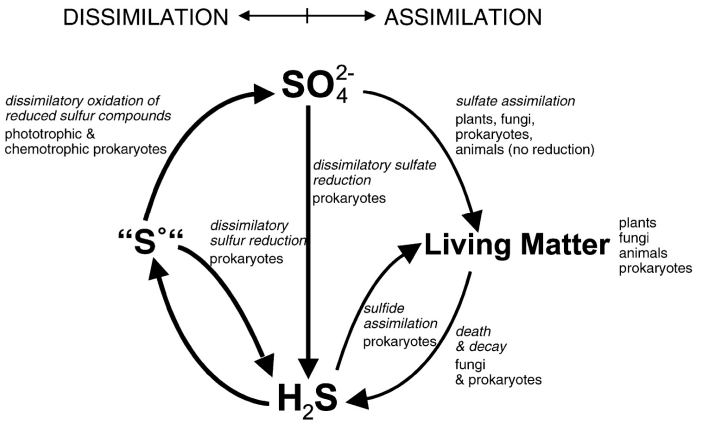


**Figure 1.1** – A simplified overview of the biological sulfur cycle (taken from (Dahl *et al.*, 2002)).

The dissimilatory reduction of oxidized sulfur compounds is linked to energy transformation *via* microbial respiratory processes (this topic will be addressed in detail in Chapter 2). In this microbial group are included the sulfate reducing organisms (SRO), but there are also examples of microbes that grow on other S-oxidized compounds, such as sulfite, thiosulfate, polythionates and sulfur, reducing them to sulfide (Rabus *et al.*, 2006), which is released to the environment. Sulfide or other reduced sulfur compounds serve as electron donors for other bioenergetic processes, namely photosynthesis and respiration, generating again oxidized sulfur compounds (**Figure 1.1**). Although in general sulfate is the major oxidation product of sulfur compound oxidation, other end-products may be formed depending on the organism. Further differences are found in the ability to use the various sulfur compounds. This

pathway of the sulfur cycle is accomplished by the sulfur oxidizing prokaryotes. Besides these reactions, disproportionation of elemental sulfur or thiosulfate is another biologically mediated process. Sulfur disproportionation yields simultaneously sulfate and sulfide under strictly anaerobic conditions as an energy generating process, and is one of the simplest inorganic life styles (Bak and Cypionka, 1987; Bak and Pfennig, 1987).

Apart from the biological activity there are also geological, chemical and physical processes that contribute to the sulfur cycle. These include the recycling of oceanic sulfate from seaspray, volcanic sulfur gases, organic S-bearing compounds released into seawater and subsequently into the atmosphere, and anthropogenic emissions of sulfur dioxide that result as acid precipitation.

## 1.2 Microbial sulfate reduction diversity and antiquity

Sulfate reducing organisms (SRO) are phylogenetically and physiologically diverse, having in common the ability to reduce sulfate to sulfide coupled with the oxidation of organic matter or hydrogen ($H_2$) (Postgate, 1984). Depending on the species, SRO couple the oxidation of $H_2$ or a variety of carbon substrates to acetate (incomplete oxidizers) or to $CO_2$ (complete oxidizers), with the majority of SRO being found in the first group. In the absence of electron acceptors, SRO are also able to perform fermentation (Rabus *et al.*, 2006) or grow syntrophically with other organisms (Stams and Plugge, 2009;

Walker *et al.*, 2009). SRO are estimated to be responsible for up to 50 % of carbon oxidation in marine anaerobic sediments (Jorgensen, 1982), emphasizing the importance of sulfate reducers.

The SRO include organisms that belong to unrelated taxonomic groups (Rabus *et al.*, 2006). These include both members of the Archaea and Bacteria domains with two and five phylogenetic lineages, respectively (Figure 1.2), including Gram-negative and Gram-positive bacteria. To the group that includes only Bacteria is commonly named sulfate reducing bacteria (SRB). Morphologically, SRO are in its majority bacilli, oval or rod shaped and with flagellar motility, and the Gram-positive are spore-forming organisms. Most studies of the phylogenetic profiles of SRO are based on the 16S ribosomal RNA gene, but other vital genes for sulfate metabolism have also been employed, such as *dsrAB* (Wagner *et al.*, 1998) that encode the dissimilatory sulfite reductase, or *aprAB* (Meyer and Kuever, 2007) that encode the adenosine phosphosulfate reductase. These functional genes are also used as a footprint to detect or find new SRO.

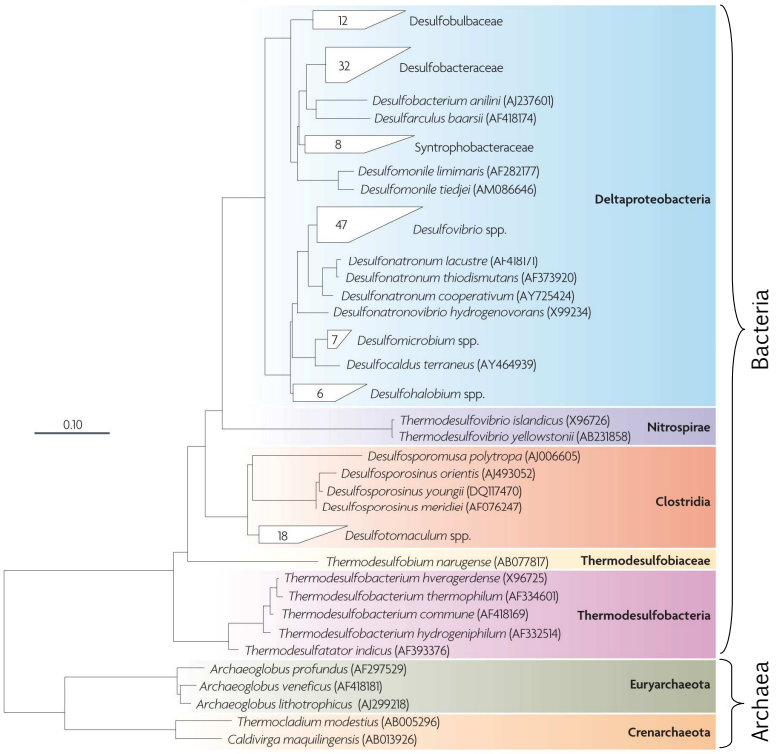


**Figure 1.2** – Phylogenetic tree based on nearly complete 16S ribosomal RNA sequences of described SRO (adapted from (Muyzer and Stams, 2008)).

Until recently the number of SRO described accounted to 230 species of 60 genera (Thabet *et al.*, 2011), of which 25 species have the genome sequenced. By far the greatest diversity of SRO resides within the Delta-subdivision of the Proteobacteria phylum, where they are distributed over more than 25 genera, including the most populated one of *Desulfovibrio* genus (with at least 70 species documented in journal publications available at *http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi*). This group is mainly constituted by mesophiles (Rabus *et al.*, 2006) and, curiously, it includes the first SRO identified by the microbiologist Martinus Beijerinck in 1895 and baptized as *Spirillum desulfuricans* (**Figure 1.4 A**)

(Beyerinck, 1895), later renamed *Desulfovibrio desulfuricans*. The second most populated group of SRO belongs to the Firmicutes phylum, namely from the *Bacillus/Clostridium* genera, including Gram-positive SRO (low G+C DNA content). Examples are *Desulfotomaculum*, *Desulfosporosinus* and *Desulfosfoporomusa* genera. Another branch of SRO is present in the Nitrospirae phylum, which holds the thermophylic *Thermodesulfovibrio* genus. SRO has already been found in two other lineages of Thermodesulfobacteria (*Thermodesulfobacterium* genus) and Thermodesulfobiaceae (*Thermodesulfobium* genus). The former is considered the deepest branch of the Bacteria domain and is classified as a thermophile (optimal growth temperature around 80 ºC) (Stackebrandt *et al.*, 1995). In the Archaea domain the most known genus is the hyperthermophilic euryarchaeote *Archaeoglobus*. Nevertheless, there are also representatives in the Crenarchaeota (genera *Caldivirga*, *Thermocladium* and *Thermoproteus*) (**Figure 1.2**) (Muyzer and Stams, 2008; Siebers *et al.*, 2011; Stackebrandt *et al.*, 1995). Examples of habitats where SRO have been detected are marine and fresh-water sediments, soils, hydrothermal vents, aquifers, oil fields, waste water treatment stations and several others (Muyzer and Stams, 2008; Widdel, 1988).

The SRO is an heterogeneous group, containing organisms from psychrophiles to hyperthermophiles (up to 105 ºC growth temperature), but with major incidence in mesophiles and thermophiles, with optimum NaCl concentrations from freshwater to hypersaline conditions, and with pH optimum ranging from 5 to 9 (Rabus *et al.*, 2006). There are also examples of SRO tolerating combined extreme conditions like “soda lakes” with

stable high pH and high salinity (*e.g. Desulfonatronospira thiodismutans* from the *Deltaproteobacteria* group (Sorokin *et al.*, 2008)). Many SRO (*e.g. Desulfovibrio*, *Syntrophobacter* and *Desulfotomaculum* spp.) can grow syntrophically with other microorganisms, such as fermentative bacteria or methanogenic archaea (Stams and Plugge, 2009) (**Figure 1.4 F**), or even in more intimate relationships, such as the case of SRO coexisting with sulfur oxidizing Gammaproteobacteria as endosymbionts in the gutless marine worm *Olavius algarvensis* (Dubilier *et al.*, 2001), providing the host with nutrients. The range of host organisms holding this kind of bacterial symbiosis is large in marine invertebrates, like ciliates, tubeworms and bivalves living in deep-sea vents, cold seeps or sediment zones (Stewart and Cavanaugh, 2006).

The diversity and versatility of SRO is also reflected in the wide spectrum of compounds that can serve as electron donors and carbon sources. The number of electron donors exceeds one hundred compounds, including monocarboxylic acids (*e.g.* formate, acetate, propionate, butyrate, lactate and pyruvate), dicarboxylic acids (*e.g.* malate, fumarate and succinate), alcohols (methanol, ethanol, propanol), hydrogen, and compounds more difficult to metabolize like aldehydes, ketones, alkanes, long-chain fatty acids, aliphatic hydrocarbons, and aromatic hydrocarbons (*e.g.* benzoate, phenol, toluene and ethylbenzene) (Hansen, 1994; Rabus *et al.*, 2006; Widdel, 1988). Regarding the electron acceptors SRO can use other compounds besides sulfate, but not all are coupled to energy transduction. Thus, most species of SRO can utilize thiosulfate and sulfite as well, and some SRO use elemental sulfur (*e.g.*

*Desulfohalobium, Desulfuromusa*, among others) (Rabus *et al.*, 2006). There are also examples of non sulfur-containing electron acceptors, such as fumarate, nitrate and nitrite that are reduced to ammonium (*e.g.* some species of *Desulfovibrio* and *Desulfotomaculum* (Mitchell *et al.*, 1986)), and also metals such as: uranyl (U(VI)) (Lovley and Phillips, 1992), ferric iron (Fe(III)) (Lovley *et al.*, 1993), chromate (Cr(VI)) (Lovley and Phillips, 1994), arsenate (As(VI)) (Newman *et al.*, 1997), selenate (Se(VI)) (Tucker *et al.*, 1998), and pertechnetate (Tc(VII)) (Lloyd *et al.*, 1999) (*e.g.* some species of *Desulfovibrio* and *Desulfotomaculum*). However, in the case of *Desulfovibrio* spp. the metal reduction processes are not coupled to microbial growth. Due to their versatility SRO are ubiquitous in anaerobic environments.

The SRO have been regarded as strict anaerobes, but a high number of sulfate reducers are present in the oxic zones and near the oxic-anoxic layers of sediments and microbial mats (Canfield and Des Marais, 1991; Teske *et al.*, 1998). These microorganisms possess multiple defence pathways, including oxygen scavenging systems, as a weapon to protect them from oxygen toxicity and its reactive species (Dolla *et al.*, 2007). Besides these enzymatic systems, SRO have developed other adaptation strategies to protect themselves against oxygen, including behavioural and molecular responses, like formation of cell aggregates or migration to anoxic regions (Cypionka, 2000; Cypionka *et al.*, 1999). Nevertheless, it was assumed that the relationship with oxygen was only a defensive one and was not related to respiration and growth (Cypionka, 2000; Dolla *et al.*, 2006). However, enzymes related to an aerobic lifestyle, such

as terminal oxygen reductases including quinol cytochrome *bd* oxidases and the cytochrome *c* oxidases were identified in *Desulfovibrio* (Kitamura *et al.*, 1995; Lemos *et al.*, 2001), and it was proposed that these organisms should have the capacity to respire oxygen either in microaerophilic (Wildschut *et al.*, 2006) or even fully aerobic conditions (Cypionka, 2000). Several studies have shown that pure cultures survive hours or even days of exposure to air, and a *Desulfovibrio* species has displayed growth in lactate/nitrate medium in the presence of nearly atmospheric oxygen levels (Lobo *et al.*, 2007), but it was still not shown that the growth is associated with aerobic respiration. Moreover, some sulfate reducers were isolated from microbial mats that can be saturated with air during the day phase (Krekeler *et al.*, 1997).

Microbial sulfate reduction is hypothesized to be a very ancient metabolism on Earth (Wagner *et al.*, 1998). To investigate the dating of biological activity within the ~4.65 billion years (Ga) of Earth's history (Goodwin, 1981), stable isotope ratio measurements are the tracer of choice, as microfossil and biomarker evidence are largely unavailable before 2.7 Ga (Waldbauer *et al.*, 2009). Several elements critical to life, like carbon, nitrogen, sulfur, hydrogen, and oxygen, are used as biogeochemical tracers to understand the co-evolution of life and the environment (Shen and Buick, 2004). This is possible, in part, because biological and non-biological processes yield different isotopic signatures in materials (rocks) that are preserved for billions of years, in contrast with microfossils or biomarker molecules that have proven to be

poorly conserved (Johnston, 2011; Shen and Buick, 2004). A prime example of this is the geological sulfur isotope record. This record is used to track the geochemical cycling of sulfur, oxygen, iron, and even carbon (Canfield, 2005). Sulfur has four stable isotopes – $^{32}S$, $^{33}S$, $^{34}S$ and $^{36}S$, with $^{32}S$ and $^{34}S$ being the most abundant (*c.* 95 % and 4 %, respectively).

The S isotope analysis is based on the fact that the microbial enzymes discriminate against the heavier sulfur isotopes during dissimilatory sulfate reduction (Shen and Buick, 2004) (at sulfate concentrations above ~0.3 mM (Habicht *et al.*, 2005)). Consequently, $H_2S$, the end-product of sulfate reduction, is enriched in $^{32}S$ and depleted in $^{34}S$ (and also in $^{33}S$ and $^{36}S$). While the evolutionary story emerging from the geological and biogeochemical record is complex, experimental work with modern sulfate reducing organisms has proven critical to our understanding of the isotope record (Shen and Buick, 2004), and detailed biochemical analysis of sulfate reducing organisms continues to lend insights to geochemistry (Bradley *et al.*, 2011).

The differences in isotopic compositions are analysed through time in well preserved rocks of different ages, providing a record of ancient environments and the organisms they hosted. The oldest S-isotopic record for microbial sulfate reduction on Earth stands at ~3.47 Ga ago (Shen *et al.*, 2001). These data are from barite ($BaSO_4$) deposits (which were originally composed of gypsum ($CaSO_4 \times 2H_2O$)) from the Dresser Formation at North Pole, Australia. This location is the best-known site of sulfate-containing sediments from the Early Archean era, which by biological dissimilatory sulfate reduction

gave origin to the barite crystal deposits found today. Shen *et al.* reported that the barite deposits contained microscopic sulfides depleted in $^{34}S$ (Shen *et al.*, 2001). Other work with sedimentary sulfides at several locations, provides evidence for microbial sulfate reduction at ~2.7 Ga ago (Shen and Buick, 2004).

During the Archean period the microbial cycling of S resulted in $H_2S$, which reacted with abundant iron in the oceans and sediments, yielding pyrite. This mineral is the main sink of reduced sulfur in marine sediments (Canfield, 2005). Later, towards the end of the Archean period (~2.45 Ga ago), the accumulation of oxygen from oxygenic photosynthesis led to a gradual biospheric oxygenation (Canfield *et al.*, 2000; Poulton *et al.*, 2004). The $O_2$-containing atmosphere contributed to increased formation of sulfate, due to the weathering of pyrite and other sulfide minerals on land, which was washed off and accumulated in the oceans. Sulfate delivery to the ocean became much higher than during the Archean, and sulfate reduction became a prominent biological process, with the oceans turning from ferruginous to an anoxic sulfidic environment (known as "the Canfield ocean") (Canfield, 1998). This situation is estimated to have persisted between ~1.8 and 0.7 Ga, before full oxygenation of the oceans occurred (Canfield, 1998; Lyons and Gill, 2010).

The geological sulfur isotope record, along with other data, has led some geobiologists to propose that microbial sulfate reduction was the earliest energy metabolism to evolve (Shen *et al.* 2001). This is depicted by the temporal marks presented in the "Tree of Life" (**Figure 1.3**), that reveal that sulfate reduction is

by far older than the evolution of oxygen-producing cyanobacteria. The timing of appearance of different types of energy metabolism in geologic history is currently under debate, and recent and growing evidence indicate that elemental sulfur and sulfite reduction may, in fact, have preceded sulfate reduction, as the most ancient energy metabolism (David and Alm, 2011). This is thought to be due to volcanic activity in the Archean, which enriched the atmosphere in sulfur species, like sulfur dioxide and elemental sulfur. This hypothesis is also reflected in the genomic evolution mapping data (David and Alm, 2011), and recent geochemical model predictions (Halevy *et al.*, 2010).

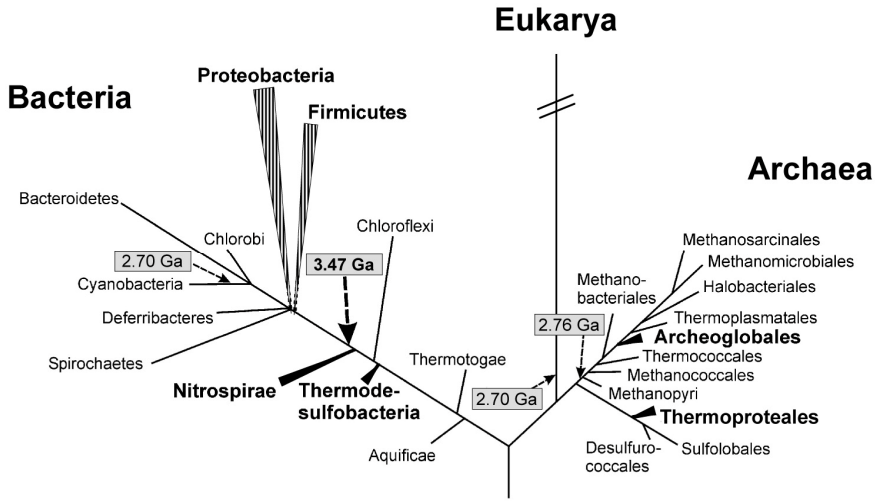


**Figure 1.3** – Small subunit ribosomal RNA phylogenetic tree ("Tree of Life") containing several of the main lineages from the Bacterial and Archaeal domains, with temporal marks in grey boxes. Lineages of sulfate reducers growing at temperatures > 70 ºC are shown by black background triangles and sulfate reducers growing at temperatures < 70 ºC are shown by triangles fulfilled with horizontal lines (modified from (Shen and Buick, 2004)).

## 1.3 The genus *Desulfovibrio*

*Desulfovibrio* spp. from the *Deltaproteobacteria* class has been the most extensively studied genus among SRO, from the metabolic and biochemical point of view, due to their widespread environmental distribution, rapid growth and easiness of manipulation. Molecular biological techniques that allow genetic manipulation of *Desulfovibrio* spp. are becoming more widespread (such as chromosomal insertions and deletions through strategies for homologous recombination, plasmid insertion and replication) (Keller *et al.*, 2011; Rabus *et al.*, 2006).

Cells of *Desulfovibrio* are Gram-negative, curved (vibrio) or rod shaped, with sizes is in the range of 0.5 - 1.3 x 0.8 - 5 μm (Holt, 1994), often motile with a single polar flagellum and non-sporulating (**Figure 1.4**). The genus is mesophilic, with optimal growth temperature between 25 - 35 ºC (Widdel, 1988).

*D. vulgaris*, *D. desulfuricans* and *D. gigas* have been commonly used models for studies on physiology and biochemistry of sulfate reducers. The work described in this thesis was performed with *Desulfovibrio vulgaris* Hildenborough (hereafter *D. vulgaris*) as a model organism for the study of energy metabolism in SRO. *D. vulgaris* was isolated from clay soil near Hildenborough, Kent (south of London) in the United Kingdom in 1946 (Postgate, 1984), and it was the first SRO to have its genome sequenced (Heidelberg *et al.*, 2004).

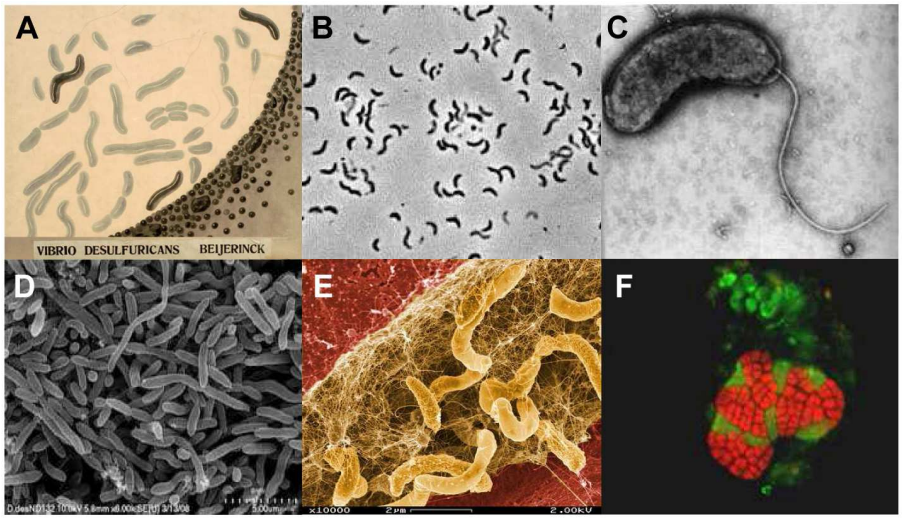


**Figure 1.4** – Several images of *Desulfovibrio* spp. A - Painting of *Vibrio desulfuricans* made by the sister of Martinus Beijerinck (taken from (Muyzer and Stams, 2008)); B - Phase-contrast micrograph of *D. hydrothermalis* (taken from (Ignatiadis, 2000)); C - Transmission electron microscopy image of a *Desulfovibrio* sp. with the polar flagellum (taken from (Todar, 2011)); D – Scanning electron microscopy image of *D. desulfuricans* (taken from (Gilmour *et al.*, 2011)); E - Color-enhanced digital micrograph of a black and white scanning electron microscope image from a biolfim of *D. desulfuricans* grown on a hematite surface (taken from (PNNL, 2009)); F - Fluorescence *in situ* hybridization image observed with a confocal laser scanning microscope of a syntrophic association between methanotrophic archaea (red) and SRB (green) (taken from (MUMM, 2005)).

With the advances of genome sequencing techniques over the past decade several genomes of SRO have also been sequenced, and presently there are 12 fully sequenced *Desulfovibrio* strains. The sequenced genomes can be accessed on genome sites such as MicrobesOnline (http://www.microbesonline.org) (Alm *et al.*, 2005), the Integrated Microbial Genomes (IMG; http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) and the Genome database from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/sites/genome).

## 1.4 Impact of SRO microbial activity

The interest in SRO goes beyond their relevance to the carbon and sulfur biogeochemical cycles, or their scientific interest, since these organisms have a strong impact on the environment and health. This impact is mainly a result of their metabolic product, hydrogen sulfide, which has both positive and negative perspectives.

The odour of $H_2S$ resembling rotten eggs denounces its toxic effects, being easily perceptible at concentrations well below 1 part per million (ppm) in the air (around 0.02 ppm). $H_2S$ exposure symptoms show up between 0 and 10 ppm, and above 500 ppm (~15 mM) it can cause death, like with cyanide (EPA, 2003). It is classified as a chemical asphyxiant, similar to carbon monoxide and cyanide gases, and human exposure should be minimised by employing adequate safe working practices. $H_2S$ is even toxic to sulfidogenic organisms, as shown recently with *D. vulgaris* (Caffrey and Voordouw, 2010).

The negative facet of $H_2S$ derives from the combination of both its toxic and corrosive properties, and SRO microbial activity has been associated with corrosion in many industrial systems such as drinking water distribution systems, and petroleum and gas industry at the level of metal and concrete structures. The sulfate reducers are considered the single most common causative organism in microbial corrosion, which may in turn represent 50 % of all sources of corrosion (Hamilton, 1985). Corrosion is an electrochemical phenomenon of metal dissolution based on oxidation and reduction reactions. The term biocorrosion or microbiologically influenced corrosion (MIC)

is applied for metal deterioration under a biological influence. The high concentrations of sulfate in marine environments, coupled with biofilm formation that helps to constitute anoxic niches, leads to corrosion rates of carbon steel that are enhanced by several orders of magnitude. Thus, SRO activity is a strong concern of seaports and shipping companies. To give an example, steel biocorrosion has an estimated financial impact with losses that amount to US$100 million per year just in the United States (Beech and Sunner, 2007).

The metal biocorrosion performed by SRO has a special impact in the petrochemical industry. There are examples of species isolated from hydrocarbon sources, such as *D. alaskensis* (formerly *Desulfovibrio desulfuricans* G20 (Hauser *et al.*, 2011)), which was isolated from a soured oil reservoir in Alaska and is known for its ability to develop biofilms (Beech *et al.*, 1998; Feio *et al.*, 2004). The long-chain hydrocarbons present in oil constitute a good carbon source, and the sulfate is present in the water used to stabilize the reservoir pressure and for flooding the reservoir in order to remove the crude oil. These factors, together with the anaerobicity present in the pipelines form an ideal niche for the development of SRO. In the case of gas reservoirs, short-chain alkanes, such as propane and butane, are plentiful as carbon sources. The microbial growth results in changes to the petroleum or gas composition, and contributes to the production of $H_2S$ (microbial souring) (Nemati *et al.*, 2009). This ability of SRO has severe consequences for the petroleum industry both in underground and at the surface, causing corrosion in the production, processing and storage facilities (including drilling,

pumping machinery, pipelines and storage tanks). Another important issue concerns the safety problems with employees on petroleum platforms or drilling activities. The impact of SRO in this industry represents a huge economical burden, and many studies have been conducted to control the biogenic sulfide production using different strategies. These include removal of sulfate from the water prior to injection or suppression of SRO with biocides or metabolic inhibitors, individually or synergistically (nitrate, molybdate, formaldehyde, bronopol, etc) (Greene *et al.*, 2006; Nemati *et al.*, 2009; Voordouw, 2011).

Another environment where anaerobic conditions prevail, and also suffers from the effects of corrosive $H_2S$, is the digestive system that includes the large intestine of humans and many animals, the rumen of ruminants and also the guts of invertebrates (Macfarlane *et al.*, 2007). However, the gastrointestinal tract is not the only place in the human body where SRB can be found, as there are reports of their presence also in other mucosal tissues, such as the mouth and vagina (Langendijk *et al.*, 2001; Tanaka *et al.*, 2010).

Every human being carries SRB as members of the large intestine flora, including species as *Desulfovibrio*, *Desulfomonas*, *Desulfobulbus* and *Desulfotomaculum* (Loubinoux *et al.*, 2002b; Macfarlane *et al.*, 2007). Patients with inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, apparently show a higher content of *Desulfovibrio* spp. in their faeces compared with healthy humans due to a deregulation of the intestinal flora equilibrium (Gibson *et al.*, 1988; O'Connell *et al.*, 2009). The impact of SRB is due to the toxicity of $H_2S$ that

causes DNA damage and inhibits the butyrate oxidation pathway, an important energetic route for colonic epithelial cells, besides other inherent toxic effects that lead to cell inflammation (Pitcher and Cummings, 1996). Other illnesses in the human gut tract have also been linked to SRB activity, like cholocystitis, abdominal abscesses, spondylitis or even colorectal cancer (in this case sulfide is considered to be an initiator of the disease by activating a number of biochemical pathways) (Macfarlane *et al.*, 2007). Besides the human gastrointestinal tract, *Desulfovibrio* have also been found in subgingival dental tissues and periodontal pockets, and SRB were detected in periodontitis patients (Langendijk *et al.*, 2001; Loubinoux *et al.*, 2002a). Presently, four *Desulfovibrio* spp. (*D. fairfieldensis*, *D. desulfuricans*, *D. piger*, and *D. vulgaris*) are suggested as opportunistic pathogens that may be involved in human intra-abdominal, oral flora, and blood stream infections (La Scola and Raoult, 1999; Loubinoux *et al.*, 2002b; Loubinoux *et al.*, 2000; Pimentel and Chan, 2007; Tee *et al.*, 1996; Urata *et al.*, 2008). *D. fairfieldensis* was reported in blood, peritoneal fluid, abdominal collection, pelvic collection, colorectal collection, liver abscess, urine, and periodontal pockets, *D. piger* was found in peritoneal fluid and intra-abdominal collection, *D. desulfuricans* in blood and peritoneal fluid, and last *D. vulgaris* was identified in peritoneal fluid and abdominal collection. To add to this list, very recently, *Desulfovibrio* spp. (*D. desulfuricans*, *D. fairfieldensis*, and *D. piger*) were found in a significant percentage in the abnormal intestinal bacterial flora of regressive autistic individuals, and the gut microflora is believed to play a role in the disease (Finegold, 2011a;

Finegold, 2011b). Another organism, *D. intestinalis* was reported in the vaginal flora, and was suggested to be involved in gynecological or obstetric pathology (Tanaka *et al.*, 2010).

In contrast with these harmful impacts, there are also situations in which the metabolic activity of SRB has a positive impact namely in the environment, through the bioremediation of several compounds. This is particularly relevant in the case of heavy metals, since in contrast to organic contaminants, these cannot be destroyed, and metal remediation through common physical-chemical techniques is expensive and unsuitable in case of large effluents. Thus, strategies have to be applied for metal removal or neutralization, and here microorganisms present a good potential. Sulfide produced during microbial sulfate reduction promotes metal precipitation, due to the low solubility of the metal sulfides (*e.g.* Cd, Co, Cu, Fe, Ni, Zn Te, Se, Rh and Pt (Macaskie *et al.*, 2001; Whiteley and Ngwenya, 2006; Whiteley and Rashamuse, 2007). Once precipitated, the metals can be recovered and reused. Thus, SRB represent appropriate candidates for bioremediation purposes of metal contaminated wastewaters or soil, resulting from mining activities or industrial discharges (Barton and Fauque, 2009). Current and past mining activities are the principal sources of metal contamination in soils and waters (Johnson and Hallberg, 2005). Several SRB-based bioremediation processes have been studied as biological treatment systems, using SRB in a range of bioreactors designed for optimum $H_2S$ production. These reactors are fed with metal-contaminated pollutant, causing rapid precipitation and recovery of metal sulfides (Malik, 2004). Moreover, the SRB biotechnological

application for metal removal have also been coupled with the use of other wastes from the food and wine industry as carbon and/or energy sources (Costa *et al.*, 2009a; Costa *et al.*, 2009b). Besides metal/radionuclides bioremediation, these bacteria can also degrade harmful organic compounds, such as 2,4,6-trinitrotoluene (TNT) among others (Ramos *et al.*, 2001).

Recently, $H_2S$ that is synthesized enzymatically from L-cysteine, has been increasingly recognised to have a role in human physiology. $H_2S$ has been shown to be involved in diverse physiological and pathological processes, such as learning and memory, neurodegeneration, regulation of inflammation and blood pressure, and metabolism (Lowicka and Beltowski, 2007; Szabo, 2007). Thus, it has been recognized as the third endogenous gaseous transmitter, along with nitric oxide and carbon monoxide. Many potential therapeutical applications, such as pharmacological $H_2S$ 'donor' molecules and 'specific' $H_2S$ synthesis inhibitors, are currently under further investigation (Olson, 2011).

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# Chapter 2

# Electron Transfer Respiratory Chains

## 2.1 Electron transfer respiratory chains

The key ingredient required to sustain life is energy, which means that mechanisms involved in the conservation or production of energy are essential for biological systems. Energy is the driving force for metabolic processes in the cells, for cell proliferation, movement and ion transport across the membrane. In living systems adenosine triphosphate (ATP) is the energetic currency and its synthesis through oxidative phosphorylation is coupled to electron transfer in respiratory chains. The respiratory process couples the electron transfer from an electron donor to a final electron acceptor (electron sink), and takes place in the cytoplasmic cell membrane of prokaryotes, in the inner membrane of mitochondria of eukaryotes, and also in the thylakoid membranes of chloroplasts. The membrane plays an essential role in any biological cell, since it works as a separation barrier allowing the development of differences in ion concentrations (*i.e.* $\Delta$pH in case of protons and $\Delta$Na for sodium ions) and also in electrochemical potential ($\Delta\Psi$), which together contribute to the proton motive force (pmf). In mitochondria the pmf is created by membrane-bound proteins called respiratory complexes that have the ability to couple the free energy released from vectorial electron transport to transmembrane proton translocation. The pmf is used for ATP synthesis, thus respiration results in energy transduction. This coupling mechanism, named chemiosmotic theory, was first proposed by Peter Mitchell (Mitchell, 1961).

Higher eukaryotes use oxygen as terminal electron acceptor for respiration, while prokaryotes (and lower eukaryotes) utilize not only oxygen but a vast array of alternative oxidants, such as nitrate, sulfate, elemental sulfur, transition metals (*e.g.* iron and manganese) and even metalloids (*e.g.* selenate and arsenate) or radionuclides (*e.g.* uranium), among others. This group of prokaryotes includes strict and facultative anaerobes that make use of a range of electron acceptors with lower redox potentials than oxygen, in processes that are thus less exergonic than aerobic respiration. Nevertheless, this represents an advantage by allowing these organisms to live in environments where oxygen is not present. This use of a range of possible electron acceptors, makes the anaerobic respiratory chains of Bacteria and Archaea highly diverse and flexible (Richardson, 2000), which allows their adaptation to environmental and bioenergetic demands (Soballe and Poole, 1999).

*Escherichia coli* is one of the most intensively studied examples of flexible respiratory chains, due to the presence of multiple electron entry-point enzymes (dehydrogenases), three different quinone types (electron carriers), and several electron exit-points (diverse oxidases and other terminal reductases) (Richardson, 2000; Unden and Bongaerts, 1997) (**Figure 2.1**). Some components of the mitochondrial aerobic respiratory chain, such as complex III and the diffusible cytochrome *c*, are absent in *E. coli*.

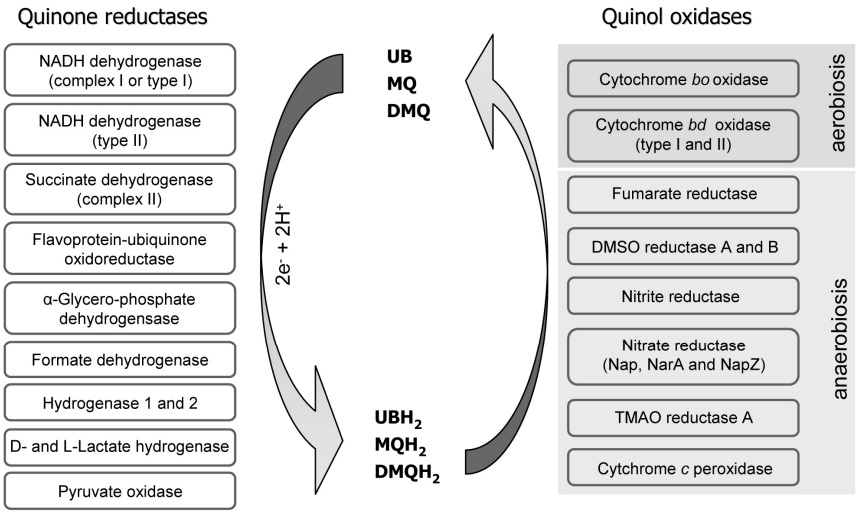


**Figure 2.1** – Schematic representation of the respiratory electron transfer chain of *E. coli*, as an example of the variety of quinone reductases and quinol oxidases membrane-bound complexes linked by the quinone pool. UQ, ubiquinone; MQ, menaquinone; DMQ, demethylmenoquinone (adapted from (Richardson, 2000)).

Quinones are small organic molecules present in the membrane due to their lipophilic character, where they can move freely, acting as redox mediators. The respiratory chain of strictly aerobic bacteria includes ubiquinone (UB, $E^{o'}$ = +113 mV), that has a more positive redox midpoint potential than menaquinones (MQ, $E^{o'}$ = -74 mV), which function in anaerobic respiratory chains (Schoepp-Cothenet *et al.*, 2009; Soballe and Poole, 1999). Quinones play an important electron carrier role connecting respiratory complexes, and they are found in all domains of Life, but there are also several exceptions (*e.g.* some orders of methanogenic Archaea, acetogenic bacteria and fermentative organisms) (Nowicka and Kruk, 2010; Thauer *et al.*, 2008).

The subunit composition of redox proteins is based in variations of a set of "building blocks" (protein subunits with

different cofactors) (Baymann *et al.*, 2003; Rothery *et al.*, 2008). Different combinations of those blocks lead to different enzymes with completely different catalytic functions (Baymann *et al.*, 2003). One example is constituted by a group of enzymes classified as complex iron-sulfur molybdoenzyme (CISM) family, which includes enzymes such as dimethyl sulfoxide (DMSO) reductase, polysulfide reductase, formate dehydrogenase and nitrate reductase (Rothery *et al.*, 2008). The CISM proteins are composed by three subunits: a catalytic subunit (with a molybdopterin cofactor), an electron transfer subunit (a four cluster protein) and a membrane anchor protein that interacts with the quinone pool (belonging to the NrfD or NarI family).

In anaerobic respiratory chains the membrane-bound respiratory enzymes can have their active side localized on different sides of the membrane. Depending on the different orientations of the dehydrogenases and reductases, and their respective quinone binding sites, several mechanisms of energy conservation can be considered (Simon *et al.*, 2008). The bioenergetic concept behind these mechanisms is the redox loop, as proposed by P. Mitchell (Mitchell, 1979). The redox loop couples electron transport to proton transfer across the membrane *via* the quinone pool, in a system that does not depend on proton pumping proteins. The redox loop needs two quinone reactive enzymes with opposite quinone oxidoreductase functions (*i.e.* a quinol oxidase plus a quinone reductase), which are called the two arms of the redox loop. It also involves different localization of the active center of electron

donor/acceptor and the quinone/quinol binding site (Simon *et al.*, 2008).

Anaerobic respiratory chains are rich in redox loops, and a well described example is present in the nitrate respiratory pathway of *E. coli*, involving the formate dehydrogenase (FdnGHI) and the dissimilatory nitrate reductase (NarGHI), both belonging to the CISM family (Jormakka *et al.*, 2003; Jormakka *et al.*, 2002) (**Figure 2.2**). In this system, formate is oxidized in the periplasm compartment to $CO_2$ and $H^+$, and the electrons are conducted across the membrane to the menaquinone binding center on the cytoplasmic side of the membrane, where the menaquinone is reduced with uptake of two protons from the cytoplasm. The quinol is oxidized by the nitrate reductase on the periplasmic side of the membrane, releasing protons to the periplasm, with the electrons being transferred to the active site of nitrate reductase on the cytoplasm for reduction of nitrate to nitrite, with consumption of two cytoplasmic protons. This redox loop allows energy conservation by this system, since there is a net movement of electrons across the membrane to the cytoplasm, and a net movement of protons to the periplasm, resulting in a coupling stoichiometry of $4H^+/2e^-$ (Jormakka *et al.*, 2003; Simon *et al.*, 2008). The movement of electrons from the donor to the acceptor is ensured by a highly efficient "wire-like" arrangement of electron transferring cofactors in both enzymes plus the quinone pool, and powered by a pronounced downhill decay of redox potential (**Figure 2.2 A**).

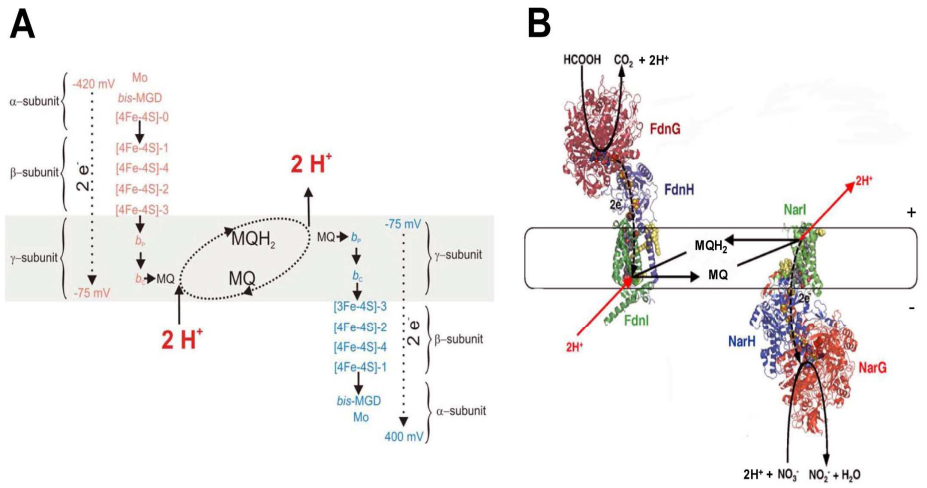


**Figure 2.2** – The nitrate respiratory pathway by a redox loop mechanism composed by formate dehydrogenase (FdnGHI) and nitrate reductase (NarGHI) enzymes from *E. coli*. Menaquinone reduction and menaquinol oxidation occurs at opposite sites of the cell membrane and on different protein complexes, resulting in proton transfer to the periplasm and electron transfer to the cytoplasm. A - Electron pathway *via* several metal cofactors present in Fdn and Nar (from (Jormakka *et al.*, 2003)); B - Structural models of the FdnGHI and NarGHI chain (adapted from (Simon *et al.*, 2008)).

## 2.2 Respiration by reduction of sulfate

Energy transduction in anaerobic sulfate respiration involves the reduction of sulfate (electron acceptor) to hydrogen sulfide ($H_2S$), coupled with the oxidation of organic compounds (*e.g.* lactate oxidation, Eq. 1), or molecular hydrogen as electron donors (Eq. 2). Comparing with other anaerobic respiratory processes, sulfate reduction is less exergonic than denitrification (nitrate reduction to $N_2$), but more exergonic than methanogenesis ($CO_2$ reduction to $CH_4$) or acetogenesis ($CO_2$ reduction to acetate).

$SO_4^{2-} + 2CH_3CHOHCOO^- \rightarrow H_2S + 2CH_3COO^- + 2CO_2 + 2H_2O$ (Eq. 1)

$SO_4^{2-} + 4H_2 + 2H^+ \rightarrow H_2S + 4H_2O$ (Eq. 2)

Many SRB are incomplete oxidizers because they are not able to further oxidize acetate ($CH_3COO^-$) to carbon dioxide ($CO_2$). The overall reduction of sulfate requires eight electrons and ATP (Eq. 3):

$SO_4^{2-} + ATP + 8H^+ + 8e^- \rightarrow H_2S + AMP + PPi$ (Eq. 3)

### 2.2.1 From sulfate to sulfide: SAT and terminal reductases

The dissimilatory reduction of sulfate to sulfide involves three steps: the first is an activation, followed by two reduction processes performed by cytoplasmic soluble enzymes. Since sulfate reduction takes place in the cytoplasm, sulfate must be transported across the cell membrane. The sulfate dianion uptake occurs with the symport of counter cations, sodium ($Na^+$) or protons ($H^+$) in marine and freshwater strains, respectively (Cypionka, 1987; Kreke and Cypionka, 1993). From the (bio)chemical point of view, sulfate is one of the least favourable electron acceptors, because it is a thermodynamically stable compound with a reduction potential of $E^{o'}$ = -520 mV for the sulfate/sulfite pair (Thauer *et al.*, 2007). To overcome this issue the first step is to activate sulfate by reaction with ATP to form adenosine-5'-phosphosulfate (APS) and inorganic pyrophosphate (PPi). This

endergonic reaction is catalysed by ATP sulfurylase (or sulfate adenylyltransferase, Sat) and APS is a much better electron acceptor with an $E^{o'}$ of –60 mV for the redox couple APS/sulfite. To drive this reaction an inorganic pyrophosphatase is required, which removes PPi by hydrolysis generating pyrophosphate. Most SRB have soluble pyrophosphatases, but in a few cases a transmembrane protein is present (Serrano *et al.*, 2007) that couples the hydrolysis of PPi to proton translocation, contributing for the proton-motive force (*e.g.* in Gram-positive SRO, *Syntrophobacter fumaroxidans* and *Desulfococcus oleovorans*, among others) (Pereira *et al.*, 2011). The sulfate activation reaction is a common step in the dissimilatory and assimilatory sulfate reduction pathways.

After activation, APS is the first true electron acceptor of the sulfate reduction process, being reduced to sulfite by APS reductase with the release of adenosine-5'-phosphate (AMP). Subsequently sulfite is reduced to sulfide by the dissimilatory sulfite reductase (dSir or DsrAB). The first reduction involves two electrons, while the second reduction is a six-electron reaction. APS reductase is a soluble dimeric Fe-S flavoenzyme, containing two [4Fe-4S] clusters and a FAD cofactor that was first purified and characterized from *D. desulfuricans* (Peck *et al.*, 1965). The first complete structure of APS reductase was obtained from the archaeon *Archaeoglobus fulgidus* (Fritz *et al.*, 2002), and later from *D. gigas* (Chiang *et al.*, 2009), showing it can be arranged in different multimeric forms. The dissimilatory sulfite reductases of SRB can be clustered in four different types according to their UV/visible spectroscopy characteristics (absorption peaks), and molecular and cofactor content (Rabus

*et al.*, 2007). The dSir types are: 1) desulfoviridin, mainly present in the genus *Desulfovibrio*, characterized by a green colour due to the presence of a sirohydrochlorin cofactor, and an absorption peak at 628 nm (sirohydrochlorin is a demetallated siroheme prosthetic group) (Kobayashi *et al.*, 1972; Moura *et al.*, 1988); 2) desulforubidin, present in *Desulfomicrobium* and *Desulfosarcina* sp., with a characteristic absorption peak at 545 nm (Arendsen *et al.*, 1993; DerVartanian, 1994; Lee *et al.*, 1973; Moura *et al.*, 1988); 3) desulfofuscidin, found in *Thermodesulfobacterium* sp., with a characteristic absorption peak at 576 nm (Hatchikian and Zeikus, 1983); and 4) P-582 protein in *Desulfotomaculum* strains, having an absorption peak at 582 nm (Akagi *et al.*, 1974).

There are two different proposed pathways for the reduction of sulfite to sulfide: one considers a direct six electron reduction; and the other proposes that reduction occurs in three two electron steps, with formation of the intermediates trithionate and thiosulfate (the trithionate pathway), that are obtained by trithionate and thiosulfate reductases, respectively (Chambers and Trudinger, 1975; Kobayashi *et al.*, 1969; Peck *et al.*, 1982) (**Figure 2.3**). There are contradictory reports concerning this issue, with indications that the intermediate species detected are chemical side reaction artefacts of the *in vitro* experiments (Chambers and Trudinger, 1975), whereas other studies observe the formation of the intermediates in growing cultures (Fitz and Cypionka, 1990). It has not been clearly established if trithionate and thiosulfate are indeed intermediates, but the fact that there is no convincing

biochemical or genetic support for the presence of trithionate or thiosulfate reductases in all SRB argues against the trithionate pathway. The assimilatory sulfite reductase (aSir), which is closely related to DsrAB (Crane and Getzoff, 1996; Oliveira *et al.*, 2008; Schiffer *et al.*, 2008) carries out a single six electron reduction step forming sulfide as a single product (Crane and Getzoff, 1996).

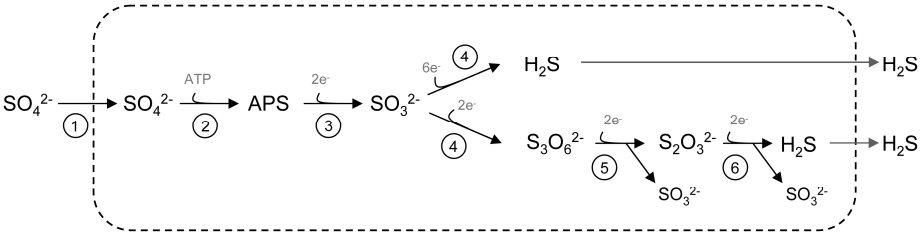


**Figure 2.3** – Proposed pathways of dissimilatory sulfite reduction. The dotted line represents the cell wall, 1, sulfate transporter; 2, ATP sulfurylase; 3, APS reductase; 4, sulfite reductase; 5, trithionate reductase; 6, thiosulfate reductase (adapted from (Bradley *et al.*, 2011; Shen and Buick, 2004)).

The dSiRs were initially reported to have an $\alpha_2\beta_2$ composition (Rabus *et al.*, 2007), but later an additional protein that co-purified with DsrAB was proposed to be a third subunit of desulfoviridin in *D. vulgaris* (Pierik *et al.*, 1992). This protein, DsrC will be addressed in point 2.2.2 of this chapter and is the target of study in Chapter 4. There was also some dispute around the content and the nature of dSir's cofactors (Rabus *et al.*, 2007), which was solved when the first crystal structures of dSiR from *D. vulgaris* and *A. fulgidus* were reported (Oliveira *et al.*, 2008; Schiffer *et al.*, 2008). In the *D. vulgaris* structure DsrAB forms a complex with DsrC (Oliveira *et al.*, 2008)

(**Figure 2.4**), forming a dimeric $\alpha_2\beta_2\gamma_2$ structure, in which the DsrC C-terminal arm protrudes into a channel formed between DsrA and DsrB, reaching the catalytic center. The *dsrA* and *dsrB* genes are paralogous and arose from an early gene duplication event (Dahl *et al.*, 1993). In fact, the DsrA and DsrB subunits are indeed structurally very similar, but in *D. vulgaris* only DsrB binds a catalytic cofactor (siroheme), whereas DsrA binds a sirohydrochlorin (iron-free siroheme) (Oliveira *et al.*, 2008). Thus, DsrA is a non-catalytic subunit, and DsrB harbours the catalytic site.

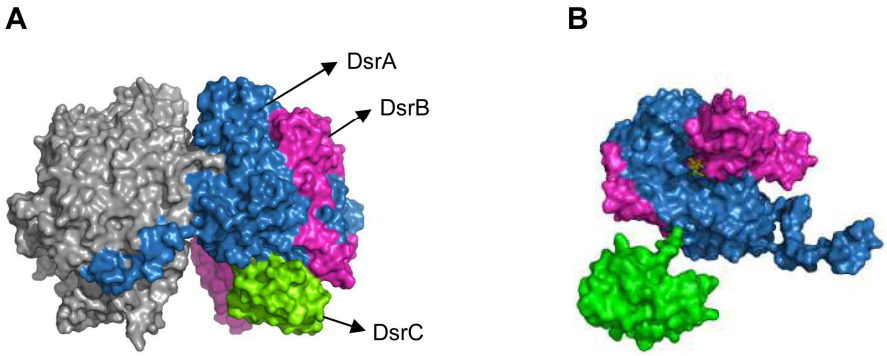


**Figure 2.4** – Structure of the *D. vulgaris* DsrAB sulfite reductase bound to DsrC. A - Molecular surface of the $\alpha_2\beta_2\gamma_2$ assembly; B - monomer of DsrAB with DsrC displaced from its binding position (adapted from (Oliveira *et al.*, 2008)).

In the *A. fulgidus* dSir crystal structure only the DsrAB proteins are present (Schiffer *et al.*, 2008), and both subunits bind siroheme. Nevertheless, only the siroheme in DsrB is considered to be catalytic, since the substrate channel leading to the DsrA siroheme is obstructed, and some residues of the sulfite binding site are missing. A similar situation is observed

for more recent dSirs structures from *D. gigas* (Hsieh *et al.*, 2010) and *Desulfomicrobium norvegicum* (Oliveira *et al.*, 2011).

A key pending issue related to the mechanism of sulfate reduction is the identification of the physiological electron donors to APS reductase and DsrAB, the terminal reductases. Two membrane complexes have been proposed to perform this function, namely the quinone-interacting membrane-bound oxidoreductase (Qmo or QmoABC) complex (Pires *et al.*, 2003) and the dissimilatory sulfite reductase complex (Dsr or DsrMKJOP) (Mander *et al.*, 2002; Pires *et al.*, 2006) (see sections 2.2.3 and 2.2.4).

### 2.2.2 DsrC

DsrC is a small protein (105 amino acids, 12 kDa in *D. vulgaris*) without cofactors, that is characterized by the presence of a highly conserved C-terminal arm containing two conserved cysteine residues, one of which is the penultimate amino acid and the other is ten amino acids before. The *dsrC* gene is strictly conserved in all SRO, but is also present in sulfur oxidizers and in all organisms that include the *dsrAB* genes. These genes can be located in the same operon (*e.g.* in the sulfur oxidizer *Allochromatium vinosum dsrABEFHCMKLJOPNRS* (Dahl *et al.*, 2005; Pott and Dahl, 1998)) or not (*e.g. D. vulgaris* DVU0402/3 for *dsrAB* and DVU2776 for *dsrC*).

Several structures of isolated DsrC have been determined: two NMR structures from the archaeon *Pyrobaculum aerophilum* and from *Al. vinosum* (Cort *et al.*, 2001; Cort *et al.*, 2008), and

an X-ray structure from *A. fulgidus* (Mander *et al.*, 2005). All structures present a similar globular shape with the exception of the C-terminal arm that is extended and flexible in the solution structures (**Figure 2.5 A**), and retracted in the crystal structure (**Figure 2.5 C**).

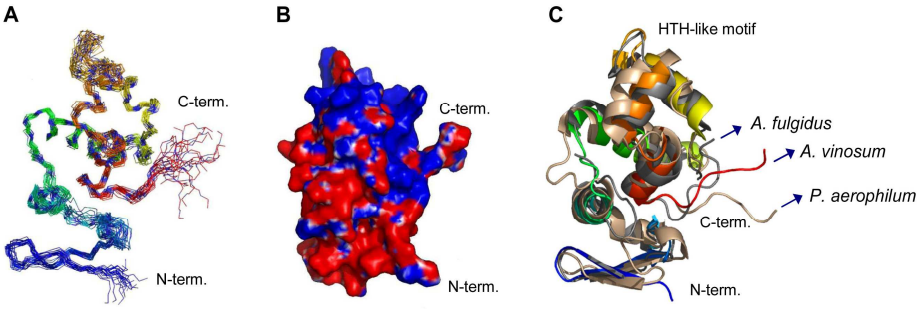


**Figure 2.5** – NMR solution structure of DsrC from *Al. vinosum* (adapted from (Cort *et al.*, 2008)) A – Stereoview of the structure ensemble and the flexible C-terminal arm; B – Molecular surface colored by electrostatic potential; and C – Superposition of three DsrC structures: *Al. vinosum* (rainbow), *P. aerophilum* (golden) and *A. fulgidus* (gray). HTH, helix-turn-helix.

The disordered nature of this arm, together with its high amino acid conservation suggested it is a site of protein-protein interaction (Cort *et al.*, 2001), which was later confirmed by the *D. vulgaris* DsrAB-DsrC crystal structure, where the C-terminal arm of DsrC projects inside DsrAB bringing the terminal cysteine of DsrC in close proximity to the substrate binding site of DsrB (Oliveira *et al.*, 2008) (**Figure 2.6 A**). In the DsrC crystal structure the two cysteines are in close proximity but not forming a disulfide bond, although this bond could be detected in solution upon treatment with an oxidizing agent (Mander *et al.*, 2005). These results suggested that the pair of conserved cysteines

are a redox-active center, and that DsrC could use a dithiol-disulfide conversion to transfer reducing equivalents. Interestingly, in the *D. gigas* dSiR structure three different conformations were observed for the C-terminal arm of DsrC

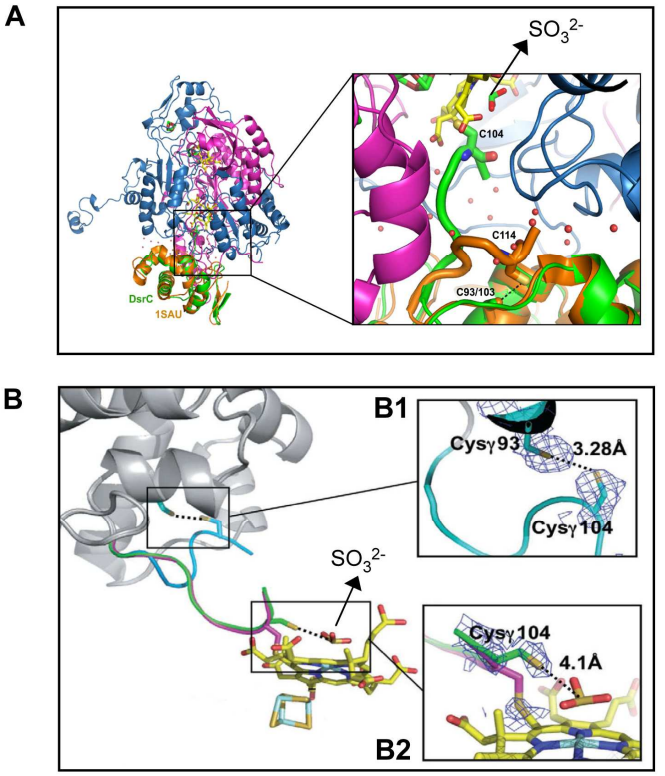


**Figure 2.6** – Structural details of DsrC bound to DsrAB. A - Secondary structure view of one DsrABC unit of *D. vulgaris* (DsrA in blue, DsrB in pink and DsrC in green,) with the *A. fulgidus* DsrC structure superposed (orange). Cys93/104 belongs to *D. vulgaris* DsrC and Cys103/114 to *A. fulgidus* DsrC. The zoomed image shows the extended C-terminal arm of the *D. vulgaris* DsrC (in green) reaching the catalytic siroheme (yellow) near a sulfite molecule, whereas in the *A. fulgidus* DsrC the arm is retracted (in orange); B - Three conformations of the dynamic arm of DsrC (blue, green and pink) inside the *D. gigas* DsrAB catalytic pocket. B1 represents the electron density of DsrC Cys93 and Cys104 in close proximity, compared with B2 where the C-terminal arm is extended and Cys104 is positioned next to the catalytic site and to the sulfite (A - adapted from (Oliveira *et al.*, 2008) and B from (Hsieh *et al.*, 2010)).

inside the catalytic pocket (Hsieh *et al.*, 2010) (**Figure 2.6 B**). In 70 % of the molecules the DsrC arm is an extended configuration, and 30 % in the retracted form (Hsieh *et al.*, 2010). This shows that the transition between the extended and retracted configurations can occur while DsrC is associated with DsrAB.

An interesting feature of the DsrC structure is a helix-turn-helix (HTH)-like structural motif (**Figure 2.5 C**) (Cort *et al.*, 2001; Cort *et al.*, 2008). This is one of the most common motifs involved in protein binding to DNA, and it is involved in a wide range of functions including transcription regulation, DNA repair and replication, RNA metabolism and also protein-protein interactions (Aravind *et al.*, 2005). The fact that the DsrC HTH motif is similar to those of transcriptional regulator proteins, has raised the hypothesis of another function for DsrC (Cort *et al.*, 2001). Recently, the DNA-binding capability of *Al. vinosum* DsrC was assessed and it was shown that can bind to a putative *dsr* promoter region located upstream of the *dsrA* gene, suggesting a possible regulatory function of DsrC (Grimm *et al.*, 2010).

DsrC belongs to a wider family of bacterial proteins in which only the last Cys is conserved. This includes *E. coli* TusE, which was shown to be involved in a cascade of sulfur transfer reactions based on persulfide chemistry (R-Cys-S-SH). These reactions transfer the thiol group from a cysteine (sulfur source) to the uridine at wobble position 34 of glutamate, lysine and glutamine transfer-RNAs, modifying this into 2-thiouridine (called 2-thiouridine of $mnm^5s^2U$, **Figure 2.7**) (Ikeuchi *et al.*, 2006). The sulfuration of this uridine is crucial for precise

codon detection and recognition by the cognate aminoacyl-tRNA synthetases. The sulfur trafficking by Tus proteins involves persulfide intermediates (**Figure 2.7**), which are a cellular strategy to bypass the toxicity of free sulfide (Beinert, 2000; Mueller, 2006; Toohey, 2011).

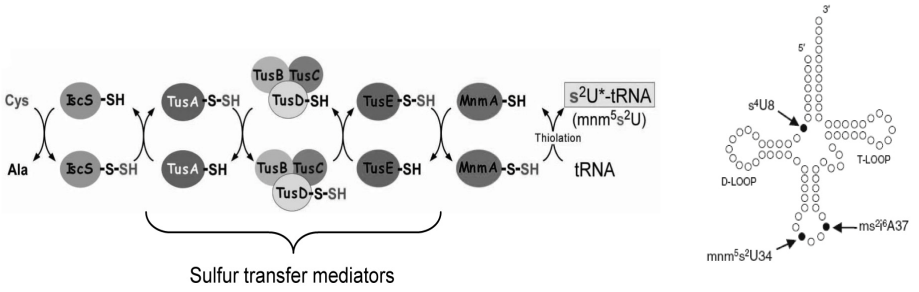


**Figure 2.7** – Sulfur relay system mediated by Tus proteins in *E. coli*. IscS is a cysteine desulfurase that catalyzes the desulfuration of L-cysteine residue generating a cysteine persulfide (IscS-S-SH). TusA interacts with IscS to stimulate its desulfurase activity, and accepts the persulfide sulfur. The sulfur is transferred to TusE through TusD of the TusBCD complex. TusE interacts with MnmA to transfer the sulfur, and this recognizes the tRNA and the wobble uridine is attacked by the persulfide sulfur resulting in the synthesis of the 2-thiouridine of $mnm^5s^2U$ (taken from (Noma *et al.*, 2009)). On the right side there is a representation of the secondary structure of tRNA with possible thio-modifications of nucleosides in *E. coli* (taken from (Hidese *et al.*, 2011)).

The role of TusE in sulfur transfer metabolism, together with the positioning of the C-terminal DsrC cysteine next to the sulfite binding site in DsrAB, led to the proposal that DsrC is involved in the reduction of sulfite *via* the formation of a persulfide intermediate for subsequent liberation of sulfide (Oliveira *et al.*, 2008). The last cysteine residue of *Al. vinosum* DsrC has been implicated in protein-protein interactions with DsrEFH (Cort *et al.*, 2008), which is homologous of the TusBCD complex. Additionally, it was shown that *in vitro* persulfurated

DsrE, from the DsrEFH complex, was able to transfer sulfur exclusively to the last cysteine of DsrC, and the reverse reaction was not observed, suggesting that the sulfur transfer system has directionality and also that DsrC could act as a sulfur-acceptor protein (Stockdreher *et al.*, manuscript *in* preparation). It should be noted that the genes for DsrEFH are not found in SRO.

The relevance of DsrC in cellular metabolism is also reflected in the fact that it is a highly expressed gene, at similar or even higher levels than other enzymes related to dissimilatory sulfate reduction (Haveman *et al.*, 2003; Keller and Wall, 2011; Wall *et al.*, 2008). Moreover, in metagenomic samples from marine oxygen minimum zones, where SRO and sulfur oxidizers abound, *dsrC* is one of the most abundantly transcribed energy metabolism genes for dissimilatory sullfur metabolism (Canfield *et al.*, 2010). The same was observed for intracellular symbionts living in the coastal bivalve *Solemya velum* (Stewart *et al.*, 2011). The metabolic importance of DsrC is also patent in the fact that an *Al. vinosum dsrC* mutant was genetically unstable and could not be maintained, or even grow with malate as electron donor and carbon source instead of sulfide (Cort *et al.*, 2008). The severity of the *dsrC* mutation was not observed for other *dsr* genes of *Al. vinosum* (Sander *et al.*, 2006), pointing to a crucial biological role of DsrC.

### 2.2.3 Qmo complex

The quinone-interacting membrane-bound oxidoreductase complex, QmoABC, was first described and isolated from the membranes of *D. desulfuricans* ATCC 27774 (Pires *et al.*, 2003). The complex has three subunits, two are cytoplasmic soluble proteins (QmoA and QmoB) and the other is membrane bound with an extra soluble domain facing the cytoplasm (QmoC) (**Figure 2.8**). QmoA binds an FAD molecule, QmoB another FAD molecule plus two [4Fe-4S]$^{2+/1+}$ centers, and QmoC binds two hemes *b* in the transmembrane domain and two [4Fe-4S]$^{2+/1+}$ centers in the soluble domain. EPR revealed that one of the iron-sulfur clusters is of the [3Fe-4S]$^{1+/0}$ type (Pires *et al.*, 2003). The Qmo subunits have several similarities with heterodisulfide reductases (Hdr), which are key enzymes in methanogens. Hdr catalyzes the last step of the respiratory chain of methanogenesis, the reduction of the heterodisulfide (CoM-S-S-CoB) to the respective CoM-SH and CoB-SH thiols ($E^{o'}$ = -140 mV) (Hedderich *et al.*, 2005; Thauer *et al.*, 2008). QmoA and QmoB are homologous to HdrA, a flavin and Fe-S protein from the soluble HdrABC trimer; and QmoC seems to be the result of a gene fusion, resulting in a protein with a hydrophobic domain homologous to HdrE (from the membrane bound HdrED dimer) and a hydrophilic domain homologous to HdrC (**Figure 2.8**). Recently, it was shown that HdrABC forms a complex with MvhADG (methyl-viologen reducing [NiFe]-hydrogenase or F420-non-reducing hydrogenase), which couples the reduction of ferredoxin with $H_2$ to the reduction of CoM-S-S-CoB through a flavin-based electron bifurcation

mechanism, where the HdrA flavin cofactor is considered to be the site of electron bifurcation (Costa *et al.*, 2010).

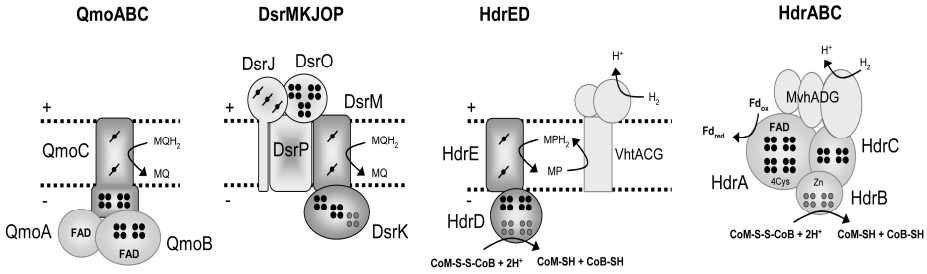


**Figure 2.8** – Schematic representation of the conserved respiratory complexes, QmoABC and DsrMKJOP, form SRB in comparison with Hdr homologues from methanogens. MP, methanophenazine; MQ, menaquinone; Fd, ferredoxin.

●● - $[4Fe\text{-}4S]^{2+}$; ●● - $[4Fe\text{-}4S]^{3+}$; ✓ - heme

The *qmoABC* genes are contiguous to *aprAB* genes coding for APS reductase in the majority of SRB, which led to the proposal that Qmo is involved in electron transfer from the membrane quinone pool to the APS reductase (Pereira *et al.*, 2007; Pires *et al.*, 2003). The QmoC membrane domain belongs to the family of heme *b*-containing subunits of respiratory oxidoreductases (the NarI protein family), which was postulated to couple electron transfer with the menaquinone pool *via* the hemes to the generation of a proton gradient (Berks *et al.*, 1995). The Qmo hemes *b* are reduced by a menaquinol analogue (Pires *et al.*, 2003), suggesting that probably QmoC transfers electrons from the menaquinone pool to QmoA/B, which direct or indirectly deliver electrons to the soluble APS reductase. Recently, this proposal was corroborated by the finding that a Δ*qmoABC* mutant of *D. vulgaris* is unable to grow

on sulfate as terminal electron acceptor, but is still able to grow on sulfite (Zane *et al.*, 2010). Thus, Qmo is essential for sulfate reduction and no other transmembrane complex is able to substitute its absence. The *qmoABC* genes are conserved in SRO genomes, reflecting its importance for energy metabolism. However, the archaeon *Caldivirga maquilingensis* lacks the *qmo* genes and in Gram-positive SRO the *qmoC* gene is missing, indicating that in these organisms Qmo is soluble, whereas APS reductase is membrane bound, and it is proposed that QmoBC receives electrons from a soluble Hdr-like protein or an electron transfer flavoprotein (Junier *et al.*, 2010). The *in vitro* electron transfer between Qmo and APS reductase has not been reported yet, but protein-protein interaction studies provide evidence for a direct interaction between the two proteins (Ramos and Pereira, manuscript *in* preparation).

### 2.2.4 Dsr complex

The DsrMKJOP complex is a transmembranar complex with subunits facing both the cytoplasm and the periplasm. The first report of the Dsr complex was in *Al. vinosum*, a sulfur oxidizing bacterium where the gene cluster *dsrABEFHCMK* containing the *dsrAB* genes was reported (Pott and Dahl, 1998). Later, this gene cluster was shown to consist of 15 *dsr* genes - *dsrABEFHCMKLJOPNRS* - and that their expression is regulated by a sulfide inducible promoter (Dahl *et al.*, 2005). Furthermore, DsrK and DsrJ from the Dsr complex were found to copurify with DsrAB, DsrEFH and DsrC (Dahl *et al.*, 2005), highlighting the probable physiological interaction between them. The *dsrAB*,

*dsrC* and *dsrMKJOP* genes are conserved among sulfate reducing and sulfur oxidizing organisms, where they are expected to perform reverse functions (Frigaard and Dahl, 2009; Schedel *et al.*, 1979).

The *dsrMKJOP* genes are present as a transcriptional unit in all SRO, except again in the Gram-positive organisms, which lack the *dsrJOP* genes (Junier *et al.*, 2010). Interestingly, in *Desulfotomaculum acetoxidans*, *dsrC* and *dsrAB* plus a ferredoxin gene are found in the vicinity of *dsrMK* (Pereira *et al.*, 2011). Other examples of this type of arrangement can be found in deltaproteobacterial SRB (see Chapter 5 for a genomic comparison). In the majority of Gram-negative SRB there is no co-localization of the *dsrAB* with the *dsrMKJOP* genes. Since the *dsrMK* genes are the only genes of the Dsr complex present in all SRO, this suggests that they are the only two vital proteins for sulfite reduction, functioning as a minimum operating module. However, all genes of the *dsrMKJOP* gene cluster were shown to be essential for sulfur oxidation of *Al. vinosum* (Sander *et al.*, 2006). *In frame* deletions of each individual gene revealed an absolute requirement for all *dsr* genes in the oxidation of stored sulfur.

Mander *et. al.* published for the first time the isolation of a Dsr complex from the archaeal sulfate reducer *A. fulgidus*, but it was named Hme complex standing for "Hdr-like menaquinol-oxidizing enzyme" (Mander *et al.*, 2002). The Hme complex contained sub-stoichiometric amounts of the DsrJ subunit. Subsequently, the DsrMKJOP complex was isolated from *D. desulfuricans* ATCC 27774 containing the expected stoichiometry of subunits (Pires *et al.*, 2006).

The Dsr complex comprises two periplasmic subunits (DsrJ and DsrO), two membrane proteins (DsrM and DsrP) and a cytoplasmic protein (DsrK) (**Figure 2.8**). Briefly, DsrJ is a membrane-anchored triheme cytochrome *c* not belonging to the cytochrome $c_3$ family; DsrO is an Fe-S cluster protein; DsrM is a membrane cytochrome *b* of the NarI family; DsrP is an integral membrane protein of the NrfD family; and DsrK is an Fe-S cluster protein related to HdrD, the catalytic subunit of HdrED that holds an unusual [4Fe-4S]$^{3+}$ catalytic center (Heiden *et al.*, 1994; Thauer *et al.*, 2008) (**Figure 2.8** and **2.9**). HdrED is a methanophenazine-dependent heterodisulfide reductase present in the cytoplasmic membrane of methanogens with cytochromes (Thauer *et al.*, 2008). The [4Fe-4S]$^{3+}$ center has an unusual rhombic EPR signal in the oxidized state upon addition of one of the thiol products (Duin *et al.*, 2002; Madadi-Kahkesh *et al.*, 2001), which is unlike those of standard [4Fe-4S]$^{2+/1+}$ clusters that are paramagnetic in the reduced state. The *A. fulgidus* Hme complex and the *D. desulfuricans* Dsr complex both display a similar EPR signal for a [4Fe-4S]$^{3+}$ cluster with *g*-values of 2.027, 1.994, and 1.943 for *D. desulfuricans* Dsr (Mander *et al.*,

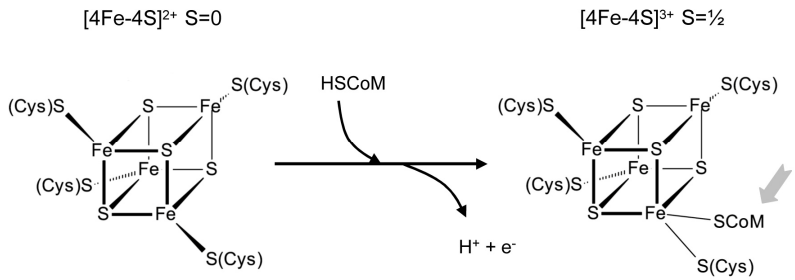


**Figure 2.9** – The ligation of CoMSH to the [4Fe-4S]$^{2+}$ cluster forms a [4Fe-4S]$^{3+}$ cluster with one extra thiolate ligand at a unique Fe site (adapted from (Walters and Johnson, 2004)).

2002; Pires *et al.*, 2006). A similar signal was observed in a HmeCD complex (DsrMK-like) from *Archaeoglobus profundus* (Mander *et al.*, 2004).

Since the Dsr complex is involved in some of the work described in this thesis (Chapter 4), its subunits will be described in more detail.

**DsrM** is predicted to contain six transmembrane helices and is related to *E. coli* NarI (membrane subunit of the NarGHI respiratory nitrate reductase) and HdrE from *Methanosarcina* sp.. NarI contains two hemes *b* on opposite sites of the membrane and a menaquinol oxidation site on the periplasmic side (Bertero *et al.*, 2003; Magalon *et al.*, 1997). DsrM, like NarI, has four conserved histidines for heme ligation, and the heme quantification of *D. desulfuricans* DsrM showed it to be a diheme cytochrome *b*. Moreover, addition of menaquinol to the Dsr complex resulted in ~40 % of heme reduction, which should correspond to the reduction of the two hemes *b* (Pires *et al.*, 2006). Recently, DsrM from the sulfur oxidizer *Al. vinosum* was heterologously expressed and exhibited a typical UV/visible heme *b* spectrum, which was partly reduced by menadiol (Grein *et al.*, 2010a). The macroscopic hemes *b* redox potentials were determined to be +60 and +100 mV (Grein *et al.*, 2010a), suitable to accept electrons from menaquinol ($E^{o'}$= -70 mV).

**DsrK** is probably a catalytic subunit of the DsrMKJOP complex, since it also contains the catalytic $[4Fe\text{-}4S]^{3+}$ cluster observed in HdrD. However, in the Dsr complex the signal of the $[4Fe\text{-}4S]^{3+}$ cluster is detected in the oxidized native state without addition of any exogenous substrate, unlike the

$[4Fe\text{-}4S]^{3+}$ cluster of HdrED that is only detectable upon binding of a thiol substrate (Duin *et al.*, 2002; Pires *et al.*, 2006) (**Figure 2.8**). The amino acid sequence of DsrK contains two classical motifs $CX_2CX_2CX_3C$ for binding of two canonical $[4Fe\text{-}4S]^{2+/1+}$ clusters, plus a CCG domain present in the conserved sequence $CX_nCCGX_mCXXC$ (Pfam protein family PF02754) (Hamann *et al.*, 2007; Hedderich *et al.*, 2005). The CCG domain was first described for Hdr from *Methanosarcina barkeri* (Kunkel *et al.*, 1997) and thiol:fumarate reductase (Tfr) from *Methanothermobacter marburgensis* (Heim *et al.*, 1998) and it is known to responsible for the binding of the catalytic $[4Fe\text{-}4S]^{3+}$ cluster in HdrD, which is also present in HdrB (**Figure 2.8**). The CCG domain is present in a large number of archaeal and bacterial proteins (Hedderich *et al.*, 2005).

Sequence analysis predicts that DsrK is cytoplasmic and does not contain transmembrane helices. However, heterologous expression of DsrK from *Al. vinosum* and *D. vulgaris* resulted in a membrane-associated protein DsrK (Grein *et al.*, 2010a) (S. Venceslau and I. Pereira, unpublished data). In *Al. vinosum* it was proposed that this membrane association was due to an in-plane helix that works as a monotopic membrane anchor (Grein *et al.*, 2010a).

**DsrJ** is a small cytochrome (15 kDa) with three heme *c* binding motifs (CXXCH) closely spaced and located close to the C-terminus. The Sec-dependent signal peptide is not cleaved off, but serves as a membrane anchor, as reported for *A. fulgidus*, *D. desulfuricans*, and more recently for *Al. vinosum* (Grein *et al.*, 2010b; Mander *et al.*, 2002; Pires *et al.*, 2006). The DsrJ sequence shows no significant homology to other cytochromes

in the database, therefore it constitutes a novel family of cytochromes *c*. From analysis of the DsrJ sequence it is evident that there are not enough histidines residues for all the hemes to have the standard bis-histidinyl ligation. On the basis of sequence alignments and spectroscopic data it was proposed that each heme *c* has a different axial coordination, namely one bis-histidine, one histidine/methionine and an unusual histidine/cysteine coordination (Pires *et al.*, 2006).

The His/Met or His/His coordination are the most common axial ligations in cytochromes *c* that participate in electron transfer. Examples of cytochromes with His/Cys coordination are very limited, with only four examples reported: 1) SoxXA diheme cytochrome complex (from the Sox enzyme system of sulfur oxidizers), which is involved in the thiosulfate oxidation step in a mechanism known as Friedrich-Kelly model (Bamford *et al.*, 2002; Cheesman *et al.*, 2001; Friedrich *et al.*, 2005; Friedrich *et al.*, 2001); 2) PufC, a trihemic cytochrome that is associated with the photosynthetic reaction center in *R. sulfidophilum*, and is responsible for mediating electron transfer between the bacteriochlorophyll and soluble carriers (Alric *et al.*, 2004); 3) GHP (that stands for green heme protein), a monohemic cytochrome *c* from the purple sulfur bacterium *Halochromatium salexigens* (van Driessche *et al.*, 2006); and 4) NaxLS complex, a heterodimeric *c*-type heme from *Candidatus* Kuenenia stuttgartiensis, an anaerobic ammonium oxidizing bacteria, whose function remains unknown (Ukita *et al.*, 2010). There is no homology between DsrJ and these His/Cys coordinated cytochromes, and the physiological roles of these proteins seem to be different. Nevertheless, some common

points with DsrJ are observed. In the SoxA subunit the heme with His/Cys ligation is characterized by a very negative midpoint potential (bellow -400 mV in all SoxXA proteins studied so far) (Cheesman *et al.*, 2001; Reijerse *et al.*, 2007), and this is also the case in NaxLS (Ukita *et al.*, 2010). Also, in DsrJ the His/Cys ligated heme could not be completely reduced (Pires *et al.*, 2006). On the other hand, in SoxXA the cysteine from the His/Cys coordination is modified to a persulfide that ligates the heme, and the substrate thiosulfate is bound to the ligating cysteine resulting in a SoxA-thiocysteine-S-sulfate intermediate (Cheesman *et al.*, 2001). This raises the question whether the His/Cys heme in DsrJ might be involved in sulfur chemistry. The physiological importance of the cysteine was recently addressed in *Al. vinosum* DsrJ, where mutation of this residue caused a tremendous effect, since the ability to metabolize stored sulfur was dramatically impaired (Grein *et al.*, 2010b).

DsrO is a ferredoxin-like protein predicted to contain three [4Fe-4S] cluster binding sites in *Desulfovibrio* spp. (Pires *et al.*, 2006), and four [4Fe-4S] cluster in other organisms. This subunit contains a typical signal peptide to allow translocation across the membrane via the Tat pathway (Berks *et al.*, 2005), and no transmembrane helix is predicted to be present. DsrO is related to the four cluster subunits of several CISM enzymes, including tetrathionate reductase (TtrB), polysulfide reductase (PsrB) and nitrite reductase (NrfC), which are associated with integral membrane subunits and transfer electrons from this subunit to a catalytic one (Rothery *et al.*, 2008).

**DsrP** is another integral membrane protein with ten predicted transmembrane helices. It belongs to the NrfD/PsrC protein family, which includes the membrane-spanning, quinone-interacting subunits of a number of respiratory enzymes of the CISM family (Rothery *et al.*, 2008; Simon *et al.*, 2008), including PsrC subunit from several organisms with which DsrP shares the highest sequence similarity. Besides PsrC, DsrP is related to the membrane subunit of *E. coli* hydrogenase-2 (HybB), which works as a menaquinone reductase, and where the presence of hemes has been debated (Menon *et al.*, 1994).

In *Desulfovibrio* DsrP there are no conserved histidine residues to serve as ligands to putative hemes, and these were also not observed spectroscopically (Pires *et al.*, 2006). In addition, in the first structure of a protein from this family, the polysulfide reductase Psr from *Thermus thermophilus*, the PsrC subunit does not contain heme (Jormakka *et al.*, 2008). However, the heterologous expression of *Al. vinosum* DsrP in *E. coli* resulted in a heme *b*-containing protein (Grein *et al.*, 2010a). Furthermore, in sulfur oxidizing organisms three possible heme-ligating residues are conserved (two His and one Met). Based on this finding the authors propose that a menaquinone/menaquinol recycling occurs between the two Dsr membrane proteins, with DsrM working as a quinol oxidase and DsrP as a quinone reductase (Grein *et al.*, 2010a).

The involvement of the Dsr complex in the sulfite reduction pathway is consensual, but the molecular and mechanistic details of its involvement are not yet known. Thus, the Dsr complex is a challenging piece of the sulfate respiratory chain. The most recent data, based on genomic analysis to SRO (see

Chapter 5), also suggests that the Dsr complex may be composed of two modules: the DsrMK, strictly conserved among all SRO, and the DsrJOP (conserved in all the deltaproteobacteria group and in the *Archaeoglobus* genus). The similarity between DsrMK and HdrED points to a role of DsrMK in electron transfer from menaquinol oxidation to the reduction of a cytoplasmic substrate, proposed to be the DsrC disulfide bond (Oliveira *et al.*, 2008; Pires *et al.*, 2006). For this reason DsrC has already been referred as a "bacterial heterodisulfide" (Cort *et al.*, 2008).

The physiological function of the second module, DsrJOP, is more puzzling, namely regarding its electron donor partners. This module faces the periplasm and has a possible menaquinone interacting subunit. The experimental evidence up to now indicates that the Dsr complex does not function as electron acceptor to the hydrogenase/Type I cytochrome $c_3$ (TpI$c_3$) couple (da Silva, 2011; Pires *et al.*, 2006). The key question regards the function of DsrJ, for which there is no hint due to its unique nature. In the case of sulfur oxidizers some sulfur transformations occur in the periplasm (Frigaard and Dahl, 2009), so it is easier to think of DsrJ as having a role in sulfur chemistry. On the other hand, in SRO all sulfur transformations are predicted to occur in the cytoplasm. Interestingly, a DsrJ protein from a sulfate reducer could complement an *Al. vinosum* Δ*dsrJ* mutant strain, showing that DsrJ from a SRO can substitute the homologous sulfur oxidizer protein (Grein *et al.*, 2010b).

## 2.3 Energy transduction models

Having described the dissimilatory reduction of sulfate that occurs exclusively in the cytoplasm, it is important to realize how this pathway is connected with the remaining metabolic pathways. The oxidation of carbon-based electron donors takes place in the cytoplasm, whereas the oxidation of some common electron donors, like $H_2$ and formate, takes place in periplasm so the reducing power must be transferred across the cell membrane.

Lactate is a standard electron donor for laboratory growth of SRO, and serves as model for short chain fatty acids, which are common energy sources for SRO in natural environments. The metabolic route of lactate oxidation in *Desulfovibrio* spp. generates equimolar amounts of acetate and ATP by substrate-level phosphorylation. Because the ATP generated in this process is equal to the number of ATP molecules needed for sulfate activation there has to be another mechanism to produce energy in this process. Moreover, as many SRB are able to grow on $H_2$ and sulfate as sole energy substrates (using $CO_2$ and/or acetate as carbon suppliers) (Peck, 1960), from which substrate-level phosphorylation is not possible, it is clear that sulfate reduction has to be associated with electron transport-linked phosphorylation. Lactate oxidation to pyruvate is catalyzed by $NAD(P)^+$-independent lactate dehydrogenases, enzymes that are mainly associated to the membrane, so it has been proposed that the electrons may be used to quinone reduction. Then pyruvate can be further oxidized to formate by pyruvate formate lyase, or oxidized by pyruvate:ferredoxin

oxidoreductase to acetyl-CoA, which is then converted to acetate by phosphotransacetylase and acetate kinase (a general scheme is shown in Figures 3.1.8 and 5.1).

Model mechanisms to explain energy conservation in *Desulfovibrio* spp. have been proposed based on cycling of intermediate metabolites such as $H_2$, formate and CO (Odom and Peck, 1981; Voordouw, 2002). A more general chemiosmotic mechanism, based on coupling of vectorial proton translocation and electron transfer, has also been proposed (Lupton *et al.*, 1984).

### 2.3.1 Hydrogen cycling model

Hydrogen is the simplest electron donor to *Desulfovibrio* spp., and it is metabolized with $CO_2$ and acetate as carbon sources (chemolithoheterotrophy) or only carbon dioxide (autotrophy). However, SRO also have the capacity to produce $H_2$ as a metabolic product during fermentative growth, in the absence of sulfate (Postgate, 1984; Rabus *et al.*, 2007). When $H_2$ is the sole energy donor, its oxidation releases electrons at the periplasm that have to be transferred to cytoplasmic sulfate reduction. But during lactate/sulfate growth, $H_2$ was also observed to be transiently formed and consumed (Tsuji and Yagi, 1980). This process, also known as the hydrogen burst or fermentation burst, is proposed to be linked to the requirement of the cell to produce ATP in order to start the reduction of sulfate (Voordouw, 2002). Odom and Peck showed that *D. gigas* spheroplasts were unable to reduce sulfate, unless the periplasmic proteins hydrogenase (enzyme responsible for $H_2$

oxidation) and Tpl$c_3$ were externally added (Odom and Peck, 1981). This led them to propose a model for energy transduction known as "hydrogen cycling model" for growth of *D. vulgaris* on lactate and it was generalized for *Desulfovibrio* spp.. In this model, the electrons produced during lactate and/or pyruvate oxidation are used by cytoplasmic hydrogenases to form $H_2$, which diffuses across the cell membrane to be utilized as electron donor to periplasmic hydrogenases. These reoxidize hydrogen to protons and electrons, and the electrons are transferred back across the membrane for cytoplasmic reduction of sulfate, resulting in a proton and charge gradient ($\Delta$pH and $\Delta\Psi$). The transmembrane nature of $H_2$ oxidation with sulfate had previously been noted by Badziong and Thauer (1980), who argued that vectorial electron transport was coupled to energy generation in this process (Badziong and Thauer, 1980). At this time it was not known how the electrons would cross the cytoplasmic membrane. Nowadays, several membrane-bound complexes that may perform this function were isolated or identified in the genomes of SRB (see section 2.4 of this chapter and Chapters 3 and 5).

At the time the model was proposed, periplasmic hydrogenases had already been demonstrated in SRB (Bell *et al.*, 1974), but cytoplasmic hydrogenases were not known. Two multi-subunit membrane-bound hydrogenases with the active site facing the cytoplasm, Ech and Coo, have been considered as possible candidates for cytoplasmic proton reduction hydrogenases (Heidelberg *et al.*, 2004; Rodrigues *et al.*, 2003). However, the genes coding for these hydrogenases are not

widespread in SRO (Pereira *et al.*, 2011), but there are other genes coding for soluble cytoplasmic hydrogenases in many SRO (Pereira *et al.*, 2011). Nevertheless, there are examples of SRO that have no hydrogenase genes at all, namely *Desulfococcus oleovorans* and *C. maquilingensis,* or that do not have cytoplasmic hydrogenases genes (*Desulfomicrobium baculatum*) (Pereira *et al.*, 2011).

The report of a *D. desulfuricans* mutant incapable of sulfate respiration with $H_2$, but growing normally on lactate/sulfate under argon (Odom and Wall, 1987), raised doubts whether $H_2$ cycling is obligate. Additionally, it is energetically unfavourable to produce hydrogen from the oxidation of lactate to pyruvate ($\Delta G'_0$ = +43.2 kJ/mol) (Thauer and Morris, 1984; Traore *et al.*, 1981). Further studies have shown the controversy of the model but this has never been refuted or confirmed convincingly. The hydrogen cycling model is probably only one of several possible pathways for energy conservation in *Desulfovibrio*. It is certainly not generally applied to all SRO, since some organisms have no hydrogenases genes.

### 2.3.2 Alternative mechanism based on proton translocation

Lupton and coworkers have formulated an alternative mechanism to $H_2$ cycling (Lupton *et al.*, 1984), where they propose that the electrons generated from lactate oxidation are transferred to a membrane-bound protein system that redirects electrons to the reductases, while concomitantly translocating protons to the periplasm creating a proton motive force.

Distinction between the two mechanisms relies on how acidification in the periplasm is achieved: hydrogen cycle considers $H_2$ diffusion across the cytoplasmic membrane and periplasmic hydrogen oxidation, while the second model is built on proton transfer into the periplasmic space by membrane proteins. The Lupton model (named as "Trace $H_2$ transformation model") considers that $H_2$ may be formed during lactate oxidation as a way to regulate the redox state of internal electron carriers like ferredoxin, and than this $H_2$ can be consumed by periplasmic hydrogenases and prevent energy loss (Lupton *et al.*, 1984).

The issue of proton translocation was addressed in studies performed by Fitz and Cypionka, where they observed that washed cells presented $H^+/H_2$ ratios above the ones produced by periplasmic hydrogenases, so the authors concluded that at least two *Desulfovibrio* spp. were able to translocate protons across the cytoplasmic membrane by substrate oxidation with sulfur compounds, and that the extracellular proton release by periplasmic hydrogenases is an additional contribution to energy conservation (Fitz and Cypionka, 1989; Fitz and Cypionka, 1991).

### 2.3.3 Formate cycling model

Oxidation of formate to carbon dioxide is catalyzed by formate dehydrogenases (Fdh) through the reaction: Formate ($H_2COO$) ⇆ $CO_2$ + $H^+$ + $e^-$. Like hydrogenases, these enzymes may have a cytoplasmic or periplasmic location, and usually several isoenzymes are present in each organism. Formate

cycling has been proposed as an alternative to $H_2$ cycling (Voordouw, 2002). Similarly to hydrogen cycling, the model for formate cycling considers that the formate formed at the cytoplasm is exported into the periplasm, where it is oxidized, generating protons to support ATP synthesis, and electrons that are transferred back to the cytoplasm for sulfate reduction, probably through a periplasmic *c*-type cytochrome network and membrane-bound complexes (Heidelberg *et al.*, 2004; Voordouw, 2002).

Many SRO have periplasmic Fdhs to oxidize formate, and these are predominantly soluble, contrary to what is observed in most bacteria (Pereira *et al.*, 2011). Fdh is a member of the DMSO reductase family of enzymes and the active site is constituted by a molybdenum (Mo) or a tungsten (W) atom coordinated by two pyranopterin cofactors, and several Fe-S clusters that function as an electric wire in electron transport (Moura *et al.*, 2004). In *D. vulgaris* there are three periplasmic Fdhs: two soluble, FdhAB and FdhABC3, and a membrane-associated enzyme facing the periplasm, FdhABD. FdhAB has the higher catalytic rate and can incorporate both Mo and W (da Silva *et al.*, 2011), as shown for the enzyme from *D. alaskensis* (Mota *et al.*, 2011), whereas FdhABC3 has a tetraheme cytochrome $c_3$ subunit and specifically incorporates molybdenum (da Silva *et al.*, 2011; Sebban *et al.*, 1995). The membrane-associated FdhABD has a membrane subunit of the NfrD family that may be involved in quinone reduction, but FdhABD is only detected at low levels in growth with tungsten (da Silva *et al.*, 2011).

### 2.3.4 Overview of electron transfer in sulfate respiration

In contrast to other anaerobic respiratory processes, the terminal reductases of SRB (APS reductase and DsrAB) are soluble proteins located in the cytoplasm, and so are not directly involved in proton pumping or redox loops. On the other hand, oxidation of formate and $H_2$, two common substrates of SRB, is performed by periplasmic enzymes not associated to the membrane, unlike the great majority of bacterial hydrogenases and Fdhs (like the *E. coli* FdnGHI in Figure 2.2). Therefore, oxidation of $H_2$/formate and sulfate reduction occur in different cell compartments, and are not directly associated to the cytoplasmic membrane. Qmo and Dsr complexes have been proposed to be involved in sulfate reduction, taking part in the respiratory electron transfer chain (Matias *et al.*, 2005; Pereira, 2008; Pires *et al.*, 2003; Pires *et al.*, 2006). However, they do not receive electrons from the Tp1$c_3$ cytochrome, the electron acceptor of periplasmic hydrogenases and Fdhs (da Silva, 2011; Pires *et al.*, 2003; Pires *et al.*, 2006). Thus, an additional entry-point for electrons to cross the membrane, or reduce the quinone pool, has to exist. On the cytoplasmic side, it is also unclear if the terminal reductases receive electrons directly or indirectly from the Qmo and Dsr membrane complexes (Matias *et al.*, 2005). In the case of Qmo, direct electron transfer to APS reductase *via* menadiol-reduced Qmo was not observed (Pires *et al.*, 2003). For the Dsr complex, a mechanism involving DsrC as an intermediate protein in electron transfer was proposed (Oliveira *et al.*, 2008). DsrC is

considered to play a pivotal role between the Dsr complex and the sulfite reductase (DsrAB), cycling through disulfide/thiol states (Cort *et al.*, 2001; Cort *et al.*, 2008; Oliveira *et al.*, 2008). Elucidation of this mechanism is crucial to understanding not only sulfate respiration, but also sulfur oxidation metabolism. A general scheme of sulfate respiration highlighting several question marks is depicted in Figure 2.10.

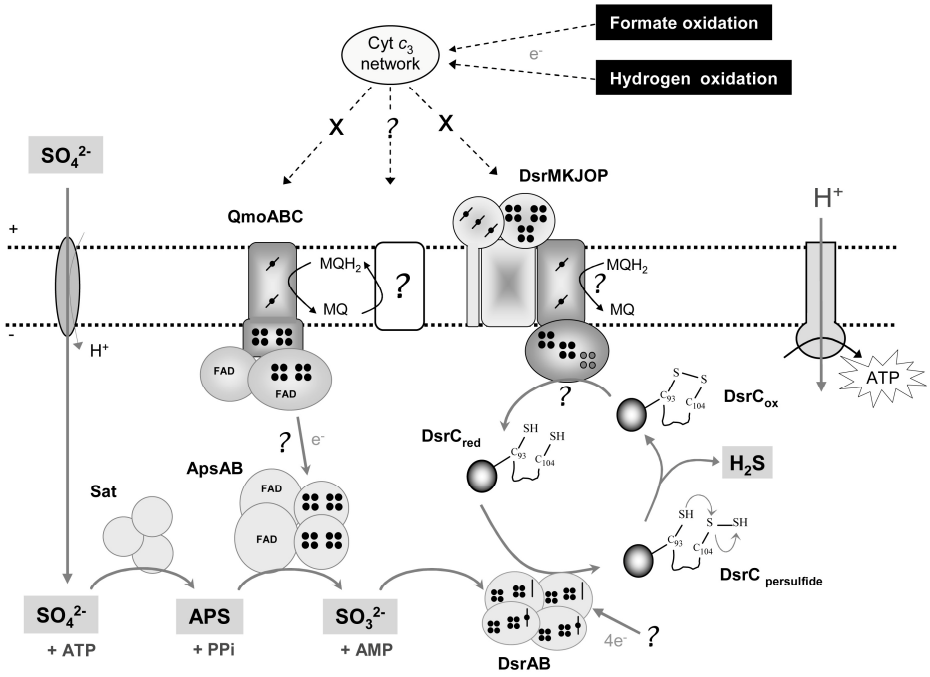


**Figure 2.10** – Electron transfer chain in sulfate reducing bacteria growing by oxidation of hydrogen and formate with cytoplasmic reduction of sulfate to sulfide. In grey boxes are the products of sulfate reduction pathway. Sat, sulfate adenylyltransferase; ApsAB, adenosine phosphosulfate reductase; QmoABC, membrane complex that is the probable electron donor to ApsAB; DsrMKJOP, membrane complex proposed to **work** as a disulfide reductase of $DsrC_{ox}$

⁘ - $[4Fe\text{-}4S]^{2+}$; ⁘ - $[4Fe\text{-}4S]^{3+}$; ✓ - heme; I - sirohydrochlorin; MQ - menaquinone.

## 2.4 Other membrane-bound electron transport complexes

Besides Qmo and Dsr complexes there are other membrane electron transport complexes (METC) in SRO, which are only present in the deltaproteobacterial organisms (and *Thermodesulfovibrio yellowstonii*). It is still unknown whether these METC transfer electrons to the menaquinone pool and/or directly to the cytoplasm for sulfate reduction, and also whether or not they are involved in proton transfer to the cytoplasm *via* redox loops or proton pumping.

The METC can be divided in two classes: the ones that have a multiheme cytochrome *c* subunit and the ones that do not have this, and contain only Fe-S centers and/or flavin cofactors. The heme-containing class is composed of Hmc, Nhc, Tmc and Ohc complexes (**Figure 2.11**) (and the Qrc complex described in Chapter 3), which are found only in SRB; while the non-heme containing group includes widely distributed bacterial complexes, such as Rnf, Nqr and Nuo, which will be discussed in Chapter 5 of this thesis.

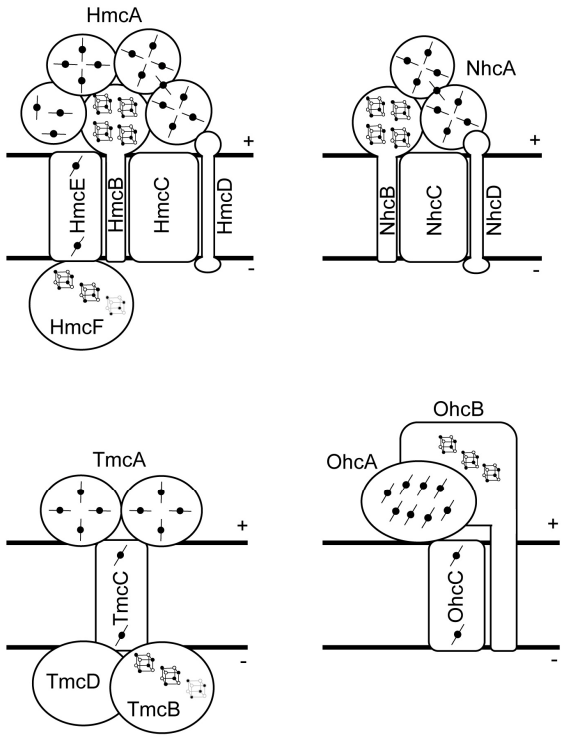


**Figure 2.11** – Schematic representation of the METC present only in SRO, namely Hmc, Nhc, Tmc and Ohc complexes. Light grey Fe-S cluster, $[4Fe\text{-}4S]^{3+}$.

## 2.4.1 HMC AND NHC COMPLEXES

The high molecular mass cytochrome *c*, HmcABCDEF, was the first complex to be identified in SRO (Rossi *et al.*, 1993), and its name derives from the HmcA subunit, which is a large protein with 16 *c*-type hemes. HmcB is a periplasmic facing protein that harbours a membrane anchor and four $[4Fe\text{-}4S]^{2+/1+}$ cluster binding motifs are present in the sequence. The complex has three membrane-bound proteins, namely HmcC (from the NfrD family), HmcE (from the NarI family), and HmcD, and a cytoplasmic protein, HmcF, homologous to HdrD and also

containing a CCG domain (**Figure 2.11**). Although this subunit description greatly resembles the Dsr composition, the sequence similarity between corresponding subunits is quite low.

The first reports documented the isolation of HmcA as a soluble protein isolated from the other subunits of the complex in several *Desulfovibrio* spp. (Chen *et al.*, 1994; Pollock *et al.*, 1991). The three-dimensional structures for several HmcA subunits have been solved, showing that it is composed by four cytochrome $c_3$-like domains, and all the hemes are bis-histidinyl coordinated except one that is high-spin (Czjzek *et al.*, 2002; Matias *et al.*, 2002; Santos-Silva *et al.*, 2007).

The HmcA was shown to be an electron acceptor of hydrogenase *via* the TpI$c_3$ cytochrome (Pereira *et al.*, 1998), in contrast to the Dsr complex. The relationship between Hmc and the menaquinone pool has not been clearly elucidated, and there are also no studies as to whether Hmc could function in proton translocation. Initially, it was postulated that the Hmc complex could link the periplasmic hydrogen oxidation and the cytoplasmic sulfate reduction (Rossi *et al.*, 1993). Transcriptional studies showed that the expression of the *hmc* operon increases when hydrogen is the electron donor comparatively to lactate or pyruvate (Rossi *et al.*, 1993; Steger *et al.*, 2002). However, the growth of the *D. vulgaris hmc* mutant was slower but not impaired in hydrogen (Dolla *et al.*, 2000). The low level of transcript abundance for this complex, namely during growth with $H_2$ (Dolla *et al.*, 2000; Keller and Wall, 2011; Pereira *et al.*, 2008), also suggests that Hmc does not play an essential role. Most probably other METC can carry out the same function, and Tmc, which has a considerably higher transcript abundance

with $H_2$ (Pereira *et al.*, 2008), has been proposed to be a likely substitute (Matias *et al.*, 2005; Pereira *et al.*, 2008). Recently, Hmc was proposed to be involved in syntrophic metabolism since it was observed that growth of a *D. vulgaris hmc* mutant in coculture with a methanogen was severely affected when compared with a wild type coculture, and on the other hand the *hmc* gene expression was increased in the syntrophic grown cells *versus* growth with sulfate. These results indicate that the Hmc complex is important for a syntrophic lifestyle when compared with a solitary one. The authors suggest that the complex functions in electron transfer to the periplasm (contrary to what had been considered), for production of hydrogen that will function as an electron donor to the hydrogenotrophic methanogen (Walker *et al.*, 2009).

The *hmc* gene cluster is present in all *Desulfovibrio* spp. with a sequenced genome, except in *D. desulfuricans* and *D. piger*, which instead have a nine-heme cytochrome complex (Nhc). The cytochrome subunit NhcA had already been discovered in the eighties in *D. desulfuricans* (Liu *et al.*, 1988), and later its crystal structure was also determined confirming the similarity with the C-terminus domain of HmcA (Matias *et al.*, 2002). The Hmc and Nhc complexes are only present in *Desulfovibrio* spp., not other SRO, and superposition of the two is not observed (see Chapter 5). The Nhc is very similar to Hmc, but has a simpler subunit composition: the Nhc lacks the cytoplasmic and NarI like subunits (the "HdrED-like" module), and the NhcA subunit lacks the N-terminal domain of HmcA (Saraiva *et al.*, 2001). Like HmcA, the NhcA is reduced by hydrogenase *via* the TpI$c_3$ (Matias *et al.*, 1999). Since Nhc has

no cytoplasmic subunits it will likely transfer electrons only to the menaquinone pool, and not directly to the cytoplasm for sulfate reduction.



### 2.4.2 Tmc

The transmembrane complex TmcABCD was isolated from *D. vulgaris* and includes a tetraheme cytochrome $c_3$ (TmcA or Type II cytochrome $c_3$), an integral membrane cytochrome *b* protein, and two cytoplasmic proteins, namely an HdrD-like Fe-S cluster protein (which includes a CCG domain of the same family as DsrK and HdrD) and a tryptophan-rich protein with no homologue in the database (Pereira *et al.*, 2006) (**Figure 2.11**). The spectroscopic characterization of Tmc revealed the presence of the unusual paramagnetic EPR signal for the catalytic $[4Fe\text{-}4S]^{3+}$ center, also observed in HdrD and DsrK, which suggests that Tmc might be an alternative to the Dsr complex and also interact with DsrC. Protein-protein interaction studies did not shown evidence that the Tmc complex interacts directly either with APS reductase or with a DsrAB-DsrC complex, under the conditions tested (Pereira *et al.*, 2006).

The TmcA cytochrome was first isolated and characterized as a soluble cytochrome that belongs to the cytochrome $c_3$ family, and was initially named as acidic cytochrome $c_3$ and later as TpII$c_3$ (Paquete *et al.*, 2007; Pieulle *et al.*, 1996; Valente *et al.*, 2001).

The TpI$c_3$ interacts with TpII$c_3$ and when coupled with hydrogenase is able to promote electron transfer to the complex, resulting in nearly complete reduction of the Tmc

complex (Pereira *et al.*, 2006). No evidence for interaction with the quinone pool was obtained, suggesting that the Tmc complex works like an electric wire to conduct electrons from the periplasm to the cytoplasm. An example of a transmembrane protein complex that conduits electrons across the outer membrane using multiheme cytochrome *c* subunits was recently described in *Shewanella oneidensis*, a bacteria that uses extracellular mineral forms of iron and manganese as respiratory electron acceptors (Clarke *et al.*, 2011). However, in this case the two cytochrome subunits face different sides of the outer membrane, and one of them is "encaged" in a β-barrel membrane protein, in order to form a heme nanowire to "import" electrons directly to the periplasm.

The relevance of Tmc has been related to the metabolism, the expression of the *tmc* genes is quite high in several growth conditions (Keller and Wall, 2011), being higher for growth in hydrogen/sulfate when compared with lactate/sulfate, pointing to a role of the Tmc complex in hydrogen oxidation (Pereira *et al.*, 2008).

### 2.4.3 Ohc Complex

The octaheme cytochrome complex, OhcBAC, was identified only at a genomic level (Pereira *et al.*, 2007). It is a membrane-bound complex facing only the periplasm, like Nhc, and both are the least represented METC among SRB (Pereira *et al.*, 2011). Ohc is composed by a cytochrome *c* from a different family than cytochrome $c_3$, an Fe-S protein and a membrane *b*-type cytochrome of the NarI family (**Figure 2.11**). The Fe-S

cluster subunit seems not to be essential since its gene is absent in half of the organisms that contain the *ohc* genes. Like Nhc, this complex is proposed to transfer electrons from the periplasm to the menaquinone pool (Pereira *et al.*, 2007). The function of Ohc is unknown, but transcriptional studies show that the *ohc* genes are among the lower abundance *D. vulgaris* genes in lactacte/sulfate, indicating perhaps a less significant role in energy metabolism (Keller and Wall, 2011).



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# Chapter 3

# The Quinone-reductase complex

# Chapter 3
## Section 3.1

# The Qrc membrane complex, related to the alternative complex III, is a menaquinone reductase involved in sulfate respiration

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### 3.1.1 Summary

Biological sulfate reduction is a process with high environmental significance, due to its major contribution to the carbon and sulfur cycles in anaerobic environments. However, the respiratory chain of sulfate reducing bacteria is still poorly understood. Here we describe a new respiratory complex that was isolated as a major protein present in the membranes of *Desulfovibrio vulgaris* Hildenborough. The complex, which was named Qrc, is the first representative of a new family of redox complexes. It has three subunits related to the complex iron-sulfur molybdoenzyme family, and a multiheme cytochrome *c*, and binds six hemes *c*, one [3Fe-4S]$^{1+/0}$ cluster, several interacting [4Fe-4S]$^{2+/1+}$ clusters, but no molybdenum. Qrc is related to the alternative complex III, and we show that it has the reverse catalytic activity, acting as a Type I cytochrome $c_3$:menaquinone oxidoreductase. The *qrc* genes are found in the genomes of deltaproteobacterial sulfate reducers, which have periplasmic hydrogenases and formate dehydrogenases that lack a membrane subunit for reduction of the quinone pool. In these organisms, Qrc acts as a menaquinone reductase with electrons from periplasmic hydrogen or formate oxidation. Binding of a menaquinone analogue affects the EPR spectrum of the [3Fe-4S]$^{1+/0}$ cluster, indicating the presence of a quinone-binding site close to the periplasmic subunits. Qrc is the first respiratory complex from sulfate reducers to have its physiological function clearly elucidated.

## 3.1.2 Introduction

Respiratory chains rely on membrane-bound protein complexes to perform a stepwise transduction of the free energy of redox reactions into an electrochemical potential difference across the membrane that drives the synthesis of ATP. In aerobic eukaryotic organisms, a fixed and mostly linear respiratory chain is present, in contrast to bacteria and archaea whose flexible and usually highly branched respiratory chains, reflect their ability to use an array of different electrons donors and acceptors (Richardson, 2000). This flexibility enables prokaryotes to explore virtually every possible energy source and oxidant pairs that can provide energy to sustain life, and thus colonize multiple habitats on Earth, including the most extreme environments. In addition, the recycling of elements provided by bacterial energy metabolism is a crucial component for the maintenance of life in our planet (Falkowski *et al.*, 2008). The study of bacterial respiratory complexes (BRC) has highlighted the diversity of mechanisms by which energy conservation can be achieved (Jormakka *et al.*, 2003; Simon *et al.*, 2008). BRC usually have simpler compositions than their eukaryotic counterparts and are therefore useful models to understand the molecular mechanisms involved in those processes. Furthermore, bacteria contain many unique BRC, whose architecture has a highly modular character, and with different arrangements of modules giving rise to different proteins and physiological functions (Baymann *et al.*, 2003; Rothery *et al.*, 2008).

An example of a novel BRC has recently been described, which is a functional substitute for the $bc_1$ complex, or complex III (Pereira *et al.*, 1999; Pereira *et al.*, 2007b; Yanyushin *et al.*, 2005). This complex, named Alternative Complex III (ACIII), is found in many groups of bacteria whose genome lacks a complex III but includes heme-copper oxygen reductases that receive electrons from soluble periplasmic proteins (thus requiring the presence of a protein to transfer electrons from the quinone pool to these proteins). The function of ACIII as a quinol:periplasmic electron acceptor oxidoreductase has recently been demonstrated with the complex from *Chloroflexus aurentiacus* (Gao *et al.*, 2009), whereas in *Rhodothermus marinus* it was shown to transfer electrons directly to an oxygen reductase (Refojo *et al.*, 2010). The ACIII complex includes subunits with homology to those of the complex iron-sulfur molybdoenzyme (CISM) family (Rothery *et al.*, 2008), which are widespread bacterial oxidoreductases such as formate dehydrogenase or nitrate-, dimethyl sulfoxide-, thiosulfate-, polysulfide and tetrathionate reductases. Many enzymes in this family are part of anaerobic respiratory chains and have a typical three-subunit composition consisting of a catalytic subunit with a molybdopterin cofactor, an electron transferring subunit with four Fe-S centers and an integral membrane subunit that anchors the protein complex to the membrane and is involved in electron exchange with the quinone pool. The subunit composition of ACIII is more complex and includes four integral membrane proteins, two cytochromes *c*, and a large periplasmic protein with two domains, homologous to the Fe-S cluster and catalytic subunits of the

CISM enzymes. However, no molybdenum cofactor is present in the so far described ACIII complexes of *R. marinus* and *C. aurantiacus*, in agreement with its function as an electron transfer complex (Pereira *et al.*, 2007b; Yanyushin *et al.*, 2005).

A distinct BRC, related to ACIII but with a simpler subunit composition, was described to be present in the sulfate reducing bacteria *Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans* (Yanyushin *et al.*, 2005). Sulfate reduction is a widespread process in anaerobic habitats and has a very high environmental significance, being a major contributor to the sulfur and carbon cycles (Muyzer and Stams, 2008). Yet, it is one of the last few major biological processes which remain to be elucidated at a molecular level. The respiratory chain of sulfate-reducing bacteria (SRB) is very interesting and unusual due to the presence of several unique components, namely several BRC that are distinct from those found in other organisms (Pereira, 2008). The SRB complexes described to date are either involved in oxidation of the menaquinol pool or direct transmembrane electron transfer. No complex has yet been described in SRB with the function of reducing the quinone pool. Here, we describe the isolation and characterization of a new complex from *Desulfovibrio vulgaris* Hildenborough and show that it functions as a Type I cytochrome $c_3$:menaquinone oxidoreductase. We propose to name this complex as Quinone-reductase complex (Qrc), encoded by the operon *qrcABCD*. The *D. vulgaris* QrcABCD complex is the first described example of a new family of BRC that is related to the ACIII complex, but performs the reverse function of this, by reducing the menaquinone pool with

electrons transferred from a periplasmic cytochrome *c*. We show also that Qrc is efficiently reduced by periplasmic hydrogenase and formate dehydrogenase *via* the cytochrome $c_3$, and thus acts as the linker to reduce the quinone pool during growth on $H_2$ or formate. An essential role of the *qrcB* gene (previously designated as *mopB*) for growth of *D. desulfuricans* G20 in hydrogen or formate with sulfate has recently been described (Li *et al.*, 2009). The involvement of the Qrc complex in sulfate respiration is discussed within the context of genome data available for SRB.

## 3.1.3 Material and Methods

### Bacterial growth and protein purification

*Desulfovibrio vulgaris* Hildenborough (DSM 644) was grown in lactate/sulfate medium as previously described (Postgate, 1984). The cells were suspended in 20 mM Tris-HCl buffer at pH 7.6 and ruptured by passing twice through a APV 750 homogeneizer. The resulting extract was centrifuged at a force of 10000*g* for 15 min to remove cell debris, and the supernatant was centrifuged at a force of 100000*g* for 2 h in order to obtain the crude membrane fraction. The pellet was washed by suspending it in 20 mM Tris-HCl pH 7.6 with 10 % glycerol (v/v) and 1 mM EDTA and centrifuged at 140000*g* for 2 h. The washed pellet was solubilized in 20 mM Tris-HCl pH 7.6 containing 10 % glycerol (v/v) and 2 % n-dodecyl-β-D-maltoside (DDM) (w/v) in the presence of Complete protease inhibitor cocktail tablets (Roche Diagnostics). Solubilization was carried out for 2 h in an ice bath with gentle stirring. The

solubilized proteins were separated by ultracentrifugation at 140000*g* for 2 h, and the pellet was used for a second solubilisation using the same conditions as described before. All chromatographic steps were performed at 4 ºC. The purifications were followed using UV-vis spectroscopy, BN- and SDS-PAGE. The solubilized proteins were applied to a fast flow Q-Sepharose column equilibrated with 20 mM Tris-HCl buffer pH 7.6 with 10 % glycerol, 0.1 % DDM (Buffer A), and a quarter of a Complete™ tablet per liter. A stepwise gradient of increasing NaCl concentration was performed, and the fractions eluting at approximately 250 mM NaCl were concentrated and their ionic strength was lowered. This sample was subjected to a second passage on a Q-Sepharose HP using the same protocol. The heme-containing fractions were concentrated and loaded in an IMAC Sepharose HP column, saturated with $Ni^{2+}$, and equilibrated with Buffer A with 0.4 M NaCl. A stepwise gradient was performed with imidazole to elute a fraction containing the Qrc complex at 50 mM. The [FeFe] and [NiFeSe] hydrogenases, and TpI$c_3$ were isolated from *D. vulgaris* by established procedures. The FdhAB formate dehydrogenase from this organism was purified inside the anaerobic chamber following its catalytic activity.



## Biochemical analysis

Protein concentration, BN- and Tricine-SDS-PAGE, N-terminal protein sequence analysis, and covalent and non-covalent heme content determination was performed as described (Pires *et al.*, 2006). Molybdenum and tungsten were analyzed by inductively coupled plasma (ICP). The pterin cofactor extraction and

fluorescence analysis was performed according to (Sebban *et al.*, 1995). Determination of the molecular mass of the complex was performed by gel filtration in a Superdex 200 column, and of the subunits in a MALDI-TOF spectrometer.

### Spectroscopic techniques

Room temperature UV-visible spectra were obtained in a Shimadzu UV-1603 or UV-1203 spectrophotometer inside the anaerobic chamber. Fluorescence measurements were recorded on a Cary Varian Eclipse instrument. EPR spectra were recorded on a Bruker EMX spectrometer, equipped with an ESR-900 continuous flow helium cryostat.

### Redox titration

Potentiometric titrations were monitored by visible spectroscopy in 50 mM Tris-HCl and 0.1 % DDM pH 7.6 as buffer, with 1 μM of purified protein and 2 μM of the a mixture of redox mediators at 20 ºC. It was followed the changes in absorbance at the α and Soret bands of the reduced hemes, corrected for the corresponding isosbestic points, and using buffered sodium dithionite (pH 8.5) as a reductant. The mixture of redox mediators included: 1,2-naphthoquinone, phenazine methosulfate, phenazine ethosulfate, methylene blue, indigo tetrasulfonate, indigo disulfonate, 2-hydroxy-1,4-naphthoquinone, safranine, antraquinone-2-sulfonate, neutral red, benzyl viologen and methyl viologen. The reduction potentials were measured with a combined Ag/AgCl electrode calibrated against a saturated quinhydrone solution at pH 7 and referenced to the standard hydrogen electrode.

### Quinone interaction experiments

All experiments were performed in an anaerobic chamber (95 % $Ar_2$, 5 % $H_2$). The reduction of the Qrc complex with quinols was measured following the heme reduction at the Soret band. The buffer used in all cases was 20 mM Tris-HCl pH 7.6 plus 5 mM EDTA and 0.1 % DDM. $DMNH_2$ and menaquinol-4 (vitamin K2) were prepared from the respective quinones in ethanol, by reduction with metallic zinc in 5 M HCl. Menadiol was reduced from menadione according to (Pires *et al.*, 2006) and also solubilized in ethanol. For the reverse reaction the complex was reduced with a sub-stoichiometric amount of dithionite and an ethanolic solution of DMN, menadione or menaquinone-4 were added as electron acceptors. As a control experiment, the reduced protein was treated with an equal volume of ethanol.

To test the effect of HQNO by EPR a concentrated solution of HQNO in ethanol was added to a final concentration of 0.5 mM. In a control sample the same volume of ethanol was added.

### *In vitro* electron transfer assays

The assays were performed inside the anaerobic chamber. It was tested if the purified [FeFe] and [NiFeSe] hydrogenases, as well as FdhAB formate dehydrogenase were able to reduce the Qrc. Hydrogenases were first activated under $H_2$ overpressure, and when applicable incubated with TpI$c_3$ at room temperature for 10 min before being added to the Qrc in the cuvette. Fdh was activated by incubation with 20 mM sodium formate and 1 mM DTT for 5 min. In every case the enzyme activity was

first tested with methyl viologen. The assay mixture contained 50 mM Tris-HCl pH 7.6, which was $H_2$-saturated in the case of hydrogenase assays, and contained 1 mM dithiothreitol for Fdh assays. Qrc was present at 0.6 μM, and the enzymes and cytochromes were added in catalytic amounts (5 nM). Qrc reduction was measured by following the increase in absorption at 419 nm, and sodium dithionite was added in the end of each assay to verify the complete reduction of Qrc.

### Cytochrome TplC$_3$:menaquinone-4 oxidoreductase assay

The electron transfer activities were measured at 25 ºC inside an anaerobic glove box, following the oxidation of TplC$_3$ at 552 nm (absorption coefficient: 116 $mM^{-1}$ $cm^{-1}$), in 50 mM phosphate buffer pH 7.3, 0.01 % DDM. TplC$_3$ was first reduced stepwise, but not completely (to prevent excess of reductant) with a diluted dithionite solution (~1 mM). The reaction was started by addition of Qrc (100 nM) and menaquinone-4 to the reduced TplC$_3$ in the cuvette. The unspecific oxidation of TplC$_3$ by menaquinone-4 was subtracted to give the final activity. Reaction rates were determined from the initial linear oxidation rate. Kinetic parameters ($K_m$ and $V_{max}$) were determined by nonlinear curve fitting using Hyper32 software (University of Liverpool).

### Sequence analysis tools

Blast searches were performed at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Genome sequence data were retrieved and analyzed at the Integrated Microbial Genomes website (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi),

the Comprehensive Microbial Resource website (http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi) and VIMSS Comparative Genomics website (http://www.microbesonline.org/). Multiple alignments were performed using CLUSTALX. Sequence analysis and transmembrane helix predictions were done using programs available at http://us.expasy.org/.

### 3.1.4 Results

The only BRC that has been characterized so far from *D. vulgaris* is the Tmc complex (Pereira *et al.*, 2006). To further investigate the respiratory chain of this model organism, membrane protein complexes were analyzed by BN-PAGE after separation on ion-exchange chromatography.

A major band was observed at ~230 kDa, which stained positive for hemes *c* (**Figure 3.1.1 A**). This band is not present in similar analysis of membrane extracts from *D. desulfuricans* ATCC 27774, but its high abundance indicates it is one of the major proteins in the membranes of *D. vulgaris*. After optimization of a purification protocol the new complex could be obtained in a pure form. Tricine-SDS-PAGE revealed the presence of four subunits with apparent molecular masses of 70, 38, 33 and 29 kDa (**Figure 3.1.1 B**). The 29-kDa band stains poorly with Coomassie Blue, but is well visible with heme-staining. When the sample was boiled before SDS-PAGE the 38-kDa band is no longer visible, strongly indicating that this band corresponds to a hydrophobic membrane protein. A two-dimensional gel confirmed that the four subunits are present in the BN-PAGE ~230 kDa band (**Figure 3.1.1 C**). In order

to identify the new complex in the *D. vulgaris* genome, N-terminal sequences were obtained for the four subunits. The 70- and 33-kDa bands gave clear results (ALDRR and SSFKEF) which enabled their assignment as the products of genes DVU0694 and DVU0693 that are part of a predicted 3-gene operon in *D. vulgaris* (DVU0692-0694). The N-terminal sequences of the 38- and 29-kDa bands gave very weak signals, suggesting they were blocked. However, several amino acid residues of the N-terminus of the membrane protein DVU0692 could be identified.

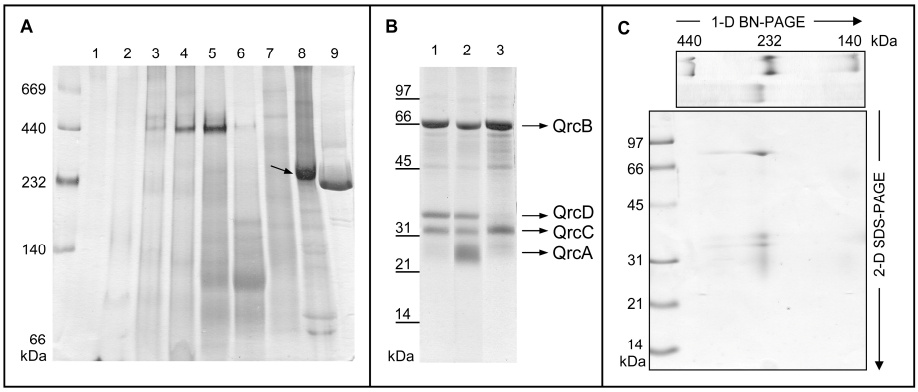


**Figure 3.1.1** – Gel electrophoresis of the Qrc complex from *D. vulgaris*. A) BN-PAGE (5-15 %) of fractions 1 to 9 from the first purification step, stained for hemes over the Coomassie Blue. The arrow points to the Qrc band. B) Tricine-SDS-PAGE of the purified Qrc (20 μg) stained with Coomassie Blue. 1, stained with Coomassie Blue; 2, as 1, but also stained for hemes *c*; 3, boiled sample. C) a two dimensional (2-D) BN/Tricine-SDS-PAGE gel of Qrc. 1-D, one-dimensional.

Since the isolated complex contains four subunits we reanalyzed the DNA sequence corresponding to the two neighbouring genes DVU0695 and DVU0696, which are annotated as hypothetical proteins. This analysis showed that a

gene (which we named 0695*) encoding for a 23-kDa multiheme cytochrome is found upstream from DVU0694. Several of the predicted N-terminal amino acids of DVU0695* are present in the N-terminal sequence analysis of the 29-kDa band (ME_RQL), confirming this assignment and showing that the gene cluster (**Figure 3.1.2 A**) encodes the four subunits present in the isolated complex. The molecular mass of the complex determined by gel filtration is 240 ± 25 kDa. This agrees with a predicted stoichiometry of 1:1:1:1 for the QrcABCD subunits (theoretical molecular mass 180 kDa), based on analogy with the CISM family, with the difference being accounted for by the detergent micelle.



## Sequence analysis of the *D. vulgaris* qrcABCD genes

The *D. vulgaris* gene cluster DVU0692-0695* was previously identified by Yanyushin and co-workers as encoding a new class of bacterial oxidoreductase that is related to the ACIII (Yanyushin *et al.*, 2005). A number of factors, discussed below, clearly distinguish the ACIII complex from the one described here. Given these differences and the fact that they perform distinct physiological functions we opted to name the new complex as Quinone-reductase complex (Qrc), and the genes as *qrcABCD*. The QrcABCD complex is present in the genomes of several organisms, which strikingly are all sulfate-reducing *Deltaproteobacteria* (with one exception, *Solibacter usitatus*, which likely acquired it through lateral gene transfer). A schematic representation of the QrcABCD complex, based on sequence analysis and the characterization described below is shown in Figure 3.1.2 B.

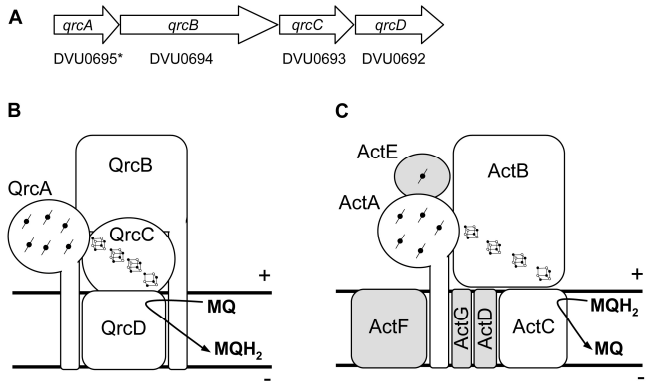


**Figure 3.1.2** – The Qrc and ACIII complexes. A) The *qrc* gene cluster in *D. vulgaris* (DVU0692-0695*). B) Schematic representation of the *D. vulgaris* Qrc complex and C) the related ACIII. Similar subunits in the two complexes are in white (ActB is a fusion of QrcB- and QrcC-like proteins). MQ, menaquinone; $MQH_2$, menaquinol.

The *qrcA* gene (DVU0695*) encodes a 23-kDa cytochrome *c* with six heme binding motifs. The N-terminal analysis of this protein shows that the Sec-translocating signal peptide is not cleaved and forms a transmembrane helix that probably serves as a membrane anchor. The closest homologues of *qrcA* are the *actA* genes that are part of the ACIII complexes (Pereira *et al.*, 2007b; Yanyushin *et al.*, 2005). Most QrcA and ActA proteins include only five heme binding sites, and there are other five strictly conserved residues (four His and one Met) that are the probable distal ligands of the five hemes. Binding of six hemes to *D. vulgaris* QrcA was confirmed by mass spectrometry (see below). Sequence analysis indicates that both QrcA and ActA proteins form a separate group when compared to other multiheme *c* cytochromes such as NrfA, NrfB, NapC/NrfH, cytochromes $c_3$, hydroxylamine oxidoreductase, cytochrome $c_{554}$ or octaheme cytochromes (data not shown).

The genes *qrcBCD* are related to the genes of the three subunit CISM molybdoenzyme family (Rothery *et al.*, 2008). The *qrcB* gene (DVU0694) encodes a 72-kDa protein similar to the molybdo-*bis*(pyranopterin guanidine dinucleotide) cofactor-containing catalytic subunit of CISM, and includes a twin-arginine signal peptide indicating it is periplasmic. Similarly to QrcA, the signal peptide is still present in the mature protein and likely serves as a membrane anchor. The *qrcC* gene (DVU0693) encodes a 29-kDa iron-sulfur protein that belongs to the family of four cluster proteins. These are electron-transferring subunits found in many respiratory enzymes such as CISM, and contain four cubane iron-sulfur clusters (FS1-FS4). The clusters form a pathway for electron transfer between the membrane quinone pool and the catalytic center, as described in the structures of Fdh-N formate dehydrogenase, Nar nitrate reductase and Psr polysulfide reductase (Bertero *et al.*, 2003; Jormakka *et al.*, 2002; Jormakka *et al.*, 2008). Sixteen Cys involved in binding of four iron-sulfur clusters are conserved in QrcC. Although QrcC has no signal peptide, it should be co-translocated with QrcB as it happens with CISM enzymes. The *qrcD* gene (DVU0692) encodes a 48-kDa integral membrane protein with ten transmembrane helices, which belongs to the family of CISM proteins that interact with the quinone pool without the involvement of hemes *b*, such as NrfD or PsrC (Simon and Kern, 2008).

The QrcB and QrcC proteins are most closely related to the two domains of the ActB subunit of ACIII that probably resulted from a gene fusion event, and QrcD to the two ActC and ActF subunits of ACIII, which are very similar and resulted from a

gene duplication event. However, a dendogram of the known sequences of QrcB, domain 1 of ActB and other proteins in the family shows clearly that QrcB forms a separate cluster from ActB (**Figure A.1** in Appendix A), even though the two proteins have a common origin as previously proposed (Yanyushin *et al.*, 2005). This is also the case for QrcC, domain 2 of ActB and related proteins (data not shown). Thus, sequence analysis indicates unambiguously that QrcB, QrcC and QrcD constitute novel groups within their respective families. In *Geobacter metallireducens* and *Thermus thermophilus* the corresponding operons encode ACIII and not Qrc complexes as previously suggested (Yanyushin *et al.*, 2005). Interestingly, in *G. metallireducens, G. uraniumreducens* and *Mariprofundus ferrooxydans* the two domains of ActB are encoded by two separate genes, as it happens in the Qrc complex, and these are the ACIII sequences most closely related to Qrc subunits.

Several of the catalytic subunits of CISM molybdoenzymes include a [4Fe-4S]$^{2+/1+}$ center named FS0 that is involved in electron transfer between the molybdopterin cofactor and the Fe-S centers of the four-cluster protein. Curiously, the four Cys that bind the FS0 cluster are only present in some QrcB proteins (but not in *D. vulgaris*) and in some ActB proteins (*e.g. G. metallireducens).* The loss of the FS0 center in several QrcB and ActB proteins agrees with the fact that a molybdopterin cofactor is not present in these proteins (see below).

## Characterization of the Qrc redox centers

We first tested whether a molybdenum (or tungsten) pterin cofactor is present in the Qrc complex, since this is absent in ACIII complexes (Pereira *et al.*, 2007b; Yanyushin *et al.*, 2005). No Mo or W were detected in the Qrc complex by ICP, and no Mo or W signals were observed by EPR spectroscopy (see below). Attempted extraction of a pterin group followed by fluorescence analysis was also negative. This shows that the QrcB protein does not bind a molybdo-*bis*(pyranopterin guanidine dinucleotide) cofactor. Besides QrcB and ActB proteins, there is a third example of a protein belonging to the family of molybdopterin oxidoreductases, which also does not contain the Mo cofactor: the Nqo3/NuoG subunit of the respiratory complex I (NADH:quinone oxidoreductase), which includes a molybdopterin oxidoreductase-like domain of unknown function (Sazanov and Hinchliffe, 2006). Using sequence analysis of QrcB, ActB and Nqo3 *versus* other molybdopterin oxidoreductases for which the structure is known, we could not identify sequence features that could explain the absence of the pterin due to a low level of sequence identity between the different families. The UV-visible spectrum of the Qrc complex confirms the presence of only hemes *c* (**Figure 3.1.3**). The absence of hemes *b* was confirmed by the pyridine hemochrome assay and HPLC analysis of extracted non-covalently bound hemes. Mass spectrometry of the Qrc complex subunits gave a mass of 27,450 Da for the QrcA protein, which confirms that six hemes are covalently bound. The macroscopic redox potentials of the hemes were determined through a redox titration monitored by visible

spectroscopy following the Soret and α bands, at pH 7.6 (**Figure 3.1.3 inset**). The hemes started to be reduced at ~+150 mV and were fully reduced at ~-250 mV. This broad potential range reflects the presence of multiple heme centers with overlapping redox potentials, and the contribution of individual hemes cannot be resolved.

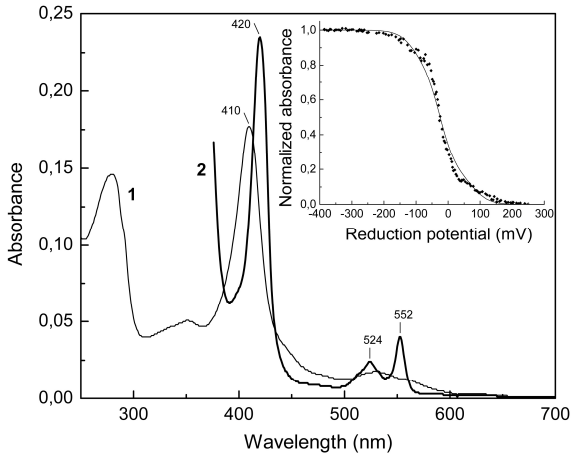


**Figure 3.1.3** – UV-vis spectra of the Qrc complex. Oxidized (1) and reduced (2) form. *Inset*: Redox titration to the Qrc hemes followed by UV-visible spectroscopy at 420 nm; the solid line corresponds to a theoretical simulation with the sum of six Nernst equations with redox potentials of +80, -25 and -110 mV in a ratio of 1:4:1.

The experimental points were simulated with the sum of six Nernst equations with midpoint redox potentials of +80, -25 and -110 mV (± 10 mV) in a 1:4:1 ratio, without taking into account possible redox interactions. These redox potentials give an indication of the macroscopic redox behaviour of the system and are not individual heme potentials. The first heme to be reduced is likely to correspond to the His/Met bound heme

and the others to the bis-His hemes, which typically have lower redox potentials.

The Qrc cofactors were also studied by EPR spectroscopy. The spectrum of the complex in the oxidized state shows a strong signal at g~2.01 typical of $[3Fe\text{-}4S]^{1+}$ clusters (**Figure 3.1.4 A**) as observed in NarGHI (Guigliarelli *et al.*, 1992; Rothery *et al.*, 1998) or FrdABCD fumarate reductase (Manodori *et al.*, 1992), both of which contain a $[3Fe\text{-}4S]^{1+/0}$ cluster. A signal corresponding to a $[3Fe\text{-}4S]^{1+/0}$ cluster is also present in the *R. marinus* ACIII complex (Pereira *et al.*, 1999). In addition, there are several overlapping signals for low-spin ferric hemes with $g_{max}$ of 3.21, 2.93 and 2.82, and $g_{med}$ of ~2.24 (**Figure 3.1.4 B**). These three signals can be well simulated with a stoichiometry of 3:2:1 (**Figure 3.1.4 B2**). The intensity of the g~2.01 signal relative to the heme signals was constant in several preparations, and was also estimated by spectral simulation giving a relative proportion of one $[3Fe\text{-}4S]^{1+/0}$ center for six low-spin hemes, in agreement with the sequence-based predictions. At high temperatures, no signals attributable to Mo or W species were detected. Reduction with ascorbate leads to almost complete reduction of the $[3Fe\text{-}4S]^{1+/0}$ signal and partial reduction of the low-spin hemes (**Figure 3.1.4 B**).

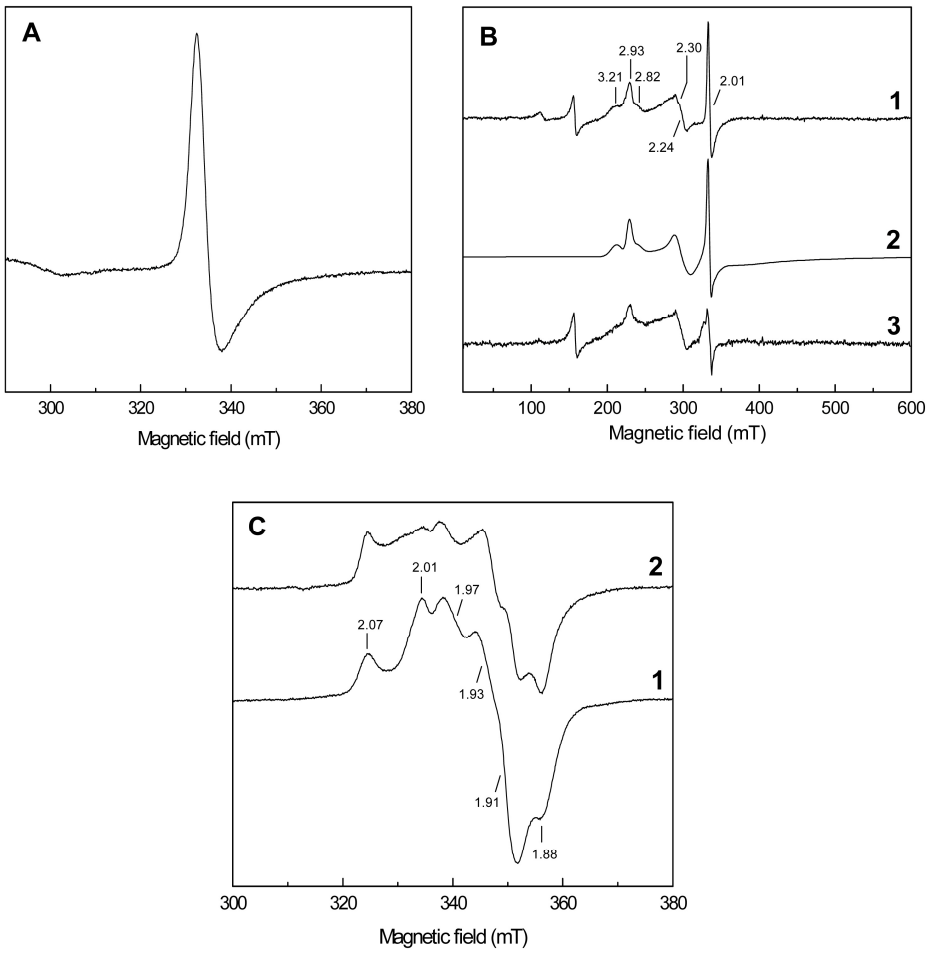


**Figure 3.1.4** – EPR spectra of Qrc complex. A) The $[3Fe\text{-}4S]^{1+}$ signal present in the oxidized sample. B) Low-spin heme signals present in the oxidized sample (1); theoretical simulation obtained by the sum of four components (three low-spin hemes and one $[3Fe\text{-}4S]^{1+}$ center) in a 3:2:1:1 ratio (hemes: $g_a$=3.16, 1.9, 1.3; $g_b$=2.93, 2.21, 1.5; $g_c$=2.825, 2.28, 1.6; and $[3Fe\text{-}4S]^{1+}$ center: g=2.01, 2.01, 1.2) (2); after reduction with ascorbic acid 0.2 M (3). C) Spectrum after dithionite reduction with some indicative g values shown for reference (1); and after reduction with 0.5 μM [NiFeSe] hydrogenase/0.5 μM TpI$c_3$ in the presence of $H_2$ (2). Spectra were recorded under the following conditions: microwave frequency, 9.38 GHz; microwave power, 20.1 mW (A) and 2.4 mW (B and C); modulation frequency, 100 kHz; modulation amplitude, 1 mT; temperature, 10 K.

Upon dithionite reduction a complex signal appears with features at 2.07, 2.01, 1.97, 1.93, 1.91 and 1.88 (**Figure 3.1.4 C**), in agreement with the presence of several reduced $[4Fe\text{-}4S]^{2+/1+}$ clusters in QrcC. The shape of the signal is indicative of magnetic interactions between the several centers, as it has been described for this protein family, where the Fe-S clusters are within close distance of each other (Guigliarelli *et al.*, 1992; Rothery *et al.*, 1998; Rothery and Weiner, 1996).

Overall, the spectral characterization indicates that all the cofactors predicted from the Qrc gene sequences (6 hemes *c* and four cubane clusters) are present in the isolated complex, with one of these clusters being a $[3Fe\text{-}4S]^{1+/0}$ one. Furthermore, no hemes *b* are present in the QrcD membrane subunit.



## Physiological function of the Qrc Complex

Given the similarity of Qrc to the ACIII complex it was first tested whether it could have a similar function of oxidizing menaquinol and reducing a periplasmic protein (Gao *et al.*, 2009). Initially, the reduction of Qrc with two menaquinol analogues (menadiol, $DMNH_2$) and with menaquinol-4 (vitamin K2) was tested in anaerobic conditions, but no reduction of the hemes *c* was observed even with large excess of the quinols. However, the opposite reaction *i.e.* oxidation of reduced Qrc complex by the menaquinones was extremely fast, suggesting that Qrc could function preferably with the reverse activity of ACIII, by acting as reducer of the menaquinone pool. This agrees with the fact that the redox potentials displayed by the hemes in *D. vulgaris* Qrc are significantly lower than those reported for *R. marinus* ACIII (+235 mV, 2 hemes; +80 mV,

1 heme; -45 mV, 2 hemes) (Pereira *et al.*, 1999). Furthermore, no complex in SRB has been positively identified to act as a menaquinone reductase, whose presence is essential when they are growing on hydrogen, since this is oxidized by periplasmic hydrogenases that in many organisms, namely *Desulfovibrio* spp., do not contain a cytochrome *b* subunit for transferring electrons to the quinone pool, but rather reduce the periplasmic Type I cytochrome $c_3$ (TpI$c_3$) (reviewed in (Matias *et al.*, 2005; Pereira *et al.*, 2007a)). A similar situation is observed for the periplasmic formate dehydrogenases. Thus, the Qrc complex could act to transfer electrons from the reduced TpI$c_3$ to the menaquinone pool. To test this hypothesis we first checked whether the Qrc complex could be reduced by a periplasmic hydrogenase and formate dehydrogenase of *D. vulgaris* Hildenborough, directly or via the TpI$c_3$. With the [FeFe] hydrogenase direct reduction of the Qrc hemes in the presence of $H_2$ was negligible, but increased dramatically when catalytic amounts of TpI$c_3$ were present (**Figure 3.1.5 A**). A similar result was observed with the [NiFeSe] hydrogenase. With the FdhAB formate dehydrogenase and formate, direct reduction of Qrc was also quite slow, and a fast rate was observed in the presence of TpI$c_3$ (**Figure 3.1.5 B**). Using the monohemic cytochrome $c_{553}$ (a proposed electron acceptor of formate dehydrogenase in *Desulfovibrio* (Sebban *et al.*, 1995)) no catalytic effect was detected, and with the two cytochromes together the rate of reduction was equivalent to that obtained for the TpI$c_3$ alone.

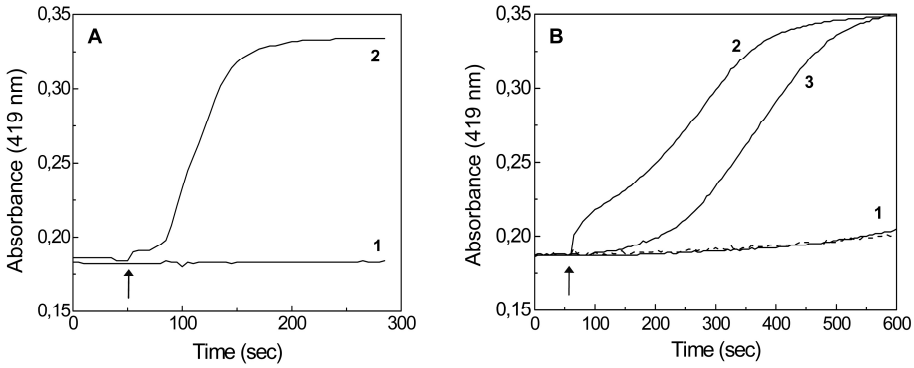


**Figure 3.1.5** – Reduction of Qrc with periplasmic proteins. A) Reduction of Qrc with [FeFe] hydrogenase under $H_2$, in the absence (1) or presence of TpI$c_3$ (2). B) Reduction of Qrc by FdhAB and formate in the absence of any cytochrome (1, dashed line) or in the presence of cytochrome $c_{552}$ (1, solid line), TpI$c_3$ (2), or both cytochromes (3). The arrow indicates the time of enzyme/cytochrome addition. Reduction was monitored by the increased absorption at 419 nm.



Reduction of the Qrc Fe-S clusters by hydrogenase/TpI$c_3$/$H_2$ was also checked by EPR (**Figure 3.1.4 C**), which showed that all the hemes, the [3Fe-4S]$^{1+/0}$ cluster and at least two of the [4Fe-4S]$^{2+/1+}$ clusters of the Qrc complex are reduced. These results show that reduction of the Qrc complex by the TpI$c_3$ indeed occurs, and that this complex can receive electrons from oxidation of either periplasmic $H_2$ or formate.

Based on these experiments, we next tested whether Qrc could catalyze the reduction of menaquinone from TpI$c_3$. For this activity we used menaquinone-4 as substrate and followed the oxidation of reduced TpI$c_3$ in strictly anaerobic conditions. The direct oxidation of reduced cytochrome $c_3$ by menaquinone-4 was very slow, but the Qrc complex did indeed catalyze this reaction (**Figure 3.1.6 A**). A kinetic analysis of the TpI$c_3$:menaquinone activity of Qrc showed that it followed Michaelis-Menten kinetics with a maximal activity of

92 nmol TpI$c_3$/min/mg (16.6 $min^{-1}$, assuming a molecular mass of 180 kDa) and a $K_m$ of 0.8 μM for the cytochrome and 4.0 μM for menaquinone-4 (**Figure 3.1.6 B** and **C**).

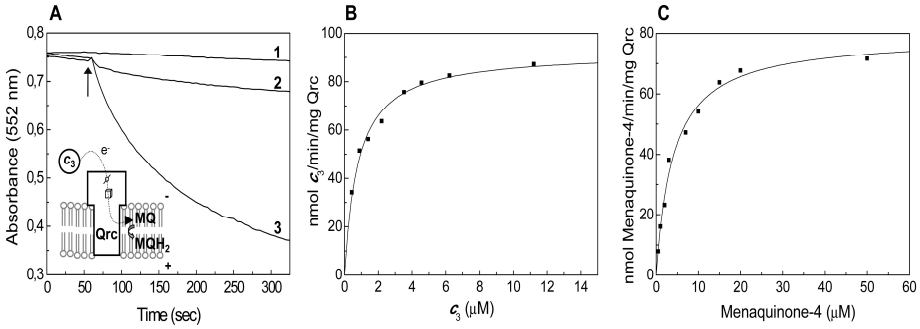


**Figure 3.1.6** – Catalytic activity of the Qrc complex. A) Oxidation of TpI$c_3$ (7 μM) after addition of menaquinone-4 (20 μM) (1), Qrc (2), or both (3). The arrow indicates the time of menaquinone-4 and/or Qrc addition. Reaction rates of the Qrc TpI$c_3$:menaquinone-4 oxidoreductase activity varying the cytochrome concentration with 20 μM menaquinone-4 (B), or the quinone concentration with 3.5 μM TpI$c_3$ (C). Each point corresponds to the initial linear rate and the solid line is the Michaelis-Menten fitted curve obtained with nonlinear regression analysis. MQ, menaquinone; $MQH_2$, menaquinol.

Overall, these results confirm that the Qrc complex is a menaquinone reductase and provide the first functional characterization of this novel family.

## The Qrc complex quinone binding site

The location of the quinone-binding site relative to the membrane is a crucial aspect of quinone-interacting proteins, as the site of proton release or proton uptake upon quinone oxidation or reduction may be crucial to determine whether the reaction will be electrogenic or not (Jormakka *et al.*, 2003; Simon *et al.*, 2008). Since there are no redox cofactors bound to the membrane QrcD subunit, it seems most plausible that

the quinone-binding site of Qrc will be situated on the periplasmic side of the membrane, within suitable distance for electron-transfer from the Qrc redox centers. In *E. coli* fumarate reductase and in a mutant of DMSO reductase (where the [4Fe-4S] FS4 cluster has been turned into a [3Fe-4S] one), a close proximity between the quinone-binding site in the membrane subunit and the FS4 cluster has been observed through the effect of HQNO, a menasemiquinone analogue, on the EPR signal of the $[3Fe\text{-}4S]^{1+}$ center (Cheng *et al.*, 2005; Rothery *et al.*, 2005; Rothery and Weiner, 1996). We performed a similar experiment and incubated the Qrc complex with HQNO before analysis by EPR. Relative to a control to which only ethanol was added, we observed no difference in the heme signals of the HQNO-treated sample, but an effect was detected in the g value of the $[3Fe\text{-}4S]^{1+}$ signal, which shifted from 2.010 to 2.024 (**Figure 3.1.7**).



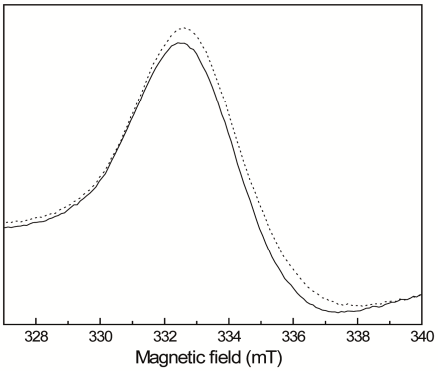


**Figure 3.1.7** – Effect of HQNO on the $[3Fe\text{-}4S]^{1+}$ center of Qrc. EPR spectra of the Qrc $[3Fe\text{-}4S]^{1+}$ center in the absence (solid line) and in the presence of 0.5 mM HQNO (dotted line). In a control sample to which only ethanol was added no effect was observed. Spectra were recorded under the following conditions: microwave frequency, 9.38 GHz; microwave power, 20.1 mW; modulation frequency, 100 kHz; modulation amplitude, 1 mT; temperature, 10 K.

This effect is of the same order of magnitude as that observed for the mutated DMSO reductase (Cheng *et al.*, 2005; Rothery and Weiner, 1996), and indicates that the menaquinone binding site of the Qrc complex is within close distance of the [3Fe-4S]$^{1+}$ center of the QrcC subunit, and thus located on the periplasmic side of the membrane.

### 3.1.5 Discussion

We report here the isolation and detailed characterization of a new membrane complex from *D. vulgaris*, which constitutes the first example of a novel family of BRC belonging to the superfamily of CISM proteins (Rothery *et al.*, 2008). This complex named QrcABCD (for quinone-reductase complex) is a major component among membrane proteins in *D. vulgaris*. The QrcABCD complex is composed of four subunits: a multiheme cytochrome *c*, and three subunits related to the subunits of CISM family, including a protein related to the molybdopterin-containing subunit (but which has no molybdenum cofactor), an electron-transfer four cluster protein, and an integral membrane protein. These subunits show highest similarity to some of the subunits of ACIII, a recently described family of BRC (Gao *et al.*, 2009; Pereira *et al.*, 2007b; Yanyushin *et al.*, 2005). ACIII is widespread in bacteria that lack a $bc_1$ complex, but only two examples have been characterized so far, from *R. marinus* (Pereira *et al.*, 1999; Pereira *et al.*, 2007b) and *C. aurantiacus* (Gao *et al.*, 2009; Yanyushin *et al.*, 2005). Besides the different composition, there are several factors that indicate that the Qrc complex should have a different physiological function from the

ACIII complex: i) in most organisms the ACIII genes are found next to genes coding for heme copper cytochrome oxidases (which will oxidize the cytochrome *c*, or another periplasmic protein, reduced by ACIII), whereas this is never observed for Qrc genes; ii) several organisms (*e.g. Desulfohalobium retbaense* or *Syntrophobacter fumaroxidans*) have a Qrc complex, but lack genes coding for a cytochrome oxidase; iii) at least one organism, *Desulfobacterium autotrophicum* HRM2 has genes coding for both Qrc and ACIII complexes, with only the latter being next to genes coding for an oxidase. In addition, sequence analysis clearly indicates that the Qrc proteins form a separate group from the related ACIII proteins. Yanyushin *et al.* proposed that the ACIII complex arose from Qrc by the acquisition of additional subunits. In this respect it is interesting to note that in the ACIII complex of the *Deltaproteobacteria Geobacter* spp. the molybdopterin-like subunit and the Fe-S subunit are not fused as in other ACIII, but are encoded by separate genes as in Qrc, supporting the evolutionary link between the two complexes. The Qrc complex may in turn have evolved from a CISM enzyme by acquisition of a cytochrome *c* subunit and loss of the molybdopterin cofactor. It is thus a most interesting evolutionary cross point in the CISM family, in which one function has been lost while another is evolving.

Our results show that the Qrc complex is a cytochrome $c_3$:menaquinone oxidoreductase, whereas ACIII oxidizes menaquinol. These contrasting functions agree with the lower redox potentials observed for the hemes of QrcA relative to *R. marinus* ActA (Pereira *et al.*, 1999), as in Qrc the electrons are transferred from the low-redox potential hemes of TpI$c_3$

(-325 to -170 mV) to menaquinone (-70 mV), whereas in ACIII they go from menaquinol to a high potential cytochrome *c*. This study provides the first spectroscopic characterization of the redox centers from a Qrc complex, which is an important contribution since only a few CISM proteins have been studied in detail (Rothery *et al.*, 2008) and only partial characterization of the redox centers is available for one ACIII (Pereira *et al.*, 1999; Pereira *et al.*, 2007b). We obtained evidence for the presence of six hemes *c*, one $[3Fe\text{-}4S]^{1+/0}$ and three $[4Fe\text{-}4S]^{2+/1+}$, but no molybdopterin cofactor, in the *D. vulgaris* Qrc complex. Based on the crystal structures of the CISM enzymes Fdh-N, Nar and Psr (Bertero *et al.*, 2003; Jormakka *et al.*, 2002; Jormakka *et al.*, 2008), it is likely that the cubane clusters of QrcC form a wire that electrically links the hemes *c* of QrcA to a menaquinone-binding site in QrcD situated close to the $[3Fe\text{-}4S]^{1+/0}$ center. In the absence of a catalytic site, the function of the large QrcB subunit is unknown and may be to provide a structural scafold for the other subunits.

Sulfate respiration is an ancient mode of energy metabolism (Shen *et al.*, 2001) that is ubiquitous in anaerobic habitats and has very important environmental and economical consequences (Muyzer and Stams, 2008). Despite this importance there is still a major gap in our understanding of bioenergetics in SRB, since the sites of energy conservation have not been clearly established, even though a quinone-based chemiosmotic mechanism is likely present (Pereira, 2008; Rabus *et al.*, 2007). Most SRB lack important respiratory complexes such as complex I or the $bc_1$ complex, but two unique BRC are conserved, QmoABC (Pires *et al.*, 2003) and DsrMKJOP (Oliveira

*et al.*, 2008; Pires *et al.*, 2006). These are possible sites of energy conservation, although this could not yet be experimentally verified. The subunit composition of Qmo indicates that it transfers electrons from the quinone pool to the cytoplasm, whereas this is less clear for the Dsr complex, which is probably involved in direct electron transfer from the periplasm to the cytoplasm. One of the major energy sources for SRB in their natural habitats is hydrogen, which is oxidized by periplasmic hydrogenases that are very abundant in SRB (Matias *et al.*, 2005). In most bacteria, the periplasmic uptake hydrogenases include a membrane cytochrome *b* subunit that transfers electrons directly to the quinone pool. Strikingly, most SRB are very unusual in that their periplasmic hydrogenases and formate dehydrogenases lack the cytochrome *b* subunit and instead transfer electrons to a pool of soluble cytochromes *c*, of which the characteristic TpI$c_3$ is the most abundant (Heidelberg *et al.*, 2004; Pereira *et al.*, 2007a). Thus, a membrane protein is required to transfer electrons from TpI$c_3$ to the quinone pool, or directly to the cytoplasm for reduction of sulfate. This last function may be performed by the Hmc (Rossi *et al.*, 1993) and Tmc complexes (Pereira *et al.*, 2006), which are reduced by the TpI$c_3$ (Pereira *et al.*, 1998; Valente *et al.*, 2001), in contrast to Dsr, but no complex had yet been described in SRB that could reduce the quinone pool. We show here that the Qrc complex is a TpI$c_3$:menaquinone oxidoreductase and is efficiently reduced by either hydrogenases or formate dehydrogenase through this cytochrome. All organisms that contain the Qrc complex are deltaproteobacterial SRB (with a single exception), indicating

that its function is specifically associated with sulfate respiration. Analysis of available SRB genome sequences show that the presence of *qrc* genes correlates with the presence of periplasmic hydrogenases lacking membrane subunits for quinone interaction, and the same is observed for the periplasmic formate dehydrogenases (with a single exception for *Desulfatibacillum alkenivorans*). Conversely, in the few deltaproteobacterial SRB that lack the Qrc complex, either the hydrogenases and formate dehydrogenases include membrane subunits for quinone reduction (*e.g. Desulfotalea psycrophila* or *D. desulfuricans* ATCC 27774) or an alternative complex is present, such as the nine-heme cytochrome complex (Nhc) (Pereira, 2008) (*e.g. D. piger*).

A recent study in *D. alaskensis* (formerly *D. desulfuricans* G20) provided important evidence to support this physiological function of the Qrc complex (Li *et al.*, 2009). In this study a mutant library of *D. alaskensis* was screened to identify genes involved in $H_2$ metabolism based on a deficiency to grow syntrophically with *Methanospirrillum hungatei* on lactate. Three mutants were identified, with transposon insertions in genes coding for the [FeFe] hydrogenase small subunit (*hydB*, Dde_0082), TpI$c_3$ (*cycA*, Dde_3182) and gene Dde_2933, that is annotated as a putative molybdopterin oxidoreductase molybdopterin-binding subunit (and thus named *mopB*), and which corresponds to *qrcB*. The *mopB/qrcB* mutant is unable to grow with $H_2$ or formate as electron donors, but grows normally with lactate. The three mutants also accumulate higher levels of hydrogen and formate during growth on lactate/sulfate. Moreover, the phenotypes of the monoculture and syntrophic

culture were restored by the complementation with *qrcB* and *cycA* genes. In *D. vulgaris* a Δ*qrcBDC* delection mutant was constructed and the results were in agreement with the ones just described for *D. alaskensis*, no cell growth with formate and hydrogen with sulfate (Keller and Wall, 2011). The very interesting result in *D. vulgaris qrc* mutant is that the mutant grows in formate and sulfite, indicating that Qrc complex acts before sulfite reduction pathways. All these results further support the presence of a metabolic pathway involving periplasmic hydrogenases or formate dehydrogenases, TpI$c_3$ and the Qrc complex, and show an essential role of the Qrc complex during growth on $H_2$ or formate. In several transcriptomic studies (Haveman *et al.*, 2003; Haveman *et al.*, 2004; Pereira *et al.*, 2008; Stolyar *et al.*, 2007) the expression of the *qrc* genes follows the same behaviour as the genes for TpI$c_3$, [FeFe] hydrogenase, and genes related to sulfate respiration (*dsrAB*, *aprAB*, *qmoABC*, *dsrMKJOP*, and genes for ATP synthase), suggesting a co-regulation of the *qrc* and sulfate reduction genes. A similar effect was observed in a proteomic study (Zhang *et al.*, 2006).

A model for the electron transfer chain of sulfate respiration in *D. vulgaris* is shown in Figure 3.1.8. Periplasmic oxidation of hydrogen or formate, during growth on these substrates, or when they are formed as intermediaries in the metabolism of carbon compounds, such as lactate, results in reduction of the TpI$c_3$. This transfers electrons to the Qrc complex, which reduces menaquinone at the periplasmic side of the membrane close to the QrcC [3Fe-4S]$^{1+/0}$ center. If the protons required for

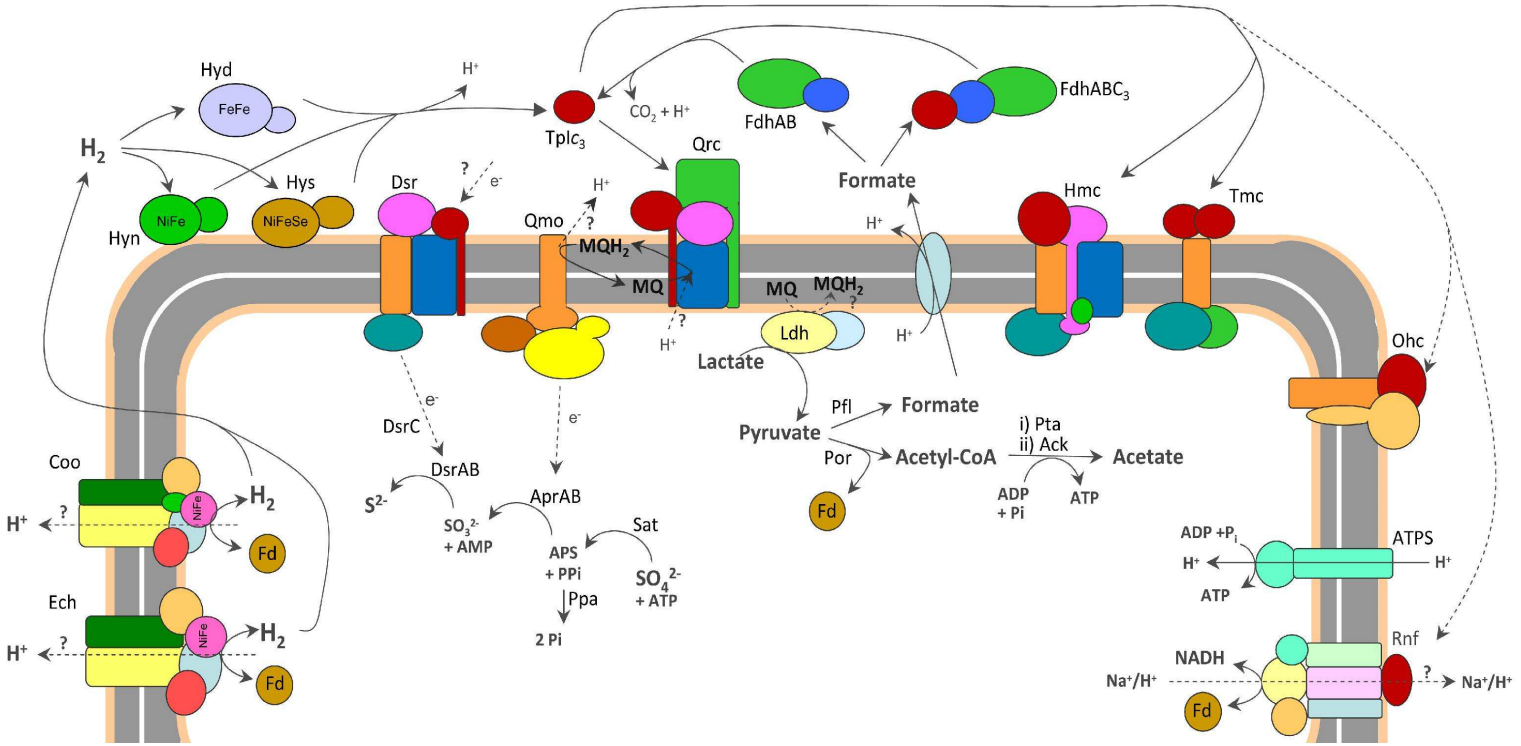


**Figure 3.1.8** – Model for the respiratory chain of *D. vulgaris* during growth on hydrogen, or lactate, and sulfate. The model is based on previous observations (reviewed in (Pereira, 2008; Pereira *et al.*, 2007a)) and the present results as discussed in the text. Abbreviations: Ldh, lactate dehydrogenase; Por, pyruvate:ferredoxin oxidoreductase; Pta, phosphate acetyltransferase; Ack, acetate kinase; Fd, ferredoxin; Ech and Coo, membrane-bound cytoplasmic-facing hydrogenases; Hyn1, [NiFe] hydrogenase isozyme 1; Hyd, [FeFe] hydrogenase; Hys, [NiFeSe] hydrogenase; Ohc, octaheme cytochrome complex; Rnf, putative NAD(H):ferredoxin oxidoreductase; ATPS, ATP synthase; MQ, menaquinone; $MQH_2$, menaquinol.

quinone reduction are taken up from the periplasm then no energy conservation will occur during this process. However, based on what has been proposed for Psr (Jormakka *et al.*, 2008), which includes a membrane subunit of the same family as QrcD, it is possible that there are channels in QrcD for proton uptake from the cytoplasm, in which case quinone reduction by the Qrc complex would be electrogenic. Further experiments have to be carried out to elucidate this point. The reduced menaquinone pool will be oxidized by the Qmo complex, which should transfer electrons to the cytoplasmic APS reductase. It is most likely that oxidation of menaquinol by Qmo occurs at the periplasmic side of the membrane releasing protons to the periplasm, since this complex includes two hemes *b* in its membrane subunit with suitable redox potentials to perform transmembrane electron transfer for the cytoplasmic subunits (Pires *et al.*, 2003). Thus, the Qrc and Qmo complexes likely constitute the two arms of an energy conserving redox loop (Jormakka *et al.*, 2003; Simon *et al.*, 2008), contributing to proton motive force generation during sulfate reduction.

But why do most SRB prefer to have a pathway involving the soluble TpI$c_3$ and the Qrc complex, rather than using the more usual mechanism of direct quinone reduction through a membrane subunit of hydrogenases and formate dehydrogenases? One possible explanation is that the involvement of TpI$c_3$ increases the metabolic flexibility of the organism, as electrons may be shuttled through several alternative pathways, as indicated by the presence of other membrane redox complexes that are associated with a cytochrome *c* subunit such as Tmc, Hmc, Ohc and Rnf (Pereira

*et al.*, 2007a), of which Hmc and Tmc have already been shown to act as electrons acceptors to TpI$c_3$ (Pereira *et al.*, 1998; Pereira *et al.*, 2006; Valente *et al.*, 2001). Indeed, sulfate reducers such as *Desulfotalea psycrophila* or *Archaeoglobus fulgidus* that lack the TpI$c_3$, also do not contain these various complexes, and probably have a less plastic metabolism. Both Tmc and Hmc have a cytoplasmic subunit that is homologous to DsrK, and so may be part of alternative pathways for sulfite reduction involving DsrAB (Oliveira *et al.*, 2008). The Rnf complex, present in several other organisms, may provide a link to the ferredoxin and NAD(H)/NADP(H) pools (McInerney *et al.*, 2007).

In conclusion, Qrc is the first BRC in the respiratory chain of SRB to have its physiological function clearly elucidated. It is related to bacterial CISM, but together with ACIII forms a group that does not bind Mo and performs electron transfer. This complex is a novel addition to the family of characterized bacterial redox complexes, typically constituted by modular subunits. It provides a striking example of how a totally different physiological function can be achieved with a minimal modification of subunits, a strategy that forms the basis for the diversity and flexibility of bacterial energy metabolism.

## 3.1.6 Acknowledgements

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# Chapter 3
## Section 3.2

# EPR characterization of the new QRC complex from sulfate reducing bacteria and its ability to form a supercomplex with hydrogenase and TpI$c_3$

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## 3.2.1 Summary

The Quinone-reductase complex (Qrc) is a respiratory complex with Type I cytochrome $c_3$:menaquinone reductase activity, recently described in sulfate reducing bacteria. Qrc is related to the complex iron–sulfur molybdoenzyme family and to the alternative complex III. In this work we report a detailed characterization of the redox properties of the metal cofactors of Qrc using EPR spectroscopy, which allowed the determination of the reduction potentials of five out of six hemes c, one [3Fe-4S]$^{1+/0}$ center and the three [4Fe-4S]$^{2+/1+}$ centers. In addition, we show that Qrc forms a supercomplex with [NiFe] hydrogenase and TpI$c_3$, its physiological electron donors.

## 3.2.2 Introduction

In the main group of sulfate reducing bacteria (SRB), of the *Deltaproteobacteria* class, most organisms are characterized by the presence of a high number of multiheme cytochromes *c*, of which the most abundant and well characterized is the tetraheme Type I cytochrome $c_3$ (TpI$c_3$). In addition, this group has a considerable number of membrane-associated redox complexes containing a cytochrome *c* subunit, which are responsible for quinone reduction or transmembrane electron transfer for the cytoplasmic reduction of sulfate (reviewed in (Pereira *et al.*, 2011)). Recently, a new respiratory membrane complex present in SRB was described in the previous section and named Qrc. Qrc was shown to transfer electrons from the TpI$c_3$ to the quinone pool, with the periplasmic hydrogenases

(Hase) and/or formate dehydrogenases (Fdh) as primary electron donors. The presence of Qrc is associated with the presence of Hases and/or Fdhs lacking a membrane subunit for direct quinone reduction. Qrc is the only described respiratory complex in SRB with a quinone reductase function, and it is essential for growth on $H_2$ or formate, as recently shown in *D. desulfuricans* G20 (Li *et al.*, 2009).

Qrc comprises three subunits in the periplasm and an integral membrane subunit. It includes a membrane-anchored hexaheme cytochrome *c* (QrcA), a periplasmic-facing large protein of the molybdopterin oxidoreductase family (QrcB), a periplasmic iron-sulfur four cluster protein (QrcC) and a membrane NrfD-like protein (QrcD) (Venceslau *et al.*, 2010). The three QrcBCD subunits are closely related to the bacterial respiratory enzymes of the complex iron-sulfur molybdoenzyme (CISM) family (Rothery *et al.*, 2008), although no molybdopterin is present in QrcB. The absence of Mo is also observed in the Nqo3/NuoG subunit of the respiratory complex I (Sazanov and Hinchliffe, 2006) and in the related protein of the alternative complex III (ACIII) (Gao *et al.*, 2010; Pereira *et al.*, 2007b; Yanyushin *et al.*, 2005). ACIII, a functional substitute of the $bc_1$ complex, is closely related to Qrc (Venceslau *et al.*, 2010; Yanyushin *et al.*, 2005), but has a higher number of subunits and acts reversibly of Qrc, by oxidizing menaquinol (Gao *et al.*, 2009; Refojo *et al.*, 2010).

Previous EPR studies of Qrc, allowed the detection of six low-spin hemes *c*, one $[3Fe\text{-}4S]^{1+/0}$ cluster and at least three interacting $[4Fe\text{-}4S]^{2+/1+}$ clusters (Venceslau *et al.*, 2010). Here, we report a more detailed study of the Qrc hemes *c* and Fe-S

centers through potentiometric titrations followed by EPR spectroscopy, providing a better characterization of the redox centers responsible for electron transfer in Qrc. In addition, we report that Qrc can form a supermolecular structure (supercomplex) with Hase and TpI$c_3$, its physiological electron donors.

## 3.2.3 Material and Methods

### Protein purification

Qrc was purified from *D. vulgaris* Hildenborough as described in section 3.1.

### Redox titration monitored by EPR

A potentiometric titration was performed inside an anaerobic chamber (95 % Ar, 5 % $H_2$) at 20 ºC using 40 μM of Qrc and 70 μM of a mixture of redox mediators (the same of previous section), in 100 mM MOPS pH 7, 5 mM EDTA. The redox potential spontaneously decreased slowly down to –200 mV, and sodium dithionite was used after that. Samples were transferred to EPR tubes under anaerobic conditions, capped and immediately frozen in liquid nitrogen upon removal from the chamber. EPR spectra were obtained using a Bruker EMX spectrometer equipped with an ESR-900 continuous flow of helium cryostat. Spectra were recorded under the following conditions: microwave frequency, 9.38 GHz; microwave power, 20.1 mW (for the Fe-S centers) and 2.4 mW (for the hemes); modulation frequency, 100 kHz; modulation amplitude, 1 mT; temperature, 10 K.

### Electrophoretic techniques

Proteins were separated on 10 % polyacrylamide Tricine-SDS-PAGE or 7-13 % high resolution Clear Native (hr CN-PAGE) containing 0.02 % (w/v) dodecyl maltoside, with running buffer also containing 0.02 % DDM and 0.05 % deoxycholic acid, as described in (Wittig *et al.*, 2007). Gels were stained with Coomassie Blue, with heme-staining using 3,3'-dimethoxybenzidine dihydrochloride (Francis and Becker, 1984) or for hydrogenase activity (da Silva *et al.*, 2008). Bands from the first-dimension CN-PAGE were excised and used for a second-dimension Tricine-SDS-PAGE, after denaturation by incubation with 5 % SDS and 5 % β-mercaptoethanol at 37 ºC for 1 h, followed by a washing step, and thiol blockage with a 4 % iodoacetamide solution.

### Mass spectrometry

The supercomplex band from the CN-PAGE was subjected to in-gel tryptic digestion followed by peptide identification in a MALDI-TOF/TOF analyzer (Applied Biosystems 4800). The data were analyzed in a combined mode using Mascot search engine and NCBI database.

## 3.2.4 Results and Discussion

### EPR characterization of Qrc cofactors

EPR spectroscopy is a most useful tool to selectively characterize the redox properties of both hemes and Fe-S centers of respiratory proteins. A few detailed EPR studies on CISM enzymes have been reported, which were most informative regarding the properties of the Fe-S clusters (reviewed in (Rothery *et al.*, 2008)). These clusters provide a linear pathway to transfer electrons between the quinone pool and the catalytic site.

To generate samples for the EPR study, we titrated Qrc in the presence of redox mediators, inside an anaerobic chamber containing 5 % $H_2$. It was observed that the redox potential of the sample slowly decreased without addition of any reductant down to ~–200 mV. This decrease was due to trace amounts of Hase in the Qrc sample (see below), which slowly reduced the complex, but this decrease was slow enough to allow us to take several samples at defined redox potentials. The Qrc hemes were reported to give rise to three EPR signals with $g_{max}$ of 3.21, 2.93 and 2.82 (**Figure 3.2.1 A**), which by spectral simulation account for 3, 2 and 1 hemes, respectively (results from section 3.1, **Figure 3.1.4 B**) (Venceslau *et al.*, 2010).

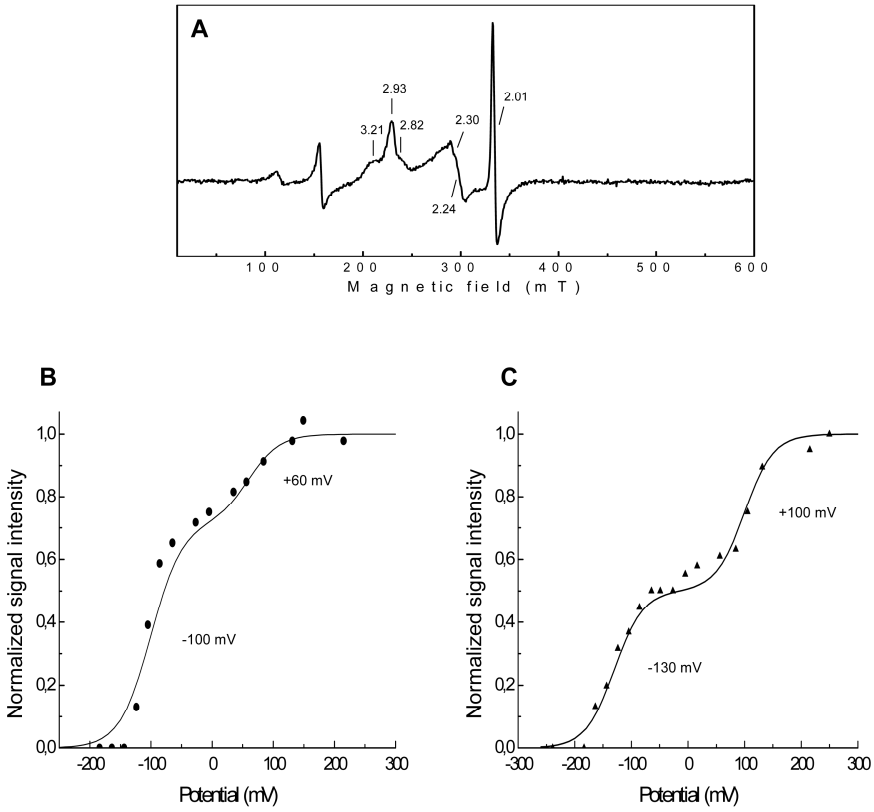


**Figure 3.2.1** – Potentiometric titration of the Qrc hemes *c*. (A) EPR spectrum of the oxidized sample where the *g* values of several heme signals are identified. Redox titration curves of the peaks at *g* values 3.21 (B) and 2.93 (C). The points represent the experimental data and the solid line represents the best theoretical fit using Nernst equation(s), with the $E_m$ values depicted.

The intensity of these peaks was followed along the redox titration. The data points for the signal with $g_{max}$ = 3.21 was simulated by the sum of three Nernst equations with midpoint redox potentials ($E_m$) of +60 and –100 mV in a 1:2 ratio approximately (**Figure 3.2.1 B**), in accordance with the fact that this signal corresponds to 3 hemes. The data points for the signal with $g_{max}$ = 2.93 could be simulated with two different redox potentials with 50 % contribution each, with $E_m$ values of

+100 and –130 mV (**Figure 3.2.1 C**). The low intensity of the signal with $g_{max}$ = 2.82 together with partial overlap with the 2.93 signal, did not allow the determination of a satisfactory redox titration curve.

In the oxidized state a strong EPR signal at g~2.01 due to a $[3Fe\text{-}4S]^{1+/0}$ center is also observed (**Figure 3.2.2 inset**), as described in other ClSM and related complexes such as NarGHI nitrate reductase (Rothery *et al.*, 1998), FrdABCD fumarate reductase (Manodori *et al.*, 1992), or ACIII (Pereira *et al.*, 1999). This signal titrates with $E_m$ value of +160 mV (**Figure 3.2.2**), which is close to the +140 mV or +180 mV redox potential determined for the $[3Fe\text{-}4S]^{1+/0}$ cluster of ACIII and NarGHI, respectively (Pereira *et al.*, 1999; Rothery *et al.*, 1998). The $[3Fe\text{-}4S]^{1+/0}$ center is close to the menaquinone binding site as reported in previous section.

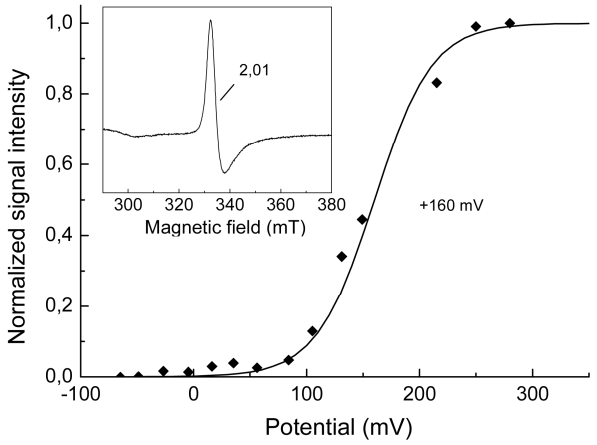


**Figure 3.2.2** – Potentiometric titration of the Qrc $[3Fe\text{-}4S]^{1+/0}$ center monitored at *g* value 2.01. *Inset*, EPR spectrum of the $[3Fe\text{-}4S]^{1+/0}$ center in the oxidized sample.

At least three $[4Fe\text{-}4S]^{2+/1+}$ clusters were reported in previous section in *D. vulgaris* Qrc. These clusters give rise to a complex EPR signal due to magnetic interactions, as reported for other four cluster proteins (Cheng *et al.*, 2005). Five signals with *g* values of 2.07, 2.01, 1.98, 1.93 and 1.91 (**Figure 3.2.3 A**) were monitored in the redox titration.

The data points obtained from signals *g* 2.07, 1.98 and 1.91 can be fitted with a single component with $E_m$ = -150 mV for the first two and -130 mV for the latter. For both signals with *g* 2.01 and 1.93, two components have to be considered with $E_m$ of –130 and –240 mV for g = 2.01 and -150 mV and -230 mV for g = 1.93 (**Figure 3.2.3**). These data suggest the presence of three clusters, two of which with $E_m$ of -130 to -150 mV and the third with a lower $E_m$ of -240 mV. This is supported by a plot of the total signal intensity *versus* the potential, which can be fitted with these same three potentials (**Figure 3.2.3 G**). Thus, one of the Qrc $[4Fe\text{-}4S]^{2+/1+}$ clusters has a lower potential than the other two, a similar situation to what is described for CISM enzymes (Cheng *et al.*, 2005).

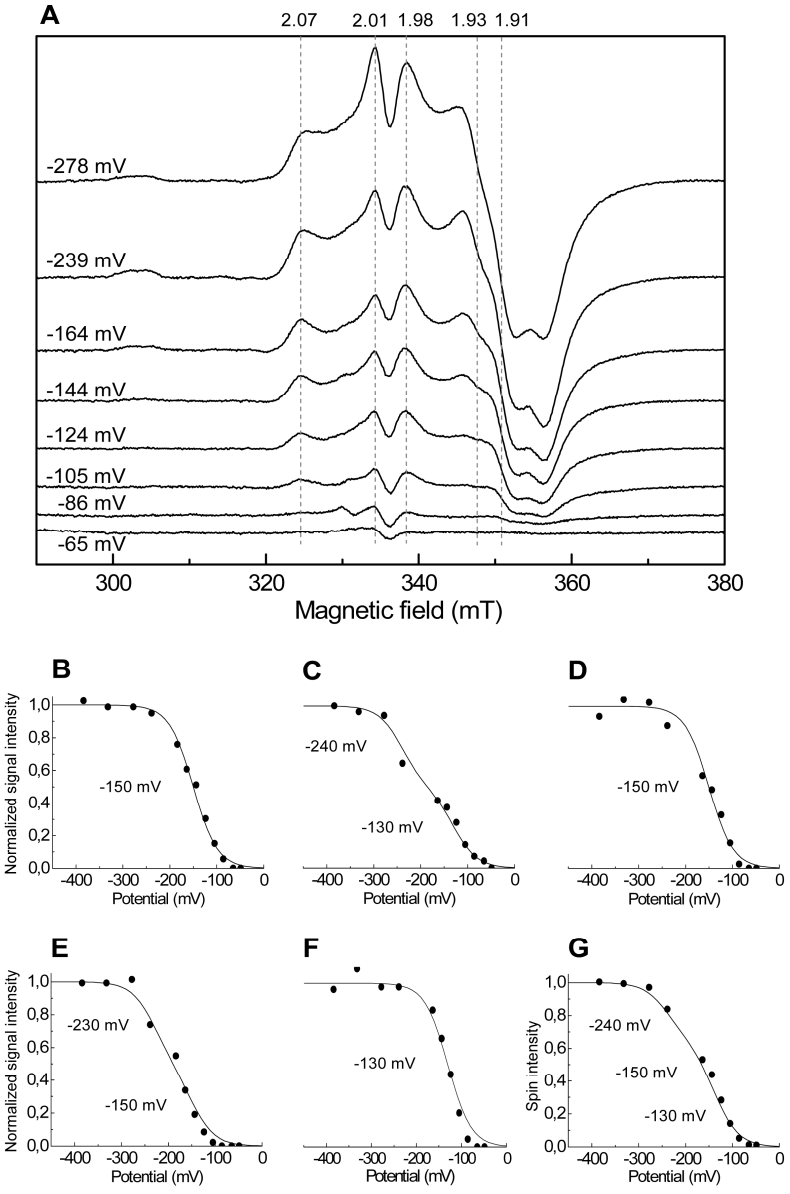


**Figure 3.2.3** – Potentiometric titration of the $[4Fe\text{-}4S]^{2+/1+}$ centers of the Qrc complex. EPR spectra obtained during the redox titration (A) and the respective redox titration curves followed at *g* values of 2.07, 2.01, 1.98, 1.93 and 1.91 (B, C, D, E and F, respectively), and total signal intensity (G). The $E_m$ value(s) used in the Nernst fittings are depicted in the plots. No further changes are observed below -280 mV.

The midpoint potentials of the Qrc cofactors are arranged in a scale of potentials that is suitable for electron transfer from the low redox-potential TpI$c_3$ (-325 to -170 mV) to the menaquinone ($E_m$= -70 mV). All the potentiometric results are summarized in Table 3.2.1.

**Table 3.2.1** – Summary of the midpoint potentials obtained for the Qrc cofactors in the EPR titration

| | $E_m$ (mV) | | |
|---|---|---|---|
| Hemes *c* | **g = 3,21**<br>+60<br>-100<br>-100 | **g = 2,93**<br>+100<br>-130 | **g = 2,82**<br>ND |
| $[3Fe\text{-}4S]^{1+/0}$ | **g = 2,01**<br>+160 | | |
| $[4Fe\text{-}4S]^{2+/1+}$ | **g = 2,07**<br>-150 | **g = 2,01**<br>-130<br>-240 | **g = 1,98**<br>-150 |
| | **g = 1,93**<br>-150<br>-230 | **g = 1,91**<br>-130 | |

ND–not determined

## Qrc forms a supercomplex with the [NiFe] hydrogenase

During the purification of Qrc, we observed that it was difficult to separate it from the [NiFe] Hase, as they eluted together in the first two ion-exchange columns, and also in a subsequent gel filtration. We could only separate these two proteins using a metal chelating IMAC column (**Figure A.2** in Appendix A). This suggested the presence of a strong interaction between Qrc and Hase, which could result in the formation of

a supercomplex. Analysis of the protein fractions by high resolution clear native gels (hr CN-PAGE) (Wittig *et al.*, 2007) stained for $H_2$ uptake activity, indeed revealed the presence of a high molecular weight band, besides the band for the isolated Hase at 87 ± 9 kDa (**Figure 3.2.4 A**). This band, with an estimated molecular mass of 360 ± 10 kDa, was found in fractions containing both Qrc and the Hase, but was no longer detected in the fractions from the IMAC column, which can separate the two proteins. The band for the isolated Qrc complex is present at 250 ± 24 kDa and stains with Coomassie and for hemes (**Figure 3.2.4 B**), but does not stain for $H_2$ uptake activity, while the supercomplex band is very well detected by Hase activity staining (which is more sensitive), but stains weakly for hemes.

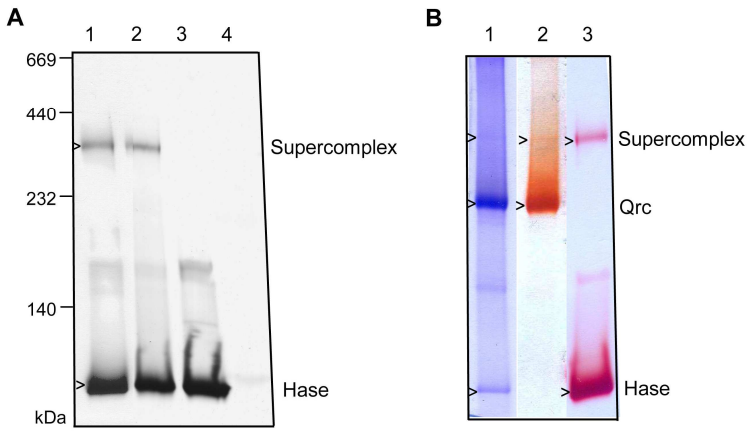


**Figure 3.2.4** – (A) High resolution CN-PAGE stained for $H_2$ uptake activity of the Qrc-containing fraction from the first chromatographic step (lane 1), of the Qrc-containing fraction from the second chromatographic step (lane 2), of the Hase-containing fraction from the IMAC column (lane 3); and of the purified Qrc (lane 4). (B) Hr CN-PAGE of the Qrc fraction from the second chromatographic step stained by Coomassie Blue (lane 1), by heme-staining (lane 2) and by $H_2$ uptake activity (lane 3).

To define the composition of the supercomplex, a two dimension Tricine-SDS-PAGE was performed on the 360 kDa band (**Figure 3.2.5 B**). Several subunits from the Qrc complex were identified, as well as the large subunit of the Hase, by comparison with the 2D gel of the isolated Qrc and Hase native gel bands. Furthermore, the 360 kDa band was excised from the hr CN-PAGE gel, digested with trypsin and analyzed by mass spectrometry. Two proteins were clearly identified to be present: the large subunit of Qrc (QrcB) and the large subunit of the soluble periplasmic [NiFe] Hase (HynA1) (**Table A.1** in Appendix A), showing that this high molecular mass band is indeed formed by a supercomplex between Qrc and the [NiFe] Hase. The identification of this Hase was also confirmed by Western blot (**Figure A.2** in Appendix A).

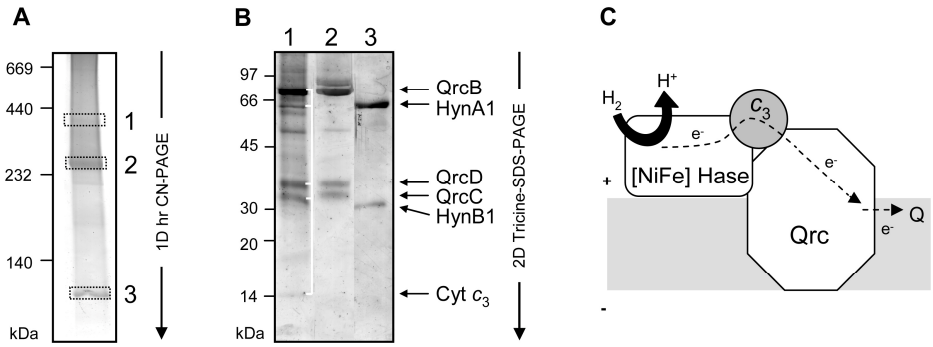


**Figure 3.2.5** – Hr CN-PAGE of (A) a supercomplex-containing fraction, and the 2D Tricine-SDS-PAGE (B) of the respective bands: supercomplex (1), Qrc (2) and Hase (3). Gel A was stained with Coomassie and Gel B with silver nitrate. C) Schematic representation of the supercomplex and its electron transfer pathway (dashed arrows). Hyn, [NiFe] Hydrogenase; Q, menaquinone.

The electron transfer between Hase and Qrc was shown to be functional only in the presence of the TpI$c_3$ cytochrome (**Figure 3.1.5 A**, section 3.1) (Venceslau *et al.*, 2010), the Hase physiological electron acceptor. Like the Hase, the TpI$c_3$ was detected in the Qrc fraction from the second Q-Sepharose column as a heme-staining band of 15 kDa (**Figure A.2** in Appendix A). Additionally, a tenuous band of this molecular mass was also observed in the 2D gel of the supercomplex, but was absent in the Qrc and Hase controls of the 2D gel (**Figure 3.2.5 B**). Moreover, the molecular weight difference between the supercomplex band and the sum of the Hase plus the Qrc native bands, estimated from several CN gels, is about 22 kDa, which likely corresponds to the TpI$c_3$. Altogether, these results indicate that the 360 kDa supercomplex is constituted of three proteins: Qrc, [NiFe] Hase and TpI$c_3$. The formation of organized respiratory enzyme complexes in supermolecular structures has been well described in mitochondria (Dudkina *et al.*, 2010) (sometimes called respirasomes), and also in bacteria (Prunetti *et al.*, 2010).

In summary, the redox studies reported here provide the first detailed characterization of a new bacterial respiratory complex, and support the function of Qrc in electron transfer between the low redox-potential TpI$c_3$ and menaquinone. The assembly of the [NiFe] Hase, TpI$c_3$ and Qrc in a supermolecular structure is likely to optimize the oxidation of $H_2$, a key step in energy metabolism of SRB.

## 3.2.5 Acknowledgments

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# Chapter 4

# Proteins involved in the dissimilatory sulfite reduction: Studies on DsrC and DsrMKJOP

The process of prokaryotic dissimilatory sulfate reduction occurs entirely in the cytoplasm and starts with a sulfate activating step to adenosine-5'-phosphosulfate (APS), followed by two consecutive reducing steps. These include the reduction of APS to sulfite performed by APS reductase, and the reduction of sulfite to sulfide performed by the dissimilatory sulfite reductase (DsrAB). The reduction of sulfite by DsrAB involves also the protein DsrC, which was initially considered as a third subunit of DsrAB (Pierik *et al.*, 1992). The electron donor to DsrAB is still unknown, but the conserved DsrMKJOP transmembrane complex has been proposed to be involved in this process (Pereira *et al.*, 2007; Pereira *et al.*, 2011; Pires *et al.*, 2006). The electron transfer chain for DsrAB, and the mechanism and products of sulfite reduction have long been the subject of controversy. Recently, the crystal structure of *D. vulgaris* DsrAB in complex with DsrC was reported, which represented a major achievement towards our understanding of the mechanism of dissimilatory sulfite reduction and how energy conservation may be achieved in this process (Oliveira *et al.*, 2008). In the crystal structure the C-terminal arm of DsrC projects inside the DsrAB structure in a way that brings its last Cys right next to the siroheme-[4Fe-4S] active site in DsrB, pointing to a direct involvement of DsrC in sulfite reduction. The structure of DsrC has also been determined separately, and in solution its C-terminal arm has a disordered structure (Cort *et al.*, 2001; Cort *et al.*, 2008), but it can also adopt a more rigid conformation where the two C-terminal conserved Cys are close enough for formation of a disulfide bond (Mander *et al.*,

2005). This conformation has also been reported in a recent structure of *D. gigas* DsrAB bound to DsrC (Hsieh *et al.*, 2010).

Based on the structural information, a new mechanism for dissimilatory sulfite reduction was proposed, involving redox cycling of DsrC (**Figure 4.1**) (Oliveira *et al.*, 2008). This mechanism considers that upon a four-electron sulfite reduction a DsrC persulfide is formed as a key intermediate. Through an internal reduction by the other DsrC cysteine, the persulfide is converted to an intramolecular disulfide bond with concomitant hydrogen sulfide liberation. The oxidized DsrC form is proposed to be subsequently reduced by the membrane DsrMKJOP complex. The involvement of the Dsr complex provides a link

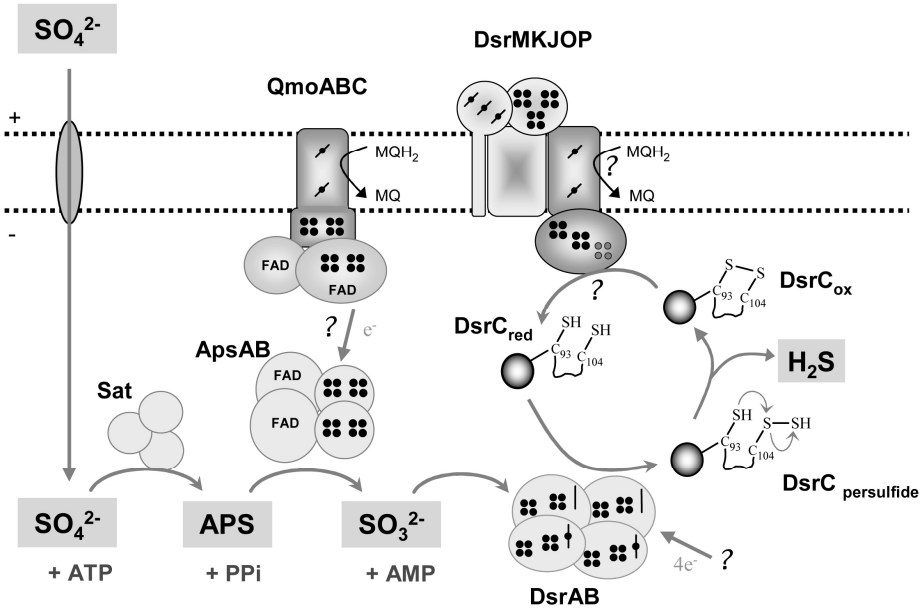


**Figure 4.1** – Schematic representation of the proposed model for sulfate reduction mechanism Sat, sulfate adenylyltransferase; ApsAB, adenosine phosphosulfate reductase; QmoABC, membrane complex that is the probable electron donor to ApsAB; DsrMKJOP, membrane complex proposed to work as a disulfide reductase of $DsrC_{ox}$ (adapted from (Oliveira *et al.*, 2008)).

⁘ - $[4Fe\text{-}4S]^{2+}$; ⁘ - $[4Fe\text{-}4S]^{3+}$; ✓ - heme; I - sirohydrochlorin; MQ - menaquinone

between quinol oxidation or transmembrane electron transfer and sulfite reduction that may explain how energy conservation is achieved in this step. Thus, DsrC, and its persulfide are suggested to be key intermediates in the sulfite reduction process.

The next two sections describe work on the DsrC and DsrMKJOP proteins, aimed at testing the proposed mechanism (**Figure 4.1**). In section 4.1 studies of the redox behaviour of DsrC are reported, to develop methods of obtaining the protein in the several redox states. In section 4.2, protein-protein interaction studies and electron transfer assays involving DsrC, DsrMKJOP and menaquinol are described.

# Chapter 4
## Section 4.1

# Redox studies of DsrC

### 4.1.1 Summary

The dissimilatory reduction of sulfur compounds is a widespread biological process that contributes to the Earth biogeochemical carbon and sulfur cycles. Sulfate/sulfite reduction leads to oxidative phosphorylation through a still unidentified mechanism, and in particular it has not been elucidated how sulfite reduction is associated with energy conservation. DsrC is thought to have a still uncertain role related with the activity of DsrAB in sulfite reduction. DsrC is a major protein in the cell and includes two redox active conserved cysteines. A DsrC persulfide has been proposed as a key intermediate in the reduction of sulfite. Here we report studies, using a gel-shift assay, of the redox behaviour of DsrC. In oxidizing conditions DsrC shows a great tendency to form a dimer through its penultimate cysteine. The dimer can be resolved *via* internal reduction by the other conserved cysteine. Using an arginine treatment it was possible to obtain specifically the intramolecular disulfide bond in DsrC. Treatment with reducing agents cleaves the intermolecular or intramolecular bonds regenerating the monomeric reduced species. We present preliminary results for the formation of persulfurated DsrC upon incubation with sulfide. Production of DsrC in different redox forms (oxidized, reduced, persulfide) is essential for further studies of protein interactions and electron transfer assays with DsrAB and the DsrMKJOP transmembrane complex.

### 4.1.2 Introduction

Sulfur is an important component of a large variety of biomolecules due to its versatile redox behaviour that can range from an electrophile (*e.g.* disulfide bond, R-S-S-R) to a nucleophile (*e.g.* thiol/sulfhydryl, R-SH). The thiol group is present in cysteine, whose unique properties of cysteine make it the most conserved residue in proteins, despite being one of the less abundant (Fomenko *et al.*, 2008). Moreover, the thiol group can be used as a sulfur acceptor, generating the persulfide form (R-S-SH), which includes a sulfane sulfur. The term sulfane covers all sulfur atoms bound reversibly to other sulfur atoms, generating a highly reactive and labile group that has been increasingly recognised as the cellular "currency" for transport of activated sulfur without its release to solution (Kessler, 2006; Mueller, 2006; Toohey, 2011; Wood, 1987). The cellular trafficking of sulfur in the form of persulfide is involved in several important biological functions such as sulfur incorporation into cofactors (iron–sulfur clusters and molybdenum cofactors) and vitamins (biotin, lipoic acid and thiamine), post-transcriptional modification of transfer RNA, and activation or inactivation of enzymes (Hidese *et al.*, 2011; Mueller, 2006; Toohey, 2011). Cysteine persulfide intermediates have also been implicated in microbial energy metabolism, such as in reactions of dissimilatory sulfur cycle enzymes, namely in sulfur oxidation (Bamford *et al.*, 2002; Sauvé *et al.*, 2007; Urich *et al.*, 2006), and more recently in sulfite reduction (Oliveira *et al.*, 2008).

A key protein in dissimilatory sulfite reduction is DsrC, which contains two conserved cysteines on a flexible C-terminal arm (C93 and C104 *Desulfovibrio vulgaris* numbering) (**Figure 4.1.1**) that may undergo thiol-disulfide interchanges (Cort *et al.*, 2001; Cort *et al.*, 2008; Mander *et al.*, 2005). DsrC is strictly conserved in all sulfate/sulfite reducing bacteria (Pereira *et al.*, 2011), and also in sulfur-oxidizing bacteria, which means that all the organisms with *dsrAB* genes also have the *dsrC* gene, although not necessarily in the same operon (Cort *et al.*, 2008). The importance of DsrC is highlighted by the fact that it is one of the most highly expressed genes in SRB (Haveman *et al.*, 2003), and also among sulfur energy metabolism genes in environmental metagenomic samples (Canfield *et al.*, 2010). DsrC is homologous to a larger family of proteins widespread in bacteria, such as *E. coli* TusE, which is involved in sulfur-transfer reactions *via* a persulfurated cysteine, as part of the biosynthesis of thio-modifications of bacterial tRNA (Ikeuchi *et al.*, 2006). In these proteins only the last C-terminal cysteine is conserved (**Figure 4.1.1**).

```
                  *        20         *        40         *        60
DsrCDv : MAE-VTYKGKSFEVDEDGFLLRFDDWCPEWVEYVKESEGIS----DISPDHQKIIDFLQD
DsrCDd : MAE-ITYKGKSFEVDEDGFLLRFDDWCPEWMDYVKESEGIS----EITADHQKILDFLQD
DsrCAf : MPE-LRVKGKKLRLDEDGFLQDWEEWDEEVAEALAKDTRFSPQPTELTEEHWKIIRYLRD
DsrCAv : MADTIEVDGKQFAVDEEGYLSNLNDWVPGVADVMAKQDNLE-----LTEEHWDIINFLRE
TusEEc : ---MLIFEGKEIETDTEGYLKESSQWSEPLAVVIAKNEGIS-----LSPEHWEVVRFVRD
TusEPa : -MTLLMLEGREIRLDKDGYLAALDDWSEPVAEALAAREELA-----LTAEHWEILQLLRE

                  *        80         *       100    ↓    *  ↓  ↓
DsrCDv : YYKKNGIAPMVRILSKN-------TGFKLKEVYELFPSGPGKGACKMAGLPKPTGCV
DsrCDd : YYKKNGIAPMVRILSKN-------TGYKLKEVYELFPSGPGKGACKMAGLPKPTGCV
DsrCAf : YFIKYGVAPPVRMLVKHC-KKEVRPDCNLQYIYKLFPQGPAKDACRIAGLPKPTGCV
DsrCAv : YYEEYQIAPAVRVLTKAVGKKLGKEKGNSKYLYSLFPYGPAKQACRFAGLPKPTGCV
TusEEc : FYLEFNTSPAIRMLVKAMANKFGEEKGNSRYLYRLFPKGPAKQATKIAGLPKPVKCI
TusEPa : FYAEFQLSPANRPLIKYVAQRLGPDKGNSLHLNHLFKGAPAKLGAKLAGLPKPSNCL
```

**Figure 4.1.1** – Sequence alignment of DsrC and TusE proteins. Conserved cysteine residues are indicated by black arrows and a conserved proline residue by a grey arrow. This proline is the residue that allows conformational changes of the arm. *Desulfovibrio vulgaris* (Dv), *Desulfovibrio desulfuricans* ATCC 27774 (Dd), *Archaeoglobus fulgidus* (Af), *Allochromatium vinosum* (Av), *Escherichia coli* (Ec), *Pseudomonas aeruginosa* (Pa).

The presence of a Cys residue in an flexible arm, compared with other functional Cys that are usually present in loops (Fomenko *et al.*, 2008), has been proposed to be a structural strategy for specific protein-protein interactions, while allowing Cys accessibility for thiol-disulfide interchange and/or persulfide formation (Cort *et al.*, 2001; Cort *et al.*, 2008; Mander *et al.*, 2005).

The penultimate cysteine residue of DsrC at the C-terminus is very prone to form intermolecular disulfide bonds in oxidizing conditions, leading to formation of the dimer form ($DsrC_{dimer}$) (Cort *et al.*, 2008). This is an undesirable situation, as the physiologically functional form of oxidized DsrC should be the monomer with an intramolecular disulfide bond ($DsrC_{ox}$). Here, we report a study of the DsrC redox behaviour aiming to produce its different states ($DsrC_{red}$ and $DsrC_{ox}$). To monitor the Cys redox state we used a method that specifically tags the thiol group with a heavy SH-reactive reagent, altering the electrophoretic protein mobility, which allows monitoring the Cys redox state through a gel-shift assay.



## 4.1.3 Materials and Methods

### Cloning and expression of DsrC from *Desulfovibrio vulgaris*

The *D. vulgaris* Hildenborough *dsrC* gene (DVU2776) was amplified by PCR using genomic DNA. The PCR product was cloned directly in pET-28a(+) vector (Novagen), which allows the insertion of a 6x-His tag tail at the N-terminus. Sequencing of the cloned plasmid was carried out to confirm the desired

sequence. After transformation into *E. coli* BL21Gold(DE3) cells (Stratagene), these were grown at 37 ºC in M9 minimal media with kanamycin (30 μg/mL) until an $OD_{600}$ of 0.4 was reached, then 100 μM of IPTG was added and growth allowed to continue for another 4 h.

### Protein purification

Cells were harvested by centrifugation for 15 min at 10000 × *g*, resuspended in 25 mM potassium phosphate, 300 mM NaCl, 30 mM imidazole pH 7.5 (buffer A), and disrupted in a French Press in the presence of DNase. To remove insoluble components the extract was centrifuged for 20 min at 20000 × *g*, followed by ultracentrifugation for 2 h at 140000 × *g* to separate the soluble fraction from the membranes. The supernatant was loaded in a HiTrap Chelating HP column (GE Healthcare), previously charged with $NiCl_2$ and equilibrated with buffer A. The protein was eluted with 100 mM imidazole, and was concentrated and dialyzed with 25 mM potassium phosphate pH 7.5. The purity of the protein was analyzed by SDS-PAGE gel and the protein concentration was determined from its UV-visible spectrum using the absorption coefficient of 18.575 $mM^{-1}.cm^{-1}$ at 280 nm.

### MalPEG gel-shift assay and redox treatments

DsrC in different redox states was analysed by a gel-shift assay using MalPEG (methoxy-polyethylene glycol maleimide, MW 5000 g/mol, Fluka). The protein was labelled by treatment with 1 mM MalPEG at 30 ºC for 15 min (adapted from (Lu and Deutsch, 2001)). The reaction was terminated by addition of an

equal volume of 2x SDS sample buffer (38 mM Tris-HCl buffer pH 6.8 with 10 % glycerol, 6 % SDS, 0.05 % bromophenol blue) and immediately subjected to 10 % polyacrylamide Tricine-SDS-PAGE without boiling the sample and in non-reducing conditions.

To obtain reduced DsrC the purified DsrC in 25 mM potassium phosphate pH 7.5 was treated with 10 mM dithiothreitol (DTT) during 30 min at 30 ºC. DTT was removed by centrifugal gel filtration using a spin column (Micro Bio-Spin 6, Bio-Rad). Oxidation of the sulfhydryl groups was tested using different oxidizing agents: $H_2O_2$ (hydrogen peroxide) 20 mM or TBHP (*tert*-butyl hydroperoxide) 10 mM, for a 30 min or 24 h period at 20 ºC (Cort *et al.*, 2008; Mander *et al.*, 2005); 1 M L-arginine for 1 h at 30 ºC; or air oxidation during two weeks at room temperature, followed by centrifugation before use, since some protein precipitation occurs. The excess of oxidizing agents was also removed by centrifugal gel filtration. After protein incubation and removal of excess of reducing or oxidizing reagents, the DsrC secondary structure was monitored by Circular Dichroism spectroscopy (190-250 nm).

The persulfide form of DsrC was prepared by incubation of the protein, previously reduced with DTT, with 4 mM of sodium sulfide at room temperature for 1 h (modified from (Forlani *et al.*, 2005)). The excess of sulfide was removed by centrifugal gel filtration device, and the MalPEG modification was performed followed by reduction with excess of DTT 50 mM. A sample was collected after MalPEG and DTT treatments and analyzed by Tricine-SDS-PAGE.

## Mass Spectrometry

To determine the number of available cysteine residues in DsrC before and after exposure to DTT or arginine, the DsrC protein was incubated with 10 mM iodoacetamide for 1 h at 30 ºC. The number of adducts formed per subunit was detected by mass spectrometry performed at the Environmental Molecular Sciences Laboratory (EMSL) of the Pacific Northwest National Laboratory (Richland, WA, USA). Samples were run on a 12T FTICR-MS (Fourier transform ion cyclotron resonance mass spectrometry) instrument with direct infusion of protein, and on an IMS-TOF MS instrument (ion mobility spectroscopy-time of flight mass spectrometry). Ion mobility spectroscopy coupled with mass spectrometry provides a gas-phase separation of ions prior to mass analysis, so that different conformations of the same molecule will have a different pattern of peaks in a two-dimensional plot of drift time *versus* mass.

## Western blot analysis

The antiserum against the purified recombinant *D. vulgaris* DsrC was produced by Davids Biotechnologie company.
*D. vulgaris* Hildenborough (DSM 644) was grown in lactate/sulfate medium (Postgate, 1984). The cells were suspended in 20 mM Tris-HCl pH 7.6 and DNase, and then disrupted in a homogenizer. Insoluble material was removed by centrifugation and the soluble fraction was separated from the membranes by ultracentrifugation. The soluble proteins were applied to a fast flow Q-Sepharose column equilibrated with 20 mM Tris-HCl buffer pH 7.6. A stepwise gradient of increasing NaCl concentration was performed. The protein content of each

fraction was determined by the Bradford assay and 30 μg of total protein was applied into a 15 % SDS-PAGE gel. Proteins were then transferred to 0.45 μm PVDF (polyvinylidene difluoride) membranes (Roche) for 15 min at 100 V in a Mini Trans-Blot wet electrophoretic transfer cell (Bio-Rad). The membranes were left to dry overnight at room temperature and were then equilibrated with TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl) without the blocking step (Mansfield, 1995). Western blot analysis was conducted using primary antibody (antiserum against DsrC) (1:5000) for 1 h in TBS plus 1 % dry milk, and secondary antibody IgG from the rabbit conjugated with alkaline phosphatase (1:15000) (Sigma). Detection was performed using a solution of nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt. The recombinant DsrC protein was used as a positive control.



## Spectroscopic Techniques

UV-visible spectra were recorded at room temperature in a Shimadzu UV-1603 spectrometer or in a NanoDrop 2000c spectrophotometer (Thermo Scientific).

Far-UV CD spectra were obtained in a Jasco J-715 spectropolarimeter using a quartz polarized 1 mm path length quartz cuvette (Hellma), and protein concentrations ranged from 0.1-0.2 $mg.ml^{-1}$.

NMR experiments were conducted at the EMSL (collaboration project with Dr. John Cort) for examination of the redox state of DsrC and to obtain a solution NMR structure of different redox states of DsrC using $^{1}H$, $^{13}C$, and $^{15}N$ chemical shifts (work still ongoing).

## 4.1.4 Results

### Most DsrC in the cell is not associated with DsrAB

DsrC from *D. vulgaris* was heterologously expressed in *E. coli* followed by a single step of purification in a metal affinity chromatography. An SDS-PAGE gel showed that recombinant DsrC was in a pure state and the UV-visible spectrum had no absorption in the visible region confirming the absence of prosthetic groups (data not shown). The recombinant protein was used to produce polyclonal antibodies in rabbits. A group of sixteen fractions obtained from separation of the *D. vulgaris* soluble fraction on an ion-exchange column was subjected to Western blot analysis with these antibodies. DrsC was detected in two distinct and well separated fractions (**Figure 4.1.2**): fraction 6 (eluted at 150 mM NaCl) and in fraction 11 (eluted at 300 mM NaCl), which corresponds to the fraction that contains the dissimilatory sulfite reductase (DsrAB). The majority of DsrC is detected in fraction 6, which does not have DsrAB since no absorption peak was detected in the spectrum at ~628 nm, a characteristic spectroscopic fingerprint from the sirohydrochlorin cofactor of the *D. vulgaris* DsrAB (Kobayashi *et al.*, 1972). Thus, the majority of DsrC is not associated with DsrAB, confirming that DsrC is not a subunit of the DsrAB sulfite reductase, as previously proposed (Pierik *et al.*, 1992), but a protein that interacts with it. Therefore, DsrC should not be called as the gamma subunit of the dissimilatory sulfite reductase.

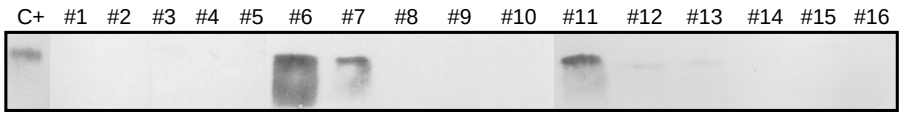


**Figure 4.1.2** – Western blot analysis of protein fractions from the first ion-exchange purification of the soluble fraction from *D. vulgaris* using anti-DsrC antibodies. All fractions (30 μg) were resolved on a 15 % SDS-PAGE gel using recombinant DsrC as a positive control (C+).

## A gel-shift assay to monitor the DsrC redox state

To monitor the redox state of the cysteine residues in DsrC, we conducted gel-shift assays using maleimide polyethylene glycol (MalPEG), a reagent that covalent and selectively labels free thiol groups (Lu and Deutsch, 2001). This thiol modification can be detected by SDS-PAGE analysis, since the apparent molecular weight of the protein is increased by ~10 kDa per each SH group modified in the protein. This assay is a simple and quick method to monitor the state of cysteine residues, since the results can be observed in a regular SDS-PAGE gel with a small quantity of protein.

This methodology was first tested with recombinant DsrC from *Allochromatium vinosum*, a sulfur oxidizing bacteria. The protein contains only the two conserved Cys in the C-terminal arm (Cys100 and Cys111, *Al. vinosum* numbering), and for which single and double Cys mutants have been previously obtained (Cort *et al.*, 2008). Treatment of the recombinant wild type protein with MalPEG originates three band-shifted forms (**Figure 4.1.3**). The most intense band at ~50 kDa is assigned to the dimeric species formed by an intermolecular disulfide bond between two terminal Cys111. The dimer species has two MalPEG groups bound to the two free Cys yielding the heaviest

band (2×15 kDa [dimer] + 2×10 kDa [MalPEG groups] = 50 kDa). The other two minor bands correspond to the monomer binding one MalPEG molecule (15 kDa + 1×10 = 25 kDa) or two MalPEG molecules (15 kDa + 2×10 = 35 kDa) (**Figure 4.1.3**, lane 2). So, the as-isolated *Al. vinosum* wild type DsrC is in a mixture of dimer and monomer forms; no evidence was observed for the existence of an intramolecular disulfide bridge, which should give a band with no shift in the gel.

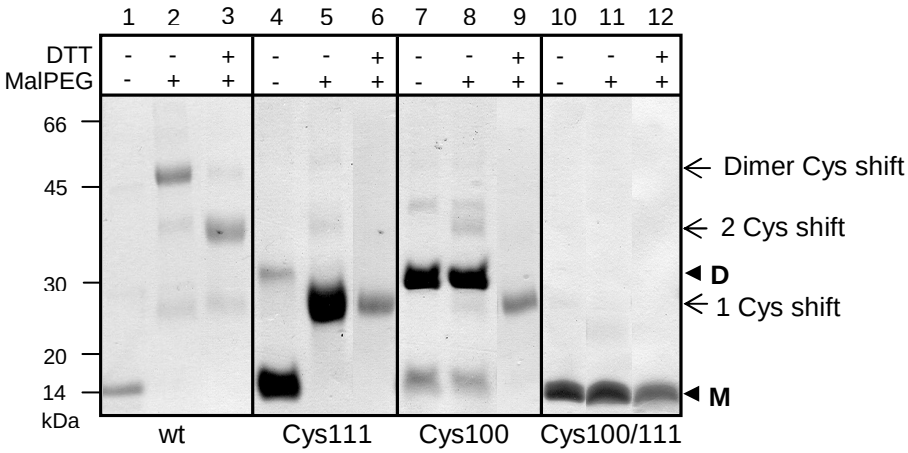


**Figure 4.1.3** – Analysis of *Al. vinosum* DsrC free sulphydryl groups with the MalPEG gel-shift assay, including the wild type (wt) protein, single (Cys111 and Cys100) and double (Cys100/111) mutants, as-isolated (lanes 1, 4, 7 and 10), after MalPEG treatment (lanes 2, 5, 8 and 11), and DTT reduction followed by MalPEG (lanes 3, 6, 9 and 12) (3 or 5 μg/lane). The arrow-heads indicate DsrC monomer (M) and dimer (D) not labelled with MalPEG, and the arrows indicate DsrC monomer labelled with one (1 Cys shift) or two (2 Cys shift) MalPEG groups, and also dimeric DsrC binding 2 MalPEG groups (Cys dimer shift). The samples are separated in a 10 % Tricine-SDS-PAGE in non-reducing conditions.

The *Al. vinosum* DsrC Cys111 mutant protein is mostly in monomeric form, and upon MalPEG labelling a single 10 kDa

band shift is observed, as expected (**Figure 4.1.3**, lane 4 and 5). In contrast, the Cys100 mutant is mostly in dimeric form, which shows no shift upon MalPEG treatment (**Figure 4.1.3**, lane 7 and 8). The much higher concentration of dimer in the Cys100 mutant *versus* the wt protein, reveals that Cys100 can reduce intramolecularly the Cys111-Cys111 disulfide bond of the dimer, as previously proposed (Cort *et al.*, 2008). As a control, the DsrC double Cys mutant was also tested and behaves as a monomer that does not react with MalPEG (**Figure 4.1.3**, lane 10 and 11). Previous treatment with the reducing agent DTT followed by MalPEG (**Figure 4.1.3**, lane 3, 6 and 9) led to the expected results: a two-cysteine shift for the wt protein, one-cysteine shift for Cys100 and Cys111 mutants, and no change for the double mutants. Thus, the study with *Al. vinosum* DsrC confirms that the gel-shift assay is adequate to monitor the solution redox state of this protein. Overall, the results show that as-isolated *Al. vinosum* DsrC contains no intramolecular disulfide bond, and that air exposure leads mainly to dimerization through an intermolecular disulfide bond involving Cys111, as reported before (Cort *et al.*, 2008).



## Redox behaviour of *D. vulgaris* DsrC

*D. vulgaris* DsrC has three cysteine residues: Cys26 close to the N-terminus, and the conserved Cys93 and Cys104 located in the C-terminal arm of the protein (equivalent to *Al. vinosum* Cys100 and Cys111). *D. vulgaris* DsrC was expressed in *E. coli* and due to the localization of the Cys residues the recombinant protein was constructed in order to have the His-tag tail positioned at the N-terminus. The MalPEG gel-shift

assay was used to study the redox behaviour of the *D. vulgaris* protein. To facilitate interpretation of the results a schematic representation is provided (**Figure 4.1.4**). Hereafter, the following nomenclature for *D. vulgaris* DsrC is employed: $DsrC_{red}$ – reduced state of the protein, with the three cysteines in thiol form; $DsrC_{ox}$ – oxidized form with an intramolecular disulfide bond; and $DsrC_{dimer}$ – oxidized form with an intermolecular disulfide bond.

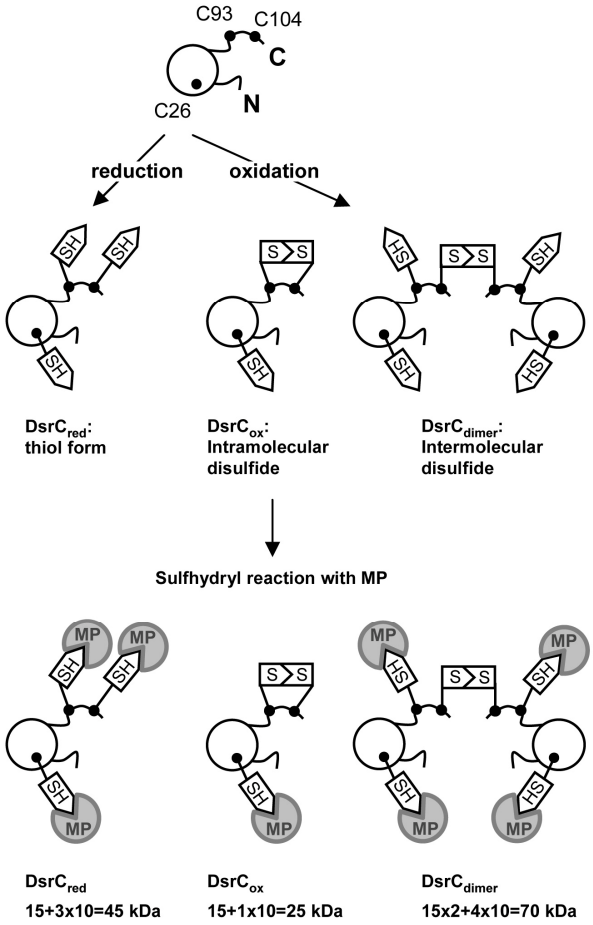


**Figure 4.1.4** – Schematic representation of the *D. vulgaris* DsrC redox states, and the corresponding MalPEG (MP) modification. This scheme does not represent the conformational rearrangements of the C-terminal arm.

MalPEG labelling of freshly purified *D. vulgaris* DsrC results in two main bands with 45 and ~70 kDa in a SDS-PAGE gel (**Figure 4.1.5**, lane 2). The 45 kDa protein band is assigned to the protein binding three MalPEG groups, indicating that the three Cys residues are reduced and structurally accessible to MalPEG modification, although the protein was purified without any reducing agent. A weaker 70 kDa band is assigned to the DsrC dimer (although this is not detected in the original sample) with four MalPEG groups bound to Cys residues not involved in the intermolecular disulfide bridge. Thus, the heterologous expression of *D. vulgaris* DsrC produces $DsrC_{red}$ as the major product, although some $DsrC_{dimer}$ is also observed as a minor product, probably as a consequence of aerobic manipulation. NMR spectroscopy (data not shown) and mass spectrometry (see below) also indicate that recombinant DsrC is produced mainly in monomeric form. This result was expected since the expression of recombinant proteins in the reducing *E. coli* cytoplasm does not allow the formation of disulfide bonds (mainly due to the presence of thioredoxin reductase and glutathione reductase) (Kadokura *et al.*, 2003). However, in one of the preparations, we observed also a band due to binding of only one MalPEG group, indicating that the other two Cys were engaged in the intramolecular disulfide bridge and thus in the $DsrC_{ox}$ state (**Figure 4.1.5**, lane 3).

Treatment of DsrC with DTT followed by incubation with MalPEG results in a single shifted band of 45 kDa corresponding to $DsrC_{red}$, as expected (**Figure 4.1.5**, lane 5). The same results were observed with a non-thiol sulfhydryl reductant, tris(2-carboxyethyl)phosphine (TCEP) (data not shown).

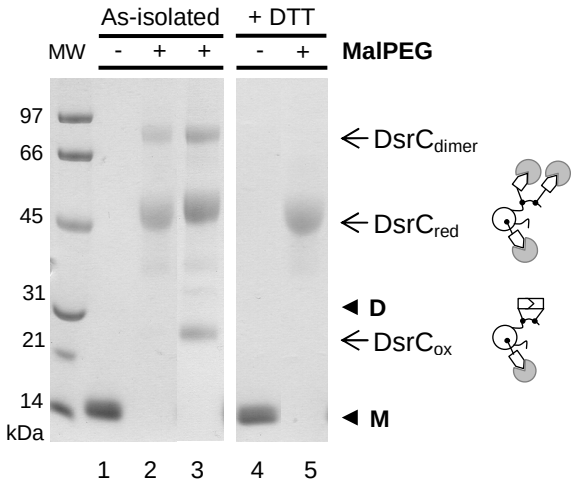


**Figure 4.1.5** – Gel-shift assay of *D. vulgaris* DsrC, as-isolated or previously reduced with 10 mM DTT. Samples treated (+) or not (-) with MalPEG 1 mM and resolved on a 10 % Tricine-SDS-PAGE gel (5 μg/lane). The arrow-heads indicate DsrC monomer (M) and dimer (D) not labelled with MalPEG and the arrows indicate DsrC labelled with one ($DsrC_{ox}$), three ($DsrC_{red}$) or four MalPEG moieties in case of dimerization ($DsrC_{dimer}$). Lanes 2 and 3 correspond to different preparations.

Since DsrC shows a tendency to dimerize in aerobic conditions, we tested other oxidizing conditions to try to obtain the $DsrC_{ox}$ form. TBHP, $H_2O_2$ and air exposure, have been previously described to produce this form, at least partially (Cort *et al.*, 2008; Mander *et al.*, 2005). We observed that protein treatment with chemical oxidizing agents such as TBHP and $H_2O_2$ enhance the dimerization of DsrC when compared with as-isolated DsrC (**Figure 4.1.6 A**, as-isolated *versus* $H_2O_2$ or TBHP, without MalPEG linkage). MalPEG labelling after treatment with either TBHP or $H_2O_2$ results in DsrC protein bands with no shift when compared with the non-labelled protein (**Figure 4.1.6 A**). This indicates that the Cys residues were oxidized into forms that could no longer react with MalPEG, which specifically reacts with

sulfhydryls (R-SH). Thus, TBHP and $H_2O_2$ can lead to formation of the intermolecular disulfide bond, but not the intramolecular one.

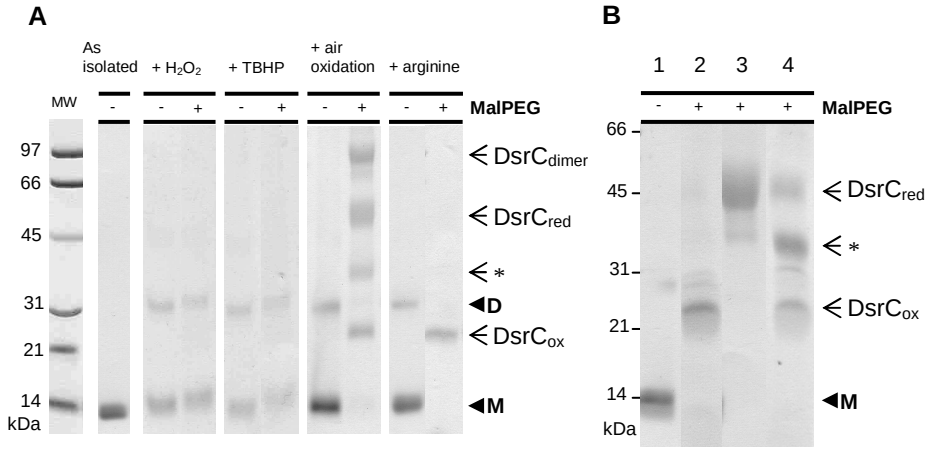


**Figure 4.1.6** – MalPEG gel-shift analysis of *D. vulgaris* DsrC with different oxidation treatments. A) chemical oxidation with 20 mM $H_2O_2$, with 10 mM TBHP, air oxidation for two weeks, or treatment with 1 M arginine. B) DsrC oxidized with arginine 1 M, followed by MalPEG treatment (lane 2), followed by reduction with 10 mM DTT (lane 3), or incubated for two weeks aerobically at 4 ºC (lane 4). Samples (5 µg/lane) were resolved on a 10 % Tricine-SDS-PAGE. DsrC binding only two MalPEG groups is indicated with (*).

We also tested prolonged air exposure as reported for *Al. vinosum* DsrC (Cort *et al.*, 2008). With *D. vulgaris* DsrC this condition resulted in quite a lot of protein precipitation. Gel-shift analysis of the air exposed protein revealed a series of bands from one to three MalPEG modifications of the DsrC monomer, plus the band corresponding to the dimer (**Figure 4.1.6 A**), indicating that non-specific oxidation is occurring with both intra and intermolecular disulfide bond formation. Molecular oxygen promotes *in vitro* disulfide formation but by a very slow and not very effective process (Kadokura *et al.*,

2003). Thus, prolonged air oxidation gives completely different results from the chemical oxidants, and generates a heterogeneous mixture of redox states in *D. vulgaris* DsrC. These results highlight the usefulness of the gel-shift assay to probe the oxidation state in solution. Overall, the data suggest that the interprotein disulfide bond ($DsrC_{dimer}$), observed in the as-isolated protein (**Figure 4.1.4**, lane 2 and 3) is the kinetic product of dimerization through the unhindered Cys104. In fact, in freshly prepared soluble fraction of DsrC from *D. vulgaris* cells the protein appears in the monomeric form, and after one purification step some dimerization is already observed, most likely due to aerobic manipulation (**Figure B.1** in Appendix B). Subsequently, the $DsrC_{dimer}$ may be converted to the monomeric $DsrC_{ox}$ form through internal reduction of the intermolecular disulfide by Cys93. Boiling of DsrC from *D. vulgaris* also promotes the intramolecular oxidation (**Figure B.2** in Appendix B). A similar process was observed to occur with *Al. vinosum* DsrC upon heat treatment of $DsrC_{dimer}$ (Cort *et al.*, 2008).

L-arginine is a low-molecular-weight additive that can suppress protein aggregation, and has been reported to facilitate *in vitro* formation of disulfide bonds through a still unknown mechanism (Chen *et al.*, 2008). Incubation of DsrC with arginine, followed by MalPEG treatment revealed a protein band with ~25 kDa (**Figure 4.1.6 A**), which corresponds to the $DsrC_{ox}$ form with two Cys residues engaged in an intramolecular disulfide bridge. The oxidation with arginine requires the presence of air (data not shown). To confirm the formation of the intramolecular disulfide bond on arginine-oxidized DsrC (**Figure 4.1.6 A** and **B**, lane 2), the $DsrC_{ox}$ protein was treated with

DTT, followed by MalPEG labelling, originating the $DsrC_{red}$ pattern (**Figure 4.1.6 B**, lane 3). Thus, arginine is a suitable catalyst to achieve specific oxidation of DsrC resulting in the intramolecular disulfide bridge. Subsequently, we tested the thermodynamic stability of the intramolecular disulfide bridge on $DsrC_{ox}$. This form of the protein was left in air at 4 ºC for 2 weeks and was then analyzed by the gel-shift assay. Despite the oxidizing environment, part of $DsrC_{ox}$ reverted back to the reduced state with two and three Cys available for MalPEG modification (**Figure 4.1.6 B**, lane 4), suggesting that the $DsrC_{ox}$ form is unstable.

In order to confirm the results obtained by the gel-shift assays we performed mass spectrometry measurements in collaboration with EMSL. Freshly prepared protein was treated with the cysteine-alkylating agent iodoacetamide that gives a mass shift of 57 Da per each thiol group available. The as-isolated DsrC not treated with iodoacetamide gives a mass peak of 13903 Da (*versus* the expected 13905 Da, lacking the amino terminal methionine residue), indicating it is in monomeric form. The treatment of DsrC with iodoacetamide resulted in protein precipitation, but the remaining soluble protein gave a mass peak of 13962 Da, which unexpectedly corresponds to DsrC containing one disulfide bond. The DTT reduced protein gave a mass peak of 14133 Da that corresponds to fully reduced cysteines (although surprisingly the mass shift corresponds to four iodoacetamide adducts, instead of three). The arginine oxidized DsrC resulted in a mass peak of 13961 Da, corresponding to DsrC with a disulfide bond. The analysis of the samples by electrospray on the IMS-TOF MS instrument showed a pattern for the arginine treated sample

different from the as-isolated and reduced forms (**Figure 4.1.7**). $DsrC_{ox}$ presents two distributions, while only one is present in the others. The extra distribution has less charged ions, meaning higher mass/charge ratio, and higher mobility.

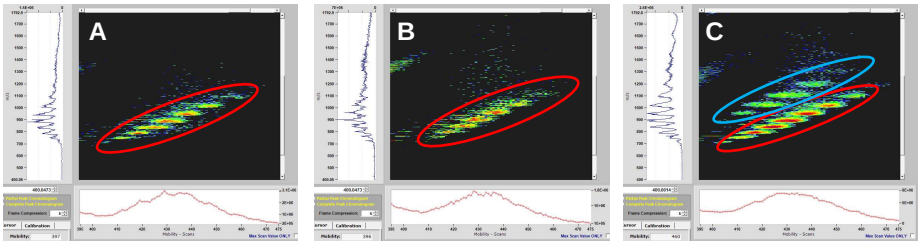


**Figure 4.1.7** – Electrospray IMS-TOF MS data of different redox states of DsrC. As-isolated (A), DTT reduced (B) and arginine oxidized DsrC (C) alkylated with iodoacetamide. The data is plotted m/z (mass over charge) *versus* mobility.

## The DsrC persulfide state

In the proposed mechanism for sulfite reduction (**Figure 4.1**) a DsrC persulfide is suggested to be an intermediate species. Therefore, we carried out preliminary experiments to address the issue of producing persulfurated DsrC. A persulfide (RS-SH) form can be distinguished from a thiol (R-SH) in the gel-shift assay by comparing MalPEG labelling followed by treatment with a reducing agent. Reduction of a labelled persulfide leads to loss of the MalPEG group, in contrast to a thiol.

To produce persulfurated DsrC the protein was incubated with excess sulfide as a sulfur donor. The assay was carried out with protein in the reduced state ($DsrC_{red}$) to guarantee that no persulfide is present before the addition of the sulfur donor. The presence of persulfide was detected by the difference of intensity in the shifted bands between non-reduced and DTT-reduced protein after sulfide treatment and labelling with

MalPEG. As shown in Figure 4.1.8, lane 4 *versus* 3, reduction of sulfide and MalPEG treated DsrC in anaerobic conditions led to increased intensity in the band for two MalPEG-modified Cys, indicating that one Cys persulfide is formed in DsrC after treatment with sulfide. However, there is also a slight increase for one MalPEG-labelled Cys or even in the no-shift band, showing that the treatment with sulfide may lead to persulfide formation in more than one Cys. Moreover, the persulfuration was not complete as there is still a band corresponding to $DsrC_{red}$.

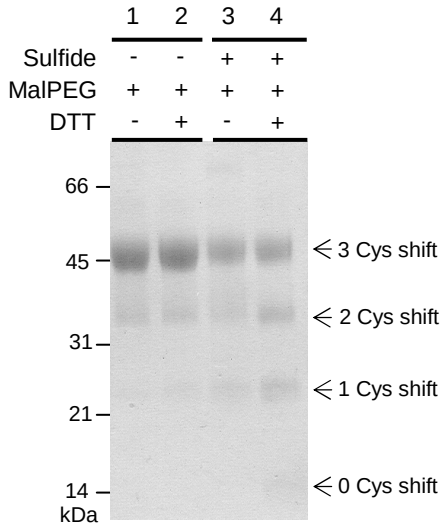


**Figure 4.1.8** – Generation of DsrC persulfide form. Reduced DsrC was treated (+) or not (-) with sulfide 4 mM, followed by MalPEG labelling, followed by reduction with excess of DTT. The experiments were performed in the anaerobic chamber and samples were subjected to a 10 % Tricine-SDS-PAGE (5 μg/lane). The arrows at the right side indicate the respective number of Cys residues labelled.

### 4.1.5 Discussion

The three-dimensional structures of DsrC have revealed a C-terminal swinging arm, carrying two conserved cysteines that may undergo thiol-disulfide interchanges as a redox active center (Cort *et al.*, 2001; Cort *et al.*, 2008; Mander *et al.*, 2005). The *dsrC* gene is strictly conserved among all sulfate reducing organisms as well as in sulfur oxidizing bacteria (Frigaard and Dahl, 2009; Pereira *et al.*, 2011), and a *dsrC* deletion mutant could not be stably produced in *Al. vinosum* pointing to an essential role in the cell (Cort *et al.*, 2008).

In *Desulfovibrio* spp. it was reported that the dissimilatory sulfite reductase (DsrAB) included a third subunit designated DsrC (Pierik *et al.*, 1992). However, in *D. vulgaris* the expression of DsrAB and DsrC was shown not to be coordinately regulated (Karkhoff-Schweizer *et al.*, 1993), and some sulfite reductases, as the one from *Archaeoglobus fulgidus* did not include DsrC (Dahl *et al.*, 1993; Molitor *et al.*, 1998). Later, Haveman *et. al.* reported that the relative expression level of *dsrC* was double that of *dsrABD* (Haveman *et al.*, 2003). Thus, several lines of evidence indicated that DsrC was an interacting partner of DsrAB forming a stable complex with this, but not a subunit of the enzyme. Our analysis of DsrC in the *D. vulgaris* soluble fraction shows that the majority of DsrC present in the cell is in fact not associated with DsrAB, and confirmed its high level of expression. This high expression is probably essential to guarantee a high turnover rate of sulfite reduction.

The role of DsrC relies on the two strictly conserved cysteines of the C-terminus, whose flexibility allows a close contact between these residues. This flexibility is conferred by

the presence of a proline residue (Pro101 in *D. vulgaris* DsrC), which is also strictly conserved and works as a hinge allowing the conformational changes observed for the C-terminal arm (Hsieh *et al.*, 2010; Oliveira *et al.*, 2011). The presence of the second conserved Cys in DsrC, which is absent in TusE, is conspicuous and highly suggestive of a thiol/disulfide interchange. The C-terminal Cys of TusE accepts sulfur from another protein, TusD of the TusBCD complex, to form a persulfide species (Ikeuchi *et al.*, 2006). By analogy, the terminal Cys of DsrC was proposed to accept a sulfane sulfur in the process of sulfite reduction, to generate a persulfide intermediate (DsrC-S-SH), which is in turn reduced by the preceding Cys (Oliveira *et al.*, 2008).

In this work we used a gel-shift assay to monitor the redox state of DsrC. This method is practical and easy to use since it is based on protein mobility in electrophoresis gels, and has been previously applied for topological studies of Cys (Lu and Deutsch, 2001). The assay was first tested with *Al. vinosum* DsrC, which has only two Cys residues for which mutants were available (Cort *et al.*, 2008). The results confirmed a high propensity of DsrC to dimerize through the terminal Cys, and showed no evidence for an intramolecular disulfide bond in the as-isolated *Al. vinosum* DsrC. Given the consistency of the results we concluded that the gel-shift assay could be used reliably.

In the case of *D. vulgaris* DsrC three Cys residues are present, which increases the number of possible modifications with MalPEG. In this case, the dimerization process also occurs quite rapidly in aerobic conditions, and it can be reversed

under reducing conditions. The recombinant *D. vulgaris* DsrC is found mainly in the reduced form, but we observed that formation of the intramolecular disulfide bond in the recombinant protein ($DsrC_{ox}$) was also possible, despite the reducing environment of the *E. coli* cytoplasm. The formation of cellular disulfide bonds usually involves catalysis (Kadokura *et al.*, 2003). The machinery involved, the Dsb proteins (disulfide bond) are thiol-disulfide oxidoreductases[1] located in the cell membrane facing the periplasm. Thus, proteins with stable disulfide bonds are located in the periplasm of bacteria (Heras *et al.*, 2007; Kadokura *et al.*, 2003). Nevertheless, there are a few examples of intracellular proteins with disulfide bonds, which are reported to be related to catalysis of redox processes and may be formed transiently in the cytoplasm (Choi *et al.*, 2001; Prinz *et al.*, 1997). Archaeal organisms seem to be an exception, since they have a high content of proteins with disulfide-bonds in the cytoplasm. This has been claimed to be a structural stabilization response to the extreme conditions where Archaea live (Mallick *et al.*, 2002). Interestingly, the archaeal DsrC proteins (from *P. aerophilum* and *A. fulgidus*) have a higher content of Cys residues, namely 5 and 4, and the non-conserved Cys were proposed to be involved in protein stabilization due to the higher growth temperatures, in contrast with mesophilic DsrC (Cort *et al.*, 2001; Mander *et al.*, 2005).

Our studies of the oxidizing conditions to promote formation of the intraprotein disulfide bridge had as a starting point

[1] The Dsb proteins are linked to the respiratory electron transfer chain, through the quinone pool. The Dsb proteins use redox active cysteines located in loops, for electron transfer during disulfide catalysis, and ultimately the electrons are conducted to the quinone pool (Kadokura *et al.*, 2003).

conditions previously reported to work with DsrC. An obvious experiment was to allow spontaneous disulfide formation through the action of molecular oxygen. This process is slow and unselective, and in this case two weeks of air oxidation were not enough to achieve total oxidation. Furthermore, two additional events occur simultaneously: dimerization and denaturation of the protein. The results from MalPEG modification clearly show that air oxidation results in a heterogeneous mixture of Cys redox states. Chemical oxidants were also tested for producing the $DsrC_{ox}$ state, namely *tert*-butyl hydroperoxide and hydrogen peroxide, as previously reported with DsrC from *A. fulgidus* and from *Al. vinosum*, respectively (Cort *et al.*, 2008; Mander *et al.*, 2005). Both oxidants give the same final result as deduced from the gel-shift assay: formation of the dimer form and oxidation of the thiol groups into higher oxidation states. Possibilities of oxidized products are sulfenic, sulfinic and sulfonic acid forms (with sulfur oxidation state of 0, +2 and +4, respectively, *versus* -2 in the thiol form). In the presence of oxidants the thiol group is first oxidized to a reversible form such as sulfenic acid (R-SOH), which can be reduced back to the sulfhydryl group. However, if oxidation of the sulfenic acid is not reverted, higher thiol oxidation states can be reached, such as sulfinic (R-SO(OH)) and sulfonic acids (R-SOH(=$O_2$)), which are irreversibly oxidized states (Reddie and Carroll, 2008). These oxidation states will not react with the thiol-reagent MalPEG, and as a result no band-shift occurs. Thus, the Cys oxidation profile of DsrC with air exposure *versus* chemical oxidants gives

completely different results, and none gives rise to the desired $DsrC_{ox}$ form.

The DsrC intramolecular disulfide bond could be formed by aerobic incubation of the protein with arginine. Arginine is the most basic amino acid, with a side-chain p$K_a$ for the guanidinium group of about 12.5. An alkaline pH will promote the formation of the thiolate anion ($R\text{-}S^-$) (the p$K_a$ of the cysteinyl thiol group ranges from 8.5 to 9.5, varying with the environment in the protein structure (Swaisgood, 2005)), which becomes a nucleophile that can attack another sulfur group, thus promoting the disulfide bond formation. The disulfide is easily formed when the Cys are close to each other, such as the CXXC motif present in the active site of the thiol oxidoreductase superfamily (includes thioltransferase, thioredoxin, glutaredoxin, and the Dsb proteins aforementioned) (Chivers *et al.*, 1997; Fomenko *et al.*, 2008). The paired vicinal cysteines contribute to a high localized thiol concentration (between 100 to 500 mM) promoting the oxidation into a disulfide bond, and some of the above referred proteins are part of the *E. coli* cytoplasmic machinery to reduce other protein's disulfide bonds (Requejo *et al.*, 2010; Ritz and Beckwith, 2001). In DsrC the two Cys are ten residues apart, but the flexibility of the C-terminal arm allows their spatial proximity.

Reduction of the arginine oxidized DsrC confirmed that a disulfide bond indeed resulted from this treatment. However, the $DsrC_{ox}$ state is apparently unstable since after two weeks at 4 ºC a heterogeneous redox state was observed. This suggests that the formation of the intramolecular disulfide bond brings

tension to the C-terminal arm and with time the S-S bond is disrupted, which also points to the fact that the oxidized form of the protein might be a transient stage.

To confirm the results observed from the MalPEG assays the as-isolated, DTT and arginine treated DsrC were also examined by mass spectrometry using an alkylating agent. The as-isolated DsrC protein surprisingly gave a result compatible with being oxidized, whereas by gel-shift assay the thiol groups were shown to be mostly in a reduced state. These contradictory results may have originated due to the iodoacetamide treatment that resulted in massive protein precipitation. This may have led to selection of the oxidized form by precipitation of the reduced one. The DTT or arginine treated samples showed mass peaks compatible with reduced and oxidized DsrC forms. Interestingly, by IMS-TOF MS the arginine oxidized DsrC showed a profile distinct from the two other forms, namely an extra set of peaks with higher mobility and less charged ions. This can be assigned to a more compact protein state, presumably a form with the intramolecular disulfide bond where the C-terminal arm is retracted. This result is very interesting, but further mass spectrometry studies will be performed to confirm this hypothesis.

The molecular mechanism of sulfite reduction is still unknown, but the DsrC persulfide is proposed to be a key intermediate. There are several examples that illustrate the importance of protein persulfides as sulfur donor in cellular processes, such as sulfur transfer into cofactors and thiolation of tRNAs (Hidese *et al.*, 2011; Kessler, 2006). So, the use of Cys persulfides has emerged as a widespread mechanism in

biological sulfur reactions, but there are few examples related with energy metabolism. An example of a persulfurated protein linked to energy metabolism is found in the SoxY protein, which is involved in dissimilatory sulfur oxidation (Sox) pathways of sulfur oxidizing bacteria (Quentmeier and Friedrich, 2001). The Sox pathway is responsible for periplasmic thiosulfate oxidation to sulfate and it is catalyzed by four proteins: SoxYZ, SoxAX, SoxB, and SoxCD. The SoxYZ complex is the central actor of the Sox enzyme system, interacting with the other three proteins. Besides playing a pivotal role, there are other aspects of SoxY that resemble DsrC: it is a redox active protein without cofactors, with a strictly conserved Cys located on a peptide swinging arm at the C-terminus (Sauvé *et al.*, 2007). The functional Cys was shown to be responsible for bearing sulfur compounds, namely a Cys persulfide intermediate, which is considered to be the substrate of SoxCD (Friedrich *et al.*, 2001). SoxYZ can carry several sulfur adduct forms (containing either a terminal sulfane or sulfone group) and exhibits interaction with distinct partner proteins, likely involving the Cys-carrier mobile arm (Friedrich *et al.*, 2008). The SoxY C-terminus is an invariant GGCGG pentapeptide, and in DsrC the carboxy-terminal is a 7-amino acid flexible arm with a highly conserved PKPTGCV sequence. The glycine residues positioned next to the carrier cysteine in both proteins are invariant, probably due to the requirement to avoid steric hindrance of the Cys (Mander *et al.*, 2005; Sauvé *et al.*, 2007).

Our preliminary data indicate that a Cys persulfide may be formed on DsrC upon sulfide donation, although this chemical process is not very efficient or specific. Other more specific

approaches will have to be tested, such as the use of cysteine and IscS (a cysteine desulfurase), which catalyzes the conversion of L-cysteine to L-alanine with the formation of a protein-bound persulfide sulfur (Hidese *et al.*, 2011; Wright *et al.*, 2006; Zheng *et al.*, 1993). The mechanism for L-cysteine desulfuration using the sulfane sulfur, present as a persulfide intermediate in the active site cysteine, is recognized in all cysteine desulfurases (*e.g.* IscS, NifS, SufS and CsdA) (Beinert, 2000; Hidese *et al.*, 2011; Mueller, 2006; Toohey, 2011). These are known to participate in biosynthethic pathways of sulfur incorporation in molecules and cofactors. Another possibility would be the use of DsrEFH instead of IscS, since in *Al. vinosum* it was recently demonstrated that DsrC accepts sulfur that is delivered by persulfurated DsrEFH (Stockdreher *et al.*, manuscript *in* preparation).

In summary, DsrC was shown to be an abundant protein in cell extracts, mostly not associated with DsrAB. We were successful in producing the physiological DsrC oxidized state with an intramolecular disulfide bond, and also the reduced form. These forms will allow future studies with physiological partners, namely DsrAB, the membrane-bound complex DsrMKJOP, and other proteins with the CCG motif, such as the Tmc and Hmc membrane-bound complexes, or also soluble proteins predicted by genome analysis (Hamann *et al.*, 2007; Pereira *et al.*, 2011). Preliminary data also indicate that DsrC Cys can form a persulfide group, an intermediate species also required to test the sulfite reduction model.

### 4.1.6 Acknowledgments

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# Chapter 4
## Section 4.2

# Studies on the interaction between the DsrMKJOP transmembrane complex and DsrC

### 4.2.1 Summary

In sulfate reducing organisms (SRO), the dissimilatory sulfite reductase DsrAB is the key enzyme responsible for the reduction of sulfite. A recently proposed mechanism for this process includes DsrC, a small soluble protein, and the DsrMKJOP transmembrane complex. The genes for these Dsr proteins are invariably present in sulfite reducing and sulfur oxidizing organisms, and in many cases they are co-localized in the genomes. DsrC is proposed to be directly involved in the process of sulfite reduction, and be the substrate for the DsrMKJOP complex, working as a disulfide reductase through a catalytic [4Fe-4S]$^{3+}$ cluster. In this study the electron transfer from menaquinol to the DsrMKJOP complex was tested using visible absorption and EPR spectroscopies. The [4Fe-4S]$^{3+}$ cluster and some of the hemes of the complex are reduced by menadiol, pointing to an electron route from the menaquinol pool to the cytoplasm. Evidence for a direct interaction between DsrC and DsrMKJOP was obtained by surface plasmon resonance studies and Far-Western blot. This interaction seems to favour the intramolecular disulfide-bonded DsrC over its reduced form, and specifically involves the DsrK subunit of the complex. These results give strong support to the proposed reduction of DsrC by the DsrMKJOP complex.

## 4.2.2 Introduction

The biogeochemical cycling of sulfur has a strong involvement of microbial life, both in the reduction of oxidized sulfur compounds, and in the oxidation of their reduced counterparts. Although these organisms perform opposite sulfur transformations, they share several proteins involved in sulfur metabolism, namely APS reductase and dissimilatory sulfite reductase (DsrAB). The first reduces the activated form of sulfate (APS, adenosine-5'-phosphosulfate) to sulfite and the second performs the reduction of sulfite. DsrAB is widespread among organisms that reduce sulfate, sulfite, and other sulfur compounds, and is found also in some phototrophic and chemotrophic sulfur oxidizers, where it is thought to work in the reverse direction (Frigaard and Dahl, 2009; Schedel *et al.*, 1979). The DsrMKJOP transmembrane complex is proposed to be involved in electron transfer to the cytoplasmic DsrAB. The Dsr complex is conserved in sulfate/sulfite reducing organisms, and it is also present in sulfur oxidizers, often in the same gene cluster as the *dsrAB* genes (Dahl *et al.*, 2005; Pereira *et al.*, 2007; Pott and Dahl, 1998). DsrC is another common protein among these sulfur-metabolising organisms (Cort *et al.*, 2008; Mander *et al.*, 2005), which is definitely involved in the process of sulfite reduction. The structure of the *D. vulgaris* DsrAB-DsrC complex showed that the C-terminal arm of DsrC protrudes inside the structure, such that one of the conserved cysteines is positioned next to the sulfite binding site (Oliveira *et al.*, 2008). A mechanism involving DsrC was proposed, in which a persulfide is formed in this last cysteine, which is

reduced intramolecularly by the other cysteine forming sulfide and an oxidized form of DsrC with a disulfide bond between the two cysteines.

The Dsr complex has been isolated from two sulfate reducing organisms, the deltaproteobacterium *Desulfovibrio desulfuricans* ATCC 27774 (Pires *et al.*, 2006) and the archaeon *A. fulgidus* (where it was named Hme) (Mander *et al.*, 2002). The complex is composed of five subunits, a periplasmic cytochrome *c* with three hemes (DsrJ), a periplasmic ferredoxin-like protein (DsrO), two integral membrane proteins (DsrM and DsrP), and a cytoplasmic Fe-S cluster protein (DsrK). In a recent genomic comparison covering several SRO, it was noticed that the *dsrMK* genes are the only strictly conserved ones among *dsrMKJOP* (Pereira *et al.*, 2011). This suggests that DsrMK is a minimum module for electron transfer, while the role of DsrJOP is unknown. The DsrMK module resembles the membrane-associated heterodisulfide reductase HdrED present in methanogens that have cytochromes and methanophenazine, a functional substitute of the bacterial menaquinone (Heiden *et al.*, 1994; Kunkel *et al.*, 1997; Thauer *et al.*, 2008). DsrM is a diheme cytochrome *b* that is embedded in the membrane (Pires *et al.*, 2006) and belongs to the superfamily of NarI (membrane subunit of respiratory nitrate reductase) (Berks *et al.*, 1995), like HdrE. DsrK lacks a signal peptide or transmembrane helices, indicating that it faces the cytoplasm. It contains two canonical $[4Fe\text{-}4S]^{2+}$ center motifs and a special cysteine rich motif designated as CCG domain at the C-terminus (Hamann *et al.*, 2007; Pires *et al.*, 2006). This CCG domain is involved in the binding of an unusual Fe-S cluster, a $[4Fe\text{-}4S]^{3+}$ center (Hamann

*et al.*, 2007; Hedderich *et al.*, 2005), that was also detected by EPR in DsrMKJOP from *D. desulfuricans* (Pires *et al.*, 2006) and in the Hme complex from *A. fulgidus* (Mander *et al.*, 2002). The midpoint redox potentials for reduction of this center are +130 mV and +90 mV in Dsr and Hme, respectively (Mander *et al.*, 2002; Pires *et al.*, 2006).

The $[4Fe\text{-}4S]^{3+}$ cluster, present in the HdrD subunit, is considered the catalytic site of HdrED, but is only observable upon addition of the coenzyme M thiol substrate (Madadi-Kahkesh *et al.*, 2001; Mander *et al.*, 2002). This catalytic redox center is responsible for the reduction of the CoM-S-S-CoB heterodisulfide into the two corresponding thiols (coenzyme M and coenzyme B) (Duin *et al.*, 2002; Hedderich *et al.*, 1998). The presence of Hdr-related subunits in the Dsr complex is very interesting and suggests the involvement of thiols-disulfides in sulfate respiration (Pires *et al.*, 2006). DsrC in its oxidized form has been proposed as a possible substrate for the complex (Cort *et al.*, 2008; Oliveira *et al.*, 2008).

In the present work, we have investigated the electron transfer between the menaquinol pool and the DsrMKJOP complex, and carried out several protein-protein interaction studies involving DsrC and the Dsr complex.



## 4.2.3 Material and Methods

### Bacterial growth and protein purification

*D. desulfuricans* ATCC 27774 (hereafter *D. desulfuricans*) was grown anaerobically according to (Postgate, 1984). Cell treatment, membrane preparation, washing and solubilization, as

well as, purification of the membrane-bound complex DsrMKJOP was performed as described earlier (Pires *et al.*, 2006). DsrC from *D. vulgaris* was expressed heterogously in *E. coli* with an His-tag tail at the N-terminal. Cloning and purification procedures were described in Section 4.1.3.

### Analytical methods

The protein purity was judged by 10 % Tricine-SDS-PAGE and protein concentration was determined by UV-visible spectroscopy using the absorption coefficient of 18.575 $mM^{-1}.cm^{-1}$ at 280 nm for DsrC, 53 $mM^{-1}.cm^{-1}$ at 630 nm for DsrAB (Wolfe *et al.*, 1994), and 132.4 $mM^{-1}.cm^{-1}$ at 555 nm for DsrMKJOP in the reduced state.

### Western blot

DsrC was detected with antibodies raised against recombinant DsrC. DsrB and DsrK were detected with antisera raised against artificial immunogenic peptides from these proteins (Davids Biotechnologie GmbH, Regensburg, Germany). Samples were subjected to 10 % Tricine-SDS-PAGE denaturing gels, and then transferred to 0.45 μm PVDF membranes during 10 min for DsrC or 1 h for DsrB and DsrK proteins at 100 V using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The transfer buffer used was 48 mM Tris, 39 mM glycine, 0.0375 % SDS pH 9.2. The membranes were dried overnight at room temperature; then incubated in buffer A (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.05 % Tween 20 and 1 % non-fat milk) together with primary antibody (DsrC, DsrK or DsrB) at 1:2000 dilution for 1 h at room temperature (Mansfield, 1995).

Membranes were incubated with anti-rabbit IgG antibody linked to alkaline phosphatase conjugate (Sigma) at 1:15000 dilution for 45 min. The colorimetric detection was performed in an alkaline phosphatase buffer plus a solution of nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Some modifications were introduced to the above protocol when Western blot was performed with non-denaturing gels (high resolution Clear Native-PAGE 5-15 % (Wittig *et al.*, 2007)), namely in the transfer conditions which was performed for 2 h, using as transfer buffer 25 mM Tris, 192 mM glycine pH 8.3.

### Far-Western blot

A total of 10 μg of purified Dsr complex was separated in a 10 % Tricine-SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was dried overnight at room temperature (following the hydrophobic protocol (Mansfield, 1995)), and was then incubated for 1 h with purified recombinant DsrC protein (25 nM) in buffer A. Unbound protein was removed from the membrane by three 10 min washes with 20 mM Tris-HCl pH 7.6, 150 mM NaCl. The membrane blot was then incubated with anti-polyHistidine primary antibody (Sigma) in a 1:3000 dilution for 1 h in buffer A, washed and incubated with secondary anti-mouse IgG linked to alkaline phosphatase conjugate (Promega) diluted 1:7500, and the detection was performed as described above. The Dsr complex was tested with anti-polyHistidine antibody as a control experiment, and no unspecific result was observed.

## Surface Plasmon Resonance (SPR) experiments

A BIAcore™ 2000 instrument coupled with CM5 or NTA (nitrilotriacetic acid) sensor chips (Biacore, GE Healthcare) were used for SPR measurements at 25 ºC using 10 mM HEPES, 150 mM NaCl, 50 μM EDTA, 0.005 % Tween 20 pH 7.4 as running buffer. DsrC was covalently immobilized using the amine coupling protocol in the case of CM5 chip. The NTA sensor chip was employed to bind histidine tagged DsrC after nickel activation of the surface. The surface of the chip was activated as recommended by the manufacturer and it was used with freshly preparations of DsrC (oxidized or reduced form, prepared as described in Section 4.1.3). Recombinant DsrC with a His-tag tail was immobilized manually in flow cell 2 in order to have ~450 Resonance Units (RU) for different redox states of the ligand, and kinetic experiments were run immediately. No regeneration was necessary as complete dissociation of the analyte was observed immediately upon stopping the injection. Flow cell 1 was used to correct for refractive index changes and nonspecific binding. DsrMKJOP complex sample was injected at a flow rate of 30 μl/min for 2 min over flow cells 1 and 2. Injections of the running buffer prior to each concentration series were conducted as a control and subtracted for baseline correction. Equilibrium dissociation constant ($K_D$) were determined from duplicate experiments, using equilibrium binding levels, with DsrMKJOP concentrations ranging from 12.5 to 400 nM, by nonlinear curve-fitting using Hyper32 software (University of Liverpool), and $K_D$ was determined according to $RU_{steady\text{-}state} = (RU_{max} \times [analyte])/(K_D + [analyte])$.

### Menadiol interaction experiments

Menadiol was obtained by reduction of menadione (Sigma) with sodium dithionite as described in (Fieser, 1940). The DsrMKJOP complex was reduced with excess of an ethanolic solution of menadiol. For a control of full reduction excess sodium dithionite was used. UV-visible was record in anaerobic conditions, and EPR samples were also prepared anaerobically and immediately frozen.

### Spectroscopic measurements

UV-visible spectra were obtained at room temperature in a Shimadzu UV-1603 or UV-1203 spectrometer, in case of anaerobic chamber. EPR spectra at X-band (9 GHz) were obtained with a Brucker EMX spectrometer equipped with an Oxford Instruments ESR900 flowing helium cryostat. EPR spectra were corrected for tube calibration and protein concentration.



## 4.2.4 Results

### Electron transfer from menadiol to the DsrMKJOP transmembrane complex

Biochemical characterization of the DsrMKJOP complex from *D. desulfuricans* membranes reported a heme content of three hemes *c* and two hemes *b*, in agreement with the expected values from sequence analysis of DsrJ and DsrM, respectively (Pires *et al.*, 2006). The interaction of DsrMKJOP with menadiol, a reduced menaquinone analogue, was investigated by UV-visible absorption and EPR spectroscopies to evaluate which

cofactors are reduced in this process. The spectrum of oxidized protein is dominated by the hemes absorption profile with an intense Soret peak at 412 nm. Upon the addition of menadiol to the oxidized Dsr complex approximately 43 % of the hemes are reduced compared with fully reduced state, achieved by addition of sodium dithionite (**Figure 4.2.1**). This corresponds to approximately two reduced hemes, and since the heme proportion is 2 heme *b per* 3 heme *c*, 43 % of heme reduction suggests that menadiol is able to reduce the hemes *b* of the DsrM membrane subunit, as previously reported (Pires *et al.*, 2006).

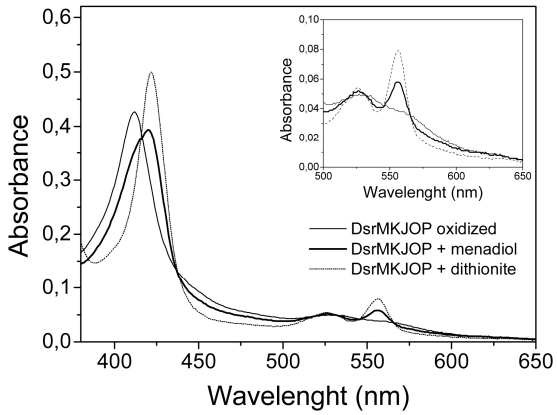


**Figure 4.2.1** – Reduction of the *D. desulfuricans* Dsr complex with menadiol observed by UV-visible spectroscopy. Oxidized state (thin line), menadiol reduced (bold line) and dithionite reduced (dashed line).

EPR spectroscopy was employed to have more detail about the Dsr cofactors reduced by menadiol, since the EPR spectrum of the Dsr complex is very informative. The oxidized spectrum shows several distinct signals assigned to low-spin hemes, and also an intense rhombic signal assigned to the $[4Fe\text{-}4S]^{3+}$ center (**Figure 4.2.2 A1**). This center displays very similar *g* values

($g_{max}$ = 2.021, $g_{med}$ = 1.985 and $g_{min}$ = 1.938) to the ones identified in Hdrs from methanogens (*g*-values of 2.013, 1.991 and 1.938 (Madadi-Kahkesh *et al.*, 2001)) and the Dsr-like complex from *A. fulgidus* (Mander *et al.*, 2002). The $[4Fe\text{-}4S]^{3+}$ center is considered the catalytic site mediating disulfide reduction in two one-electron steps (Dai *et al.*, 2007; Hedderich *et al.*, 2005). EPR spectrum of the menadiol-treated Dsr complex showed a reduced intensity of the $[4Fe\text{-}4S]^{3+}$ cluster, which is located in the cytoplasmic DsrK subunit (**Figure 4.2.2 A2**).

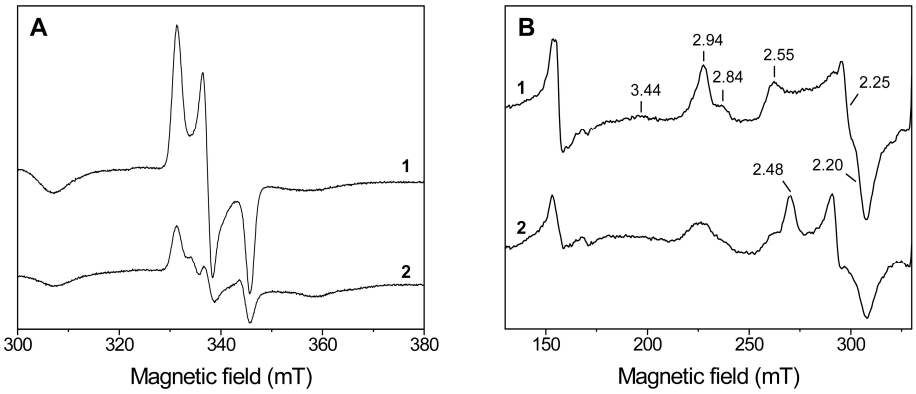


**Figure 4.2.2** – EPR spectra of the *D. desulfuricans* Dsr complex. (A) $[4Fe\text{-}4S]^{3+}$ cluster; (B) low-spin hemes; (1) oxidized state; (2) reduced with menadiol. Microwave frequency, 9.38 GHz; microwave power, 2 mW; modulation frequency, 100 kHz; modulation amplitude, 1 mT; temperature, 18 K.

Previous EPR studies indicated that the Dsr complex has three bis-histidine coordinated hemes (namely two hemes *b* and one heme *c*), with $g_{max}$ values of 2.94 and 2.84 (Pires *et al.*, 2006). The $g_{max}$ of 3.44 was assigned to a His/Met bound heme that usually have higher midpoint redox potentials than bis-His coordinated hemes (Reedy *et al.*, 2008). The $g_{max}$ = 2.55 was

attributed to an unusual His/Cys ligation in a heme *c* of DsrJ (Pires *et al.*, 2006). Similar EPR signals were displayed for other cytochromes with this peculiar axial ligation, including SoxAX and PufC from *Rhodovulum sulfidophilum* (Alric *et al.*, 2004; Cheesman *et al.*, 2001). Moreover, this EPR signal assignment was confirmed with the mutation in the corresponding cysteine axial ligand in *Al. vinosum* DsrJ (Grein *et al.*, 2010b). The broad shape of the signal at g ~ 2.55 was interpreted as resulting from the presence of several possible conformations for this heme, as observed for SoxAX, or the presence of magnetic interactions between the paramagnetic centers. This broad signal is replaced by a simple one at *g* value 2.48 upon reduction of the other hemes (Pires *et al.*, 2006).

The EPR spectrum of the oxidized Dsr complex is shown in Figure 4.2.2 B1. Upon treatment with menadiol there are three major differences in the heme signals of the Dsr complex spectrum (**Figure 4.2.2 B2**). First, the $g_{max}$ = 3.44 signal is abolished, meaning that the His/Met ligated heme is reduced, as expected by the reported redox titration (Pires *et al.*, 2006), in which this heme was the first to get reduced. The second difference regards the partial reduction of the signal with $g_{max}$ = 2.94 that corresponds to the His/His ligated hemes, which includes one or both of the DsrM hemes *b*. Thirdly, as a consequence of the reduction of these hemes, the 2.55 signal was also reduced and the 2.48 signal starts to be observed.

## Co-purification of DsrAB, DsrC and DsrMKJOP transmembrane complex

In the first step of purification of detergent-solubilized membrane proteins, aiming to isolate the DsrMKJOP complex, we noticed a fraction that contained both a signature for the complex (peaks at 422 and 555 nm in the reduced state), as well as for DsrAB (absorption at 630 nm due to sirohydrochlorin cofactor). Since the membranes were previously washed with buffer to remove unspecifically bound soluble proteins, this finding suggests that some DsrAB protein remained associated with the membrane through interaction with the DsrMKJOP complex, which is physiologically relevant.

The relative proportion of DsrAB and DsrMKJOP were roughly quantified using the absorption maxima at 630 and 555 nm and the respective absorption coefficients, and was found to be ~0.8 DsrAB: 1 DsrMKJOP. The co-purified proteins were analyzed by SDS-PAGE in parallel with pure DsrAB and DsrMKJOP proteins (**Figure 4.2.3 A**). The molecular masses of the several proteins allows a reasonably good distinction between them (DsrA, 50 kDa; DsrB, 40 kDa; DsrM, 38 kDa; DsrK, 61 kDa; DsrJ, 15 kDa (stains poorly with Coomassie); DsrO, 29 kDa. DsrP is a very hydrophobic protein of 43 kDa that does not run on the gel). The SDS-PAGE indicates that indeed the DsrAB and DsrMKJOP proteins are both present in this fraction (**Figure 4.2.3 A**, lane 3). To further check the band assignment we performed Western blot analyses using antibodies against DsrB, DsrK and DsrC (40, 61 and 11 kDa, respectively), which confirmed the presence of these proteins (**Figure 4.2.3 A**).

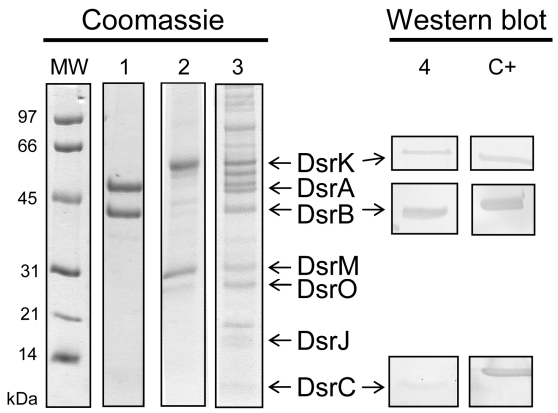


**Figure 4.2.3** – Gel electrophoresis analysis of the Dsr co-purified proteins in one of the membrane fractions of *D. desulfuricans*. 10 % Tricine-SDS-PAGE stained with Coomassie blue of pure DsrAB (lane 1), pure DsrMKJOP complex (lane 2), and fraction with co-purified proteins (lane 3); Western blot analysis of the fraction containing the co-purified proteins (lane 4, same sample as in lane 3) with antibodies against DsrK, DsrB and DsrC, respectively, using the pure proteins as positive control (lane C+).

Using high-resolution clear native gels we tried to identify if the DsrAB and DsrMKJOP proteins were forming a supercomplex, but this was not observed.

## DsrMKJOP complex and DsrC protein-protein interaction studies

To investigate the interaction between DsrC and the DsrMKJOP complex we used two different methods of studying protein-protein interactions: the biophysical technique of Surface Plasmon Resonance, and far-Western blotting.

*Surface Plasmon Resonance*

The SPR-based biosensor technology (BIAcore) allows direct monitoring of an interaction without the need for labelling

ligands, is very sensitive and requires small amounts of samples. This technique is particularly useful to test DsrC in different redox states, namely DsrC with an intramolecular disulfide bond ($DsrC_{ox}$) and DsrC with the three cysteines reduced ($DsrC_{red}$). For this, we took advantage of the His-tag tail in DsrC and used an NTA sensor chip that allows specific binding of these tags. This chip offers, additionally, the advantage of a mild immobilization of the ligand (DsrC), not requiring low pH (as widely used in CM sensor chips), as well as a controlled and homogeneous orientation of the ligand molecules. In this orientation, through binding by the His-tag positioned at the N-terminus, we can be certain that the C-terminal arm is available for interaction and not involved in binding to the chip. For the interaction experiments we used either freshly prepared $DsrC_{ox}$ (treated with arginine) or freshly prepared $DsrC_{red}$ (treated with dithiothreitol) as ligands, ensuring that we had a similar level of protein binding to the chip (similar level of resonance units) in each case. The DsrMKJOP complex was used as analyte. The assays show that there is a fast binding upon injection of the analyte, and a fast dissociation when the injection stops (**Figure 4.2.4 A**). The same type of sensorgram was observed using a CM5 sensor chip (data not shown).

Interestingly, a higher level of response is observed with $DsrC_{ox}$ as ligand compared with $DsrC_{red}$ (**Figure 4.2.4 A**). This effect is more obvious at higher DsrMKJOP concentrations (**Figure 4.2.4 B**). The kinetic parameters ($K_a$ and $K_d$) could not be

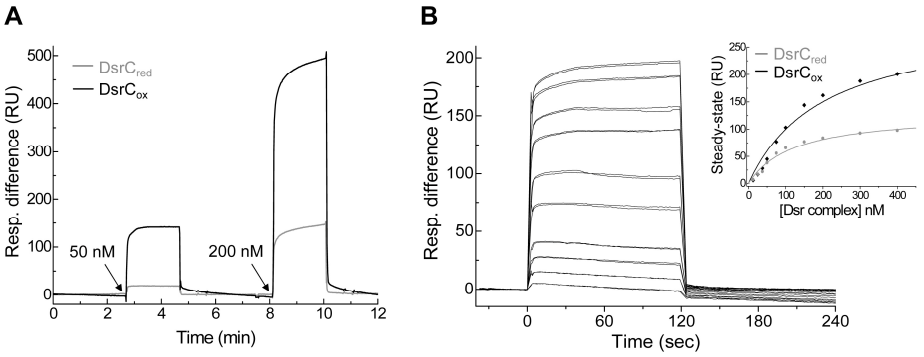


**Figure 4.2.4** – Binding of DsrMKJOP to DsrC immobilized in a NTA chip, in a reduced (grey) and oxidized (black) forms, monitored by SPR. A) Comparison of sensorgrams with $DsrC_{red}$ (grey) and $DsrC_{ox}$ (black) immobilized, at two different concentrations of DsrMKJOP. B) Interaction of DsrMKJOP with $DsrC_{ox}$ at 25 ºC with DsrMKJOP concentration ranging from 12.5 to 400 nM; inset shows a plot of the steady-state response *versus* the concentration of analyte, and the fitting for determination of the equilibrium dissociation constants. These sensorgrams have a reference channel without DsrC subtracted.

fitted globally with the algorithm for a one-to-one binding model, so we used equilibrium binding levels to determine the equilibrium dissociation constants ($K_D$) for $DsrC_{ox}$ and $DsrC_{red}$ (**Figure 4.2.4 B**). The steady-state binding levels were plotted against the concentration of the analyte, giving $K_D$ of $2.0x10^{-7}$ and $1.2x10^{-7}$ M for $DsrC_{ox}$ and $DsrC_{red}$, respectively (**Figure 4.2.4 B**). The $K_D$ values reveal a strong interaction ($K_D \leq 1x10^{-5}$ M) between DsrMKJOP and DsrC. Although, the steady-state affinity measurements are not significantly different, the maximum response for interaction ($R_{max}$) is higher for $DsrC_{ox}$ than for the reduced form, meaning that $DsrC_{ox}$ exhibits a higher ability to bind the Dsr complex.

*Far-Western blot*

To further probe the interaction between DsrC and the DsrMJOP complex we used Far-Western blot analysis, since this methodology allows identification of the specific proteins involved in the interaction. Thus, the DsrMKJOP complex was separated in a SDS-PAGE gel and transferred to a PVDF membrane. Then, the membrane was incubated with DsrC, the excess was removed by several washing steps, and the remaining DsrC bound to the membrane was detected with its antibodies (Wu *et al.*, 2007). In this case two primary antibodies were tested (anti-DsrC and anti-polyHistidine). In both cases the same result was obtained (**Figure 4.2.5**), indicating that DsrC interacts specifically and uniquely with the DsrK subunit of the complex. The control experiment without DsrC incubation did not show a band in the DsrK subunit, pointing to specific result in the Far-Western analysis (data not shown).



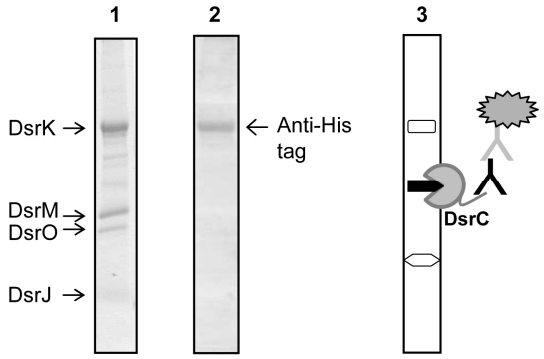


**Figure 4.2.5** – Far-Western blot analysis using DsrC as probe, and detection with anti-polyHistidine antibodies. 10 % Tricine-SDS-PAGE separation of purified DsrMKJOP complex stained with Coomassie blue (lane 1) and detection with anti-polyHistidine primary antibodies after incubation with recombinant His-tag DsrC (25 nM) (lane 2). Scheme of the Far-Western blot analysis of protein-protein interactions (lane 3).

### 4.2.4 Discussion

The proposed mechanism for sulfite reduction involving DsrAB, DsrC and the DsrMKJOP complex, demands experimental data to validate it. In the present study, we addressed the possibility of electron transfer from a reduced quinone to DsrMKJOP, and also the protein-protein interaction between the Dsr complex and DsrC.

Our results show that the Dsr complex accepts electrons from menadiol ($E^{o'}$ ~ -70 mV), an analogue of *Desulfovibrio* physiologic menaquinol. The UV-visible analysis suggests that ~40 % of the hemes are reduced, as previously reported (Pires *et al.*, 2006), and it was suggested that this corresponded to the two hemes *b* of the membrane-bound DsrM subunit. The macroscopic heme redox potentials determined previously for the DsrMKJOP complex of *D. desulfuricans* (+20, -60 and -110 mV), also indicated that not all the hemes could be reduced with menadiol (Pires *et al.*, 2006). To further investigate the reduction of DsrMKJOP by menadiol, EPR spectroscopy was also performed, which allows separate analysis of the hemes and Fe-S clusters, namely the catalytic $[4Fe\text{-}4S]^{3+}$ cluster. The EPR spectra of menadiol-reduced Dsr complex points to the reduction of one or two bis-histidine ligated hemes (most probably the hemes *b*) and the reduction of the heme with a His/Met coordination of the DsrJ subunit. Recently, the midpoint redox potentials of recombinant *Al. vinosum* DsrJ (-20, -200 and -220 mV) and DsrM (+110 and +60 mV) were determined (Grein *et al.*, 2010a; Grein *et al.*, 2010b).

The $[4Fe\text{-}4S]^{3+}$ centers substantially reduced upon addition of menadiol. This agrees with an electron transfer pathway from menadiol to the DsrM hemes *b*, and from there to the cytoplasmic $[4Fe\text{-}4S]^{3+}$ center in DsrK. In addition, the *Al. vinosum* DsrK protein was proposed to possess a monotopic membrane attachment (Grein *et al.*, 2010a), which may allow a short distance between the redox centers of DsrM and DsrK subunits.

This spectroscopic data supports the physiological role of the DsrMKJOP complex in oxidation of the menaquinol pool involving electron transfer through the $[4Fe\text{-}4S]^{3+}$ cluster of the DsrK subunit. This putative electron route highlights the hypothesis that the DsrMK module of the Dsr complex could have a function analogous to that of HdrED of methanogens.

A recent genomic analysis of all available genomes of sulfate reducing organisms showed that from the DsrMKJOP complex only the *dsrMK* genes are strictly conserved, while the *dsrJOP* genes are not present in Gram-positive sulfate reducers, and in the archaeon *Caldivirga maquilingensis* (Pereira *et al.*, 2011) (see Chapter 5). In contrast, the archaeon *A. fulgidus* has a *DsrMKJOP* gene cluster and also an extra *dsrMK* gene cluster, and in *Archaeoglobus profundus* a DsrMK complex was purified (Mander *et al.*, 2004). The invariant presence of the *dsrMK* genes among all SRO indicates that only those two proteins are essential for sulfite reduction. In many organisms (in 10 of the 25 SRO genomes analyzed) the *dsrMK* genes are located next or in the vicinities of the *dsrAB* and/or *dsrC* genes (see Chapter 5, namely **Figure 5.2**), a situation that also occurs in thiosulfate and sulfite reducers, as well as in several sulfur

oxidizing organisms. This observation reinforces the hypothesis of a direct interaction between the several Dsr proteins. One supporting evidence for such an interaction is the observation of co-elution of DsrAB and DsrC with the DsrMKJOP complex, upon purification of a membrane detergent extract, in a ratio of almost one sulfite reductase per membrane complex. The co-purification of several Dsr proteins was also reported in *Al. vinosum*, where the DsrKJO subunits of the complex were co-purified with the DsrAB sulfite reductase, DsrC and DsrEFH (Dahl *et al.*, 2005). In this sulfite oxidizing organism the Dsr encoded proteins are localized in a single operon constituted by 15 genes (Dahl *et al.*, 2005; Pires *et al.*, 2006; Pott and Dahl, 1998). In the case of *Al. vinosum* all the subunits of the DsrMKJOP complex were shown to be essential for oxidation of sulfur globules (Sander *et al.*, 2006), and very recently the same was shown with a deletion of the *dsrM* gene from *Chlorobaculum tepidum*, a phototrophic green sulfur bacterium (Frigaard *et al.*, 2011). In *Al. vinosum* a *dsrC* deletion mutant was unstable and could not be maintained, indicating that DsrC is essential for cell viability (Cort *et al.*, 2008). In *D. vulgaris,* DsrC forms a complex with DsrAB, but the majority of protein in the cell is not associated with it (Section 4.1), suggesting that indeed it is available for interaction with other functional partners, such as the DsrMKJOP complex.

Two different methods were employed to investigate protein-protein interactions between DsrC and DsrMKJOP: surface plasma resonance and Far-Western blotting. The two analyses clearly indicate a physiological association between both proteins. Actually, the two techniques complement each other

and corroborate the sulfite reduction model proposed by Oliveira *et al.*. Thus, $DsrC_{ox}$ interacts more with DsrMKJOP complex then $DsrC_{red}$, and this interaction specifically involves the DsrK subunit of the complex. The fast association and dissociation of DsrC and DsrMKJOP revealed by SPR are in agreement with the transient character of electron transfer interactions, which occur on a fast timescale. The $K_D$ parameters of $1.2x10^{-7}$ M and $2.0x10^{-7}$ M, determined by SPR for different redox states of DsrC, are very similar and indicates a high affinity between the DsrC and DsrMKJOP proteins (120 nM and 200 nM, respectively), whereas many studies by SPR report weaker protein-protein interactions in a μM range (*e.g.* (Buchele *et al.*, 2010; Neumann *et al.*, 2007)). $DsrC_{ox}$ has a higher ability to bind the Dsr complex, suggesting that this is the most likely redox form that interacts with the complex. In *Al. vinosum,* an interaction between recombinant DsrC and DsrK proteins was observed using co-elution assays (Grein *et al.*, 2010a), which is a technique that requires a high affinity binding.

In conclusion, these studies support the model proposed by Oliveira *et al.* for the reduction of sulfite. However, several questions remain to be further investigated. These include the electron donor to DsrAB, the putative catalytic activity of the $[4Fe\text{-}4S]^{3+}$ cluster on $DsrC_{ox}$, and finally the process by which proton translocation is associated with the electron transfer for sulfite reduction (Fitz and Cypionka, 1989; Fitz and Cypionka, 1991). However, given the lack of knowledge about the DsrJOP module other possibilities can not be ruled out.

### 4.2.6 Acknowledgments

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# CHAPTER 5

# A COMPARATIVE GENOMIC ANALYSIS OF ENERGY METABOLISM IN SULFATE REDUCING BACTERIA AND ARCHAEA

This chapter is part of a broader review published as:

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**S.S. Venceslau** conducted the genomic analysis of membrane redox protein complexes and participated in figures and tables preparation.

## 5.1 Summary

The number of sequenced genomes of sulfate reducing organisms (SRO) has increased significantly in the recent years, providing an opportunity for a broader perspective into their energy metabolism. In this work we carried out a comparative survey of energy metabolism genes found in 25 available genomes of SRO, focusing on proteins directly involved in the reduction of sulfate and membrane redox complexes. This analysis revealed a higher diversity of possible energy conserving pathways than classically considered to be present in these organisms, and permitted the identification of new proteins. The *Deltaproteobacteria* (and *Thermodesulfovibrio yellowstonii*) are characterized by a large number of cytochromes *c* and cytochrome *c*-associated membrane redox complexes, indicating that periplasmic electron transfer pathways are important in these bacteria. In contrast, the Archaea and *Clostridia* groups contain practically none, or few, cytochrome *c* and associated membrane complexes. Despite the absence of a periplasmic space, a few extra-cytoplasmic membrane redox proteins were detected in the Gram-positive bacteria. Several ion-translocating complexes were detected in SRO including $H^+$-pyrophosphatases, complex I homologs, Rnf, and Ech/Coo hydrogenases. In conclusion, this analysis indicates that energy metabolism of SRO is far more versatile, but more complex, than previously considered, and that both chemiosmotic and flavin-based electron bifurcating mechanisms provide possible strategies for energy conservation in SRO.

## 5.2 Introduction

Sulfate reduction is a true respiratory process, which leads to oxidative phosphorylation through a still incompletely understood electron-transfer pathway. This electron transport chain links dehydrogenases to the terminal reductases, which are located in the cytoplasm, and therefore, not directly involved in charge translocation across the membrane and generation of transmembrane electrochemical potential. In recent years, the advent of genomic information coupled with biochemical and genetic studies has provided significant advances in our understanding of sulfate respiration, but several important questions remain to be answered including the sites and mechanisms of energy conservation. These studies revealed that sulfate reduction is associated with a set of unique proteins. Some of these proteins are also present in sulfur-oxidizing organisms, whereas others are shared with anaerobes like methanogens. Most biochemical studies have focused on mesophilic sulfate reducers of the *Deltaproteobacteria*, mostly *Desulfovibrio* spp. (Matias *et al.*, 2005; Rabus *et al.*, 2007), but previous analyses indicated that the composition of energy metabolism proteins can vary significantly between different SRO (Junier *et al.*, 2010; Pereira *et al.*, 2007; Rabus *et al.*, 2007). The increasing number of SRO genomes available from different classes of both Bacteria and Archaea prompted us to perform a comparative analysis of energy metabolism proteins. In this work we report the analysis of twenty five genomes of SRO available at the Integrated Microbial genomes website. This includes 3 Archaea, 17 *Deltaproteobacteria* (of the *Desulfovibrionacae,*

*Desulfomicrobiacae, Desulfobacteraceae, Desulfohalobiacae, Desulfobulbaceae* and *Syntrophobacteraceae* families), 4 *Clostridia* (of the *Peptococcaceae* and *Thermoanaerobacterales* families) and *T. yellowstonii* DSM 11347 of the Nitrospira phylum (**Table 5.1**). This analysis extends a previous one in which only the *Deltaproteobacteria Desulfovibrio vulgaris* Hildenborough, *Desulfovibrio desulfuricans* G20 (recently renamed *Desulfovibrio alaskensis* G20 (Hauser *et al.*, 2011)) and *Desulfotalea psycrophila* were considered (Pereira *et al.*, 2007).

In SRO, redox membrane complexes are essential to link the soluble periplasmic oxidizing enzymes to the soluble cytoplasmic terminal reductases. They provide a pathway for electron flow to sulfate reduction, but may also be linked to charge separation across the membrane and ultimately with energy conservation. In this work we focus on genes coding for essential proteins for sulfate reduction and also on membrane redox complexes, including those that are present in all SRO (Qmo and Dsr), those that are present in only some SRO (*e.g.* Hmc, Nhc, Tmc, Qrc and Ohc) (Pereira *et al.*, 2007), and those that are widespread among prokaryotes (*e.g* complex I, complex III and the Rnf complex).

A general scheme depicting most of the proteins discussed is presented in Figure 5.1.

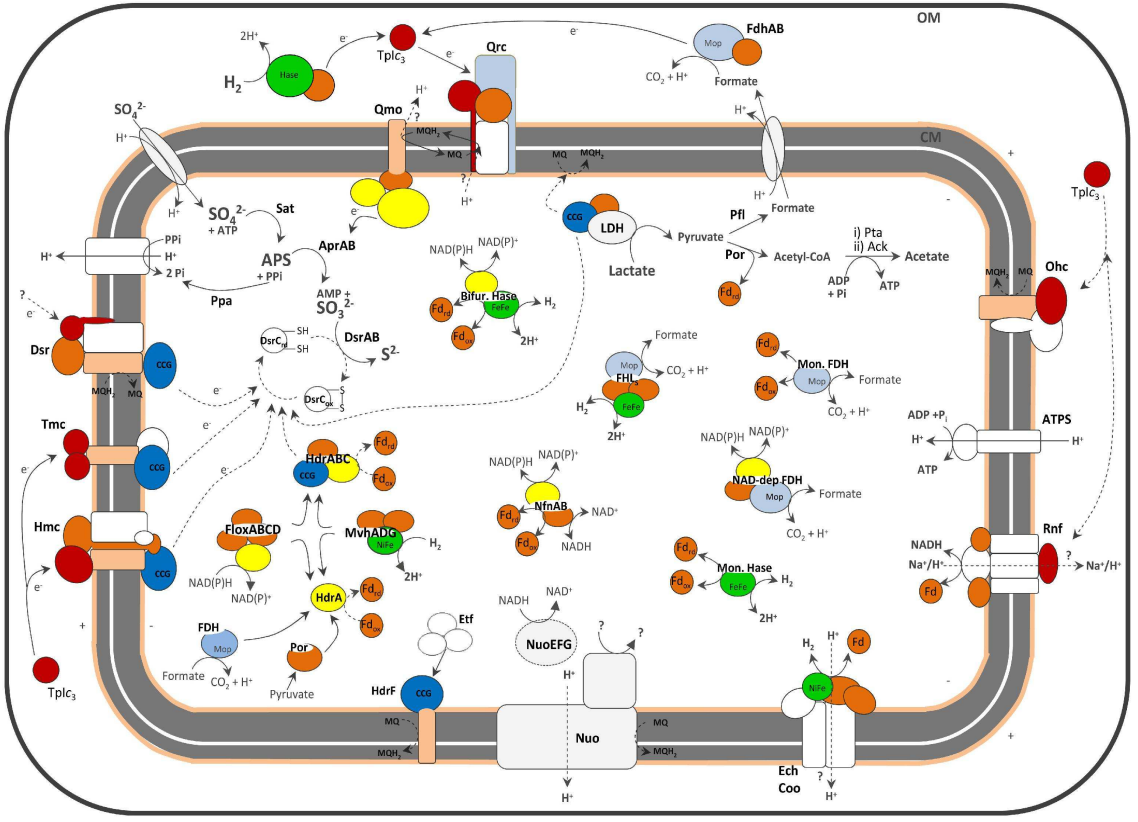


**Figure 5.1** – Schematic representation of the cellular location of SRO main energy metabolism proteins. No single organism is represented. For the exact distribution of proteins in each organism please refer to the Tables. The dashed lines represent hypothetical pathways, or (in the case of periplasmic hydrogenases (Hases) and formate dehyrogenases (Fdhs)) pathways present in only a few organisms.

## 5.3 Proteins essential for sulfate reduction

As expected, all organisms analyzed contain genes coding for those proteins long known to be directly involved in sulfate reduction (Rabus *et al.*, 2007), including sulfate transporters, ATP sulfurylase (*sat*), APS reductase (*aprAB*) and dissimilatory sulfite reductase (*dsrAB*). The hydrolysis of pyrophosphate is carried out by soluble inorganic pyrophosphatases in most cases, but in a few organisms a membrane-associated proton-translocating pyrophosphatase (Serrano *et al.*, 2007) is present, which may allow energy conservation from hydrolysis of pyrophosphate. These include the Gram-positive bacteria (Junier *et al.*, 2010), *Syntrophobacter fumaroxidans*, *Desulfococcus oleovorans*, *Desulfatibacillum alkenivorans* and *Caldivirga maquilingensis*. $F_1F_0$-ATP synthases are also present in all the SRO analyzed. Other strictly conserved proteins include ferredoxins (Fd), which are very abundant proteins in sulfate reducers (Moura *et al.*, 1994). All organisms analyzed contain FdI, which in some cases is present in multiple copies, and most contain also FdII.

One of the remaining important questions about sulfate reduction is the nature of the electron donors to the terminal reductases AprAB and DsrAB. Two membrane complexes, QmoABC and DsrMKJOP (**Figure 5.1** and **5.3**) have been proposed to perform this function (Pereira, 2008).

## The QmoABC complex

QmoABC (for Quinone-interacting membrane-bound oxidoreductase complex) was first described in *Desulfovibrio desulfuricans* ATCC 27774 (Pires *et al.*, 2003). It includes three subunits binding two hemes *b*, two FAD groups and several iron-sulfur centers. QmoA and QmoB are both soluble proteins homologous to HdrA, a flavin-containing subunit of the soluble heterodisulfide reductases (HDRs) (Hedderich *et al.*, 2005). HDRs are key enzymes in methanogens that catalyze the reduction of the CoM-S-S-CoB heterodisulfide, formed in the last step of methanogenesis, to the corresponding thiols (Hedderich *et al.*, 2005). The function of HdrA is still not clear, but it has been proposed to be involved in flavin-based electron bifurcation by an HdrABC/MvhADG complex, where the endergonic reduction of ferredoxin by $H_2$ is coupled to the exergonic reduction of the CoM-S-S-CoB heterodisulfide by $H_2$ (Costa *et al.*, 2010). QmoB includes also a domain similar to MvhD, a subunit of F420-non-reducing hydrogenase (Mvh) (Thauer *et al.*, 2010). QmoC is a fusion protein that contains a cytochrome *b* transmembrane domain related to HdrE and a hydrophilic iron-sulfur domain related to HdrC. Since the *qmo* genes are usually adjacent to *aprAB*, and both QmoC hemes are reduced by a menaquinol analogue, it has been proposed that Qmo transfers electrons from the quinone pool to AprAB, in a process that may result in energy conservation (Pires *et al.*, 2003; Venceslau *et al.*, 2010). Recently it was shown that in *D. vulgaris* Hildenborough the Qmo complex is essential for

sulfate, but not for sulfite reduction (Zane *et al.*, 2010). Our analysis confirmed that a gene locus containing *sat*, *aprAB* and the *qmoABC* genes is present in the majority of SRO analyzed, with the exception of the archaeon *C. maquilingensis* for which no *qmo* genes are detected. In the Gram-positive bacteria, namely *Desulfotomaculum acetoxidans* and *Candidatus* Desulforudis audaxviator, the *qmoC* gene is absent but is replaced by the *hdrBC* genes that code for soluble subunits of HDRs (Junier *et al.*, 2010). This suggests that in Gram-positive bacteria the reduction of APS reductase may involve soluble electron donors, rather than quinones.

## The DsrMKJOP complex

The *dsrMKJOP* genes were first reported in the sulfur-oxidizing bacterium *Allochromatium vinosum* as part of a *dsr* locus encoding also the *dsrAB* genes for the dissimilatory sulfite reductase and the *dsrC* gene, among others (Pott and Dahl, 1998). The DsrMKJOP complex was isolated from *Archaeoglobus fulgidus* (Mander *et al.*, 2002) (where it was named Hme) and *D. desulfuricans* ATCC 27774 (Pires *et al.*, 2006). It is a transmembrane complex with redox subunits in the periplasm - the triheme cytochrome *c* DsrJ, and the iron-sulfur protein DsrO; in the membrane - the cytochrome *b* DsrM (NarI family), and DsrP (NrfD family); and in the cytoplasm - the iron-sulfur protein DsrK that is homologous to HdrD, the catalytic subunit of the membrane-bound HdrED. DsrK and HdrD are both members of the CCG protein family characterized by a

conserved cysteine-rich sequence ($CX_nCCGX_mCXXC$), which includes over 2000 archaeal and bacterial proteins (Hamann *et al.*, 2007; Hedderich *et al.*, 1999). This Cys sequence binds a special [4Fe-4S] cluster, which in HDR is responsible for heterodisulfide reduction (Hedderich *et al.*, 2005), and is also present in Dsr (Pires *et al.*, 2006). Sequence analysis suggests that there may be two modules in the Dsr complex. One module, formed by DsrMK (based on its similarity to HdrED), may be involved in menaquinol oxidation and reduction of a cytoplasmic substrate, probably the DsrC disulfide (Oliveira *et al.*, 2008); a second module formed by DsrJOP may be involved in electron transfer between the menaquinone pool and a periplasmic component, but it is not clear in which direction. The *dsrMKJOP* genes are present in all SRO genomes analyzed, but in the Gram-positive bacteria (Junier *et al.*, 2010) and *C. maquilingensis* only the *dsrMK* genes are present. This indicates that only these two proteins are essential for sulfite reduction. Gram-positive bacteria lack a periplasmic space, which may explain the absence of DsrJO, and in these organisms DsrMK must transfer electrons between the menaquinone pool and the cytoplasm (as also suggested by the results presented in section 4.2). In organisms with a complete DsrMKJOP complex, electron transfer likely involves also periplasmic components. Several SRO contain both *dsrMKJOP* and one or more copies of *dsrMK*. A DsrMK protein has been isolated from *Archaeoglobus profundus* (Mander *et al.*, 2004).

## DsrC

The *dsrC* gene is also strictly conserved in all SRO. It is one of the most highly expressed genes in *D. vulgaris* Hildenborough (Haveman *et al.*, 2003; Wall *et al.*, 2008) and also in environmental samples where genes for dissimilatory sulfur metabolism were analyzed (Canfield *et al.*, 2010). All organisms containing a *dsrAB* sulfite reductase (sulfate/sulfite reducers or sulfur oxidizers) also contain the *dsrC* and *dsrMK* genes. DsrC is a small protein with a C-terminal swinging arm including two strictly conserved cysteines (Cort *et al.*, 2001; Mander *et al.*, 2005), which are proposed to be a redox-active center in the sulfite reduction pathway (Oliveira *et al.*, 2008). Our genomic analysis of SRO supports the interaction between DsrC, DsrAB and the DsrMKJOP complex: In *A. profundus* and *T. yellowstonii dsrC* is present in the same gene cluster as *dsrMKJOP*, and in the three Gram-positive organisms and *Ammonifex degensii,* a *dsrMK-dsrC* gene cluster is found (**Figure 5.2**). Strikingly, this cluster is preceded by a gene encoding a Fd. Moreover, a Fd gene is also present after the *dsrMKJOP* genes and in close proximity to *dsrAB* in three *Deltaproteobacteria* (**Figure 5.2**). This suggests that a Fd may also be involved in the electron transfer pathway between the Dsr complex, DsrC and DsrAB. The involvement of Fd provides a link between the sulfite reduction step and other soluble electron transfer pathways.

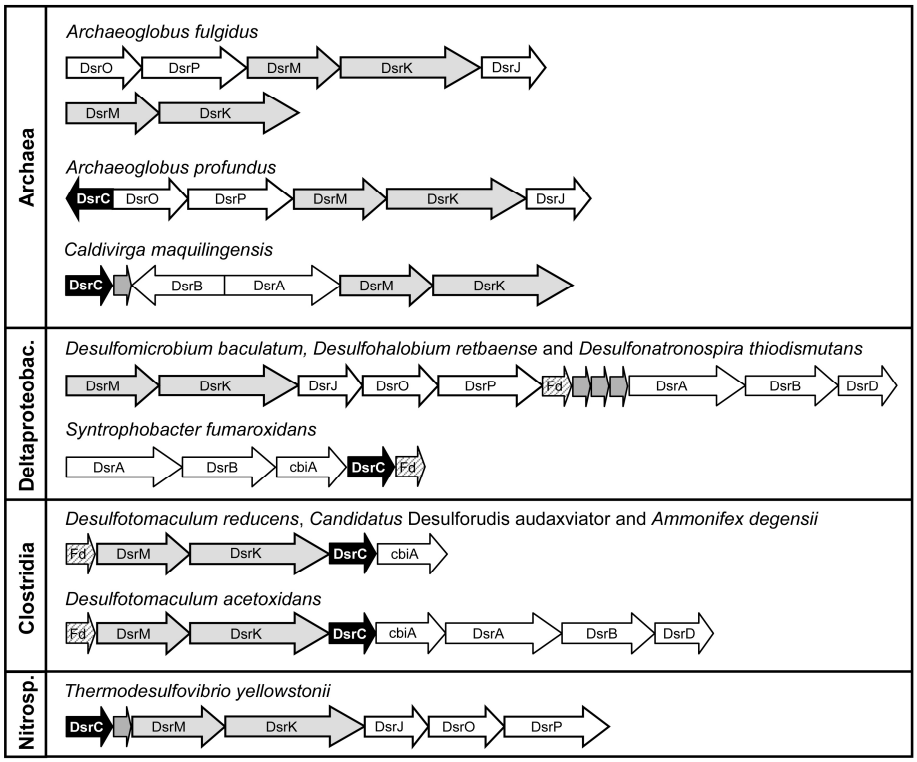


**Figure 5.2** – Co-localization of the *dsr* genes in SRO. Deltaproteobac., Deltaproteobacteria; Nitrosp., Nitrospira phylum; Fd, ferredoxin; cbiA, cobyrinic acid a,c-diamide synthase.

## 5.4 Membrane redox complexes

### Tmc, Hmc, Nhc and Ohc complexes

A family of transmembrane redox complexes that include a multiheme cytochrome *c* subunit has been described in *Desulfovibrio* (Pereira, 2008). The first complex identified was the Hmc complex composed of HmcABCDEF (Rossi *et al.*, 1993). The subunit composition of Hmc is strikingly similar to the Dsr complex in terms of the type of proteins present: a cytoplasmic CCG protein related to HdrD, two integral membrane proteins of the NarI and NrfD families, a periplasmic ferredoxin-like protein and a periplasmic cytochrome *c* (**Figure 5.1** and **5.3**). This suggests that both complexes have related functions, but the sequence identity between subunits is very low. The cytochrome *c* subunit is a large sixteen heme cytochrome in Hmc (HmcA) and a small triheme cytochrome in Dsr (DsrJ). HmcA can accept electrons from periplasmic hydrogenases *via* the TpI$c_3$ (Matias *et al.*, 2005; Pereira *et al.*, 1998), but this is not observed for DsrJ (Pires *et al.*, 2006). This cytochrome has a heme with an unusual histidine/cysteine ligation, but its function has not been elucidated (Grein *et al.*, 2010; Pires *et al.*, 2006). It is not clear if Hmc exchanges electrons with the quinone pool, or directly between the periplasm and cytoplasm. Some studies have indicated that the function of Hmc is in electron transfer to the cytoplasm during growth with hydrogen (Dolla *et al.*, 2000; Voordouw, 2002), but the *hmc* genes are downregulated under these conditions (Caffrey *et al.*, 2007; Pereira *et al.*, 2008). More recently this complex was shown to play a role during

syntrophic growth of *D. vulgaris*, where it was proposed to be implicated in the reverse electron transfer from the cytoplasm to the periplasm (Walker *et al.*, 2009).

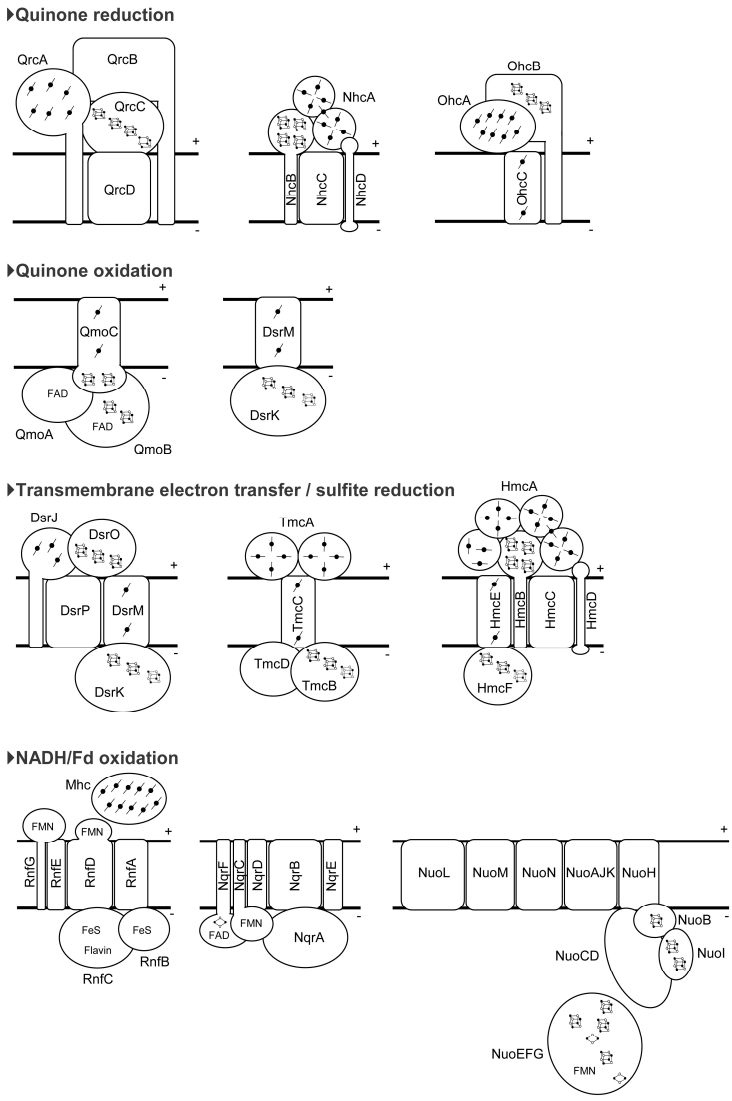


**Figure 5.3** – Schematic representation of the SRO membrane-bound electron transfer complexes, grouped in different categories according to expected function. The NuoEFG proteins are shown as one module, which is not always present.

The TmcABCD complex seems to be a simplified version of Hmc. It includes a tetraheme cytochrome $c_3$ (TmcA, first described as acidic cytochrome $c_3$ or Type II $c_3$ (Valente *et al.*, 2001)), a CCG protein homologous to HmcF (TmcB), a cytochrome *b* (TmcC) and a tryptophan-rich protein (TmcD) (Pereira *et al.*, 2006). TmcA is an efficient electron acceptor of the periplasmic Hase/TpI$c_3$ couple (Pieulle *et al.*, 2005; Valente *et al.*, 2005; Valente *et al.*, 2001). All redox centers of the Tmc complex are reduced by Hase in the presence of $H_2$ (Pereira *et al.*, 2006), and the *tmc* genes are upregulated in growth with hydrogen/sulfate *versus* lactate/sulfate (Pereira *et al.*, 2008), indicating that Tmc acts to transfer electrons from periplasmic $H_2$ oxidation to the cytoplasm.

Two other complexes related to Tmc and Hmc are present in the genomes of SRO. One includes a nine-heme cytochrome and is designated as Nhc complex (for nine-heme cytochrome complex) (Saraiva *et al.*, 2001), and the other includes an eight-heme cytochrome and was designated as Ohc (for octaheme cytochrome complex) (Pereira *et al.*, 2007). The NhcA cytochrome is similar to the C-terminal domain of the HmcA, and it is also reduced by the Hase/TpI$c_3$ couple (Matias *et al.*, 1999), whereas OhcA belongs to a different cytochrome family. OhcC is a cytochrome *b* of the NarI family, whereas NhcC membrane subunit is of the NrfD family. The subunits of the Hmc, Tmc, Nhc and Ohc complexes are homologous to each other, indicating they belong to the same family. However, the NhcC and Ohc complexes lack the cytoplasmic CCG protein,

suggesting they transfer electrons from the periplasm to the quinone pool. In contrast, both Hmc and Tmc include the CCG protein related to DsrK and HdrD, containing a binding site for a putative catalytic $[4Fe\text{-}4S]^{3+}$ center, which hints that they are implicated in similar thiol/disulfide redox chemistry as DsrK possibly involving $DsrC_{ox}$ (**Figure 5.1**).

The genomic analysis reveals that the Hmc, Tmc, Nhc and Ohc complexes (**Table 5.1**) are present in the *Deltaproteobacteria*, with the exception of the two members of the *Desulfobulbaceae* family. They are not present in the Archaea organisms or members of *Clostridia*. *T. yellowstonii* has only Hmc. This distribution correlates well with the presence of their putative electron donor, TpI$c_3$. All organisms that have Hmc usually also have Tmc, and some organisms have two copies of Tmc. In *D. desulfuricans* ATCC 27774 a three-subunit complex is found including a triheme cytochrome $c_7$, homologous to the N-terminal part of Hmc. Although its subunits are more similar to Hmc, the subunit composition is more characteristic of a Tmc. The Nhc complex has a more limited distribution, and in some organisms the cytochrome subunit has 13 hemes. In *Desulfonatronospira thiodismutans* the cytochrome subunit is not present.

**Table 5.1** – Analysis of membrane redox complexes distribution in the SRO genomes. The presence of periplasmic soluble Hases and Fdhs, and TpI$c_3$, is also indicated. MK only *dsrMK* genes present; † only *qmoAB* present; ***** *rnf* gene cluster without the multiheme cytochrome gene; ‡ $F_{420}H_2$:quinone oxidoreductase; * *nuo* gene cluster lacking *nuoEFG*; ◊ *nuo* gene cluster located separately from *nuoEFG* genes.

| | $H^+$-PPi | Dsr | Qmo | Peripl. Sol. Hase | Peripl. Sol. Fdh | $TpIc_3$ | Qrc | Tmc | Hmc | Nhc | Ohc | Rnf | Nuo | Nqr | $bc_1$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Archaea** | | | | | | | | | | | | | | | |
| *Archaeoglobus fulgidus* | | 1+2MK | 1 | | | | | | | | | | 1‡ | | |
| *Archaeoglobus profundus* | | 1 | 1 | | | | | | | | | | 1‡ | | |
| *Caldivirga maquilingensis* | 2 | MK | | | | | | | | | | | ? | | 1 |
| **Deltaproteobacteria** | | | | | | | | | | | | | | | |
| ***Desulfovibrionacae*** | | | | | | | | | | | | | | | |
| *Desulfovibrio aespoeensis* | | 1 | 1 | + | + | 2 | 1 | 1 | 1 | 1 | | 1 | 1* | | |
| *Desulfovibrio desulfuricans* G20 | | 1 | 1 | + | + | 2 | 1 | 1 | 1 | | | 1 | | | |
| *Desulfovibrio desulfuricans* ATCC 27774 | | 1 | 1 | + | + | 1 | | 1 | | 1 | | 1 | 1* | | |
| *Desulfovibrio magneticus* RS-1 | | 1 | 1 | + | + | 2 | 1 | 1 | 1 | | 1 | | 1*+1 | | |
| *Desulfovibrio piger* | | 1 | 1 | + | + | 1 | | | | 1 | | 1 | | | |
| *Desulfovibrio salexigens* | | 1 | 1 | + | + | 3 | 1 | 1 | 1 | | 1 | 2 | 1* | 1 | |
| *Desulfovibrio* sp. FW1012B | | 1 | 1 | + | + | 2 | 1 | 1 | 1 | | | | 1*+1 | | |
| *Desulfovibrio vulgaris* Hildenborough | | 1 | 1 | + | + | 1 | 1 | 1 | 1 | | 1 | 1 | | | |
| ***Desulfomicrobiacae*** | | | | | | | | | | | | | | | |
| *Desulfomicrobium baculatum* | | 1 | 1 | + | + | 2 | 1 | 1 | 1 | | 1 | 1 | | | |
| ***Desulfobacteraceae*** | | | | | | | | | | | | | | | |
| *Desulfatibacillum alkenivorans* | 1 | 1 | 1 | + | | 2 | 1 | | | 1 | 1 | 1 | | | |
| *Desulfobacterium autotrophicum* HRM2 | | 1 | 1 | + | + | 1 | 2 | 2 | | | | 1+1* | | 1 | |
| *Desulfococcus oleovorans* Hxd3 | 1 | 1 | 1 | | + | 2 | 1 | 2 | 1 | | 1 | 1+1* | | 1 | |
| ***Desulfohalobiacae*** | | | | | | | | | | | | | | | |
| *Desulfohalobium retbaense* DSM 5692 | | 1 | 1 | + | + | 4 | 1 | 1 | 1 | | 1 | 1* | | | |
| *Desulfonatronospira thiodismutans* ASO3-1 | | 1 | 1 | | + | 3 | | 2 | | 1 | | | 1* | | |
| ***Desulfobulbaceae*** | | | | | | | | | | | | | | | |
| *Desulfotalea psychrophila* | | 1 | 1 | + | | | | | | | | 1* | 1* | 1 | |
| *Desulfurivibrio alkaliphilus* | | 1 | 1 | + | | 2 | | | | | | | 1* | 1 | |
| ***Syntrophobacteraceae*** | | | | | | | | | | | | | | | |
| *Syntrophobacter fumaroxidans* MPOB | 2 | 1 | 1 | + | + | 1 | 1 | | | | 1 | 1* | 1◊ | | 1 |
| **Clostridia** | | | | | | | | | | | | | | | |
| ***Peptococcaceae*** | | | | | | | | | | | | | | | |
| *Desulfotomaculum acetoxidans* DSM 771 | 1 | MK | 1† | | | | | | | | | | 1* | | |
| *Desulfotomaculum reducens* | 1 | MK | 1† | | | | | | | | | | 1* | | |
| *C.* Desulforudis audaxviator MP104C | 1 | MK | 1† | | | | | | | | | | | | |
| ***Thermoanaerobacterales*** | | | | | | | | | | | | | | | |
| *Ammonifex degensii* KC4 | | MK | 1 | + | + | | | | | | | | | | |
| **Nitrospirae** | | | | | | | | | | | | | | | |
| *Thermodesulfovibrio yellowstonii* | | 1 | 1 | + | + | 1 | | | 1 | | | | 1* | | 1 |
| **No. of organisms** | 7 | 20/5 | 24 | 17 | 16 | 17 | 12 | 12 | 10 | 5 | 8 | 13 | 15 | 5 | 3 |

## Qrc complex

Recently, a new membrane complex named Qrc (for quinone-reductase complex) was isolated from *D. vulgaris* (Venceslau *et al.*, 2010) (work presented in Chapter 3). The Qrc complex accepts electrons from periplasmic hydrogenases (Hases) and formate dehydrogenases (Fdhs) through TpI$c_3$ and has activity as a TpI$c_3$:menaquinone oxidoreductase (Venceslau *et al.*, 2010). Our previous study showed that the *qrc* genes are present in sulfate reducers that have periplasmic Hases and/or Fdhs that lack a membrane subunit for quinone reduction (Venceslau *et al.*, 2010). The present analysis confirms this and shows that the *qrc* genes are found in many *Deltaproteobacteria*, but not in other SRO (**Table 5.1**). *Desulfovibrio piger* and *Dt. thiodismutans* both have soluble periplasmic Hases and Fdhs but lack a Qrc, but in both cases an alternative complex for quinone reduction is present, like Nhc and Ohc. An exception is *T. yellowstonii* that also has soluble periplasmic Hases and Fdhs and for which only the Hmc complex was identified. In this case maybe electrons go directly to the cytoplasm through Hmc or this is also capable of quinone reduction.

## Rnf, Nqr, and Nuo complexes for NADH and ferredoxin oxidation

Although it has long been known that NADH and ferredoxin (Fd) are important cytoplasmic components of energy metabolism in SRO, it is still not clear what role they play in the electron transfer chains of these organisms. The membrane-bound Rnf complex mediates electron transfer between NADH and Fd and is found in numerous organisms (Li *et al.*, 2006; McInerney *et al.*, 2007; Muller *et al.*, 2008; Seedorf *et al.*, 2008). The complex has been identified in the genomes of many bacteria and archaea, including aerobes, facultative anaerobes and anaerobes, some of which are pathogens (Biegel *et al.*, 2011). Examples include Gram-negative bacteria (*e.g. E. coli*, *Pseudomonas aeruginosa,* *Azotobacter vinelandii,* and *Haemophilus influenzae*), Gram-positive bacteria (*e.g. Clostridium tetani* and *Clostridium perfringens*), or the archaeon *Methanosarcina acetivorans* (Biegel *et al.*, 2011). It was first described in the purple nonsulfur bacterium *Rhodobacter capsulatus,* where six genes were identified to encode a membrane-bound electron transport complex (*rnfABCDEF*) related with electron transport to nitrogenase (and hence named *Rhodobacter* nitrogen fixation, Rnf) (Schmehl *et al.*, 1993). In nitrogen fixing bacteria, Rnf is proposed to catalyze the reverse electron transport from NADH to Fd, with this unfavourable redox reaction ($E^{o'}$ = -320 mV for NADH and $E^{o'}$ = -420 mV for ferredoxin (Thauer *et al.*, 2008)) being driven

by inward transport of sodium ions across the cell membrane (Schmehl *et al.*, 1993). In other bacteria the same enzyme apparently runs in the reverse direction: promoting electron transfer from reduced Fd to $NAD^+$ (ferredoxin:$NAD^+$-oxidoreductase), coupled to electrogenic $Na^+$ or $H^+$ translocation, *e.g.* in $Na^+$-dependent acetogens like *Acetobacterium woodii* and $H^+$-dependent ones like *Moorella thermoacetica* (Muller, 2003; Muller *et al.*, 2008). In the archaeon *M. acetivorans* ferredoxin is the electron donor and the electron acceptor is methanophenazine a quinone-like molecule (Biegel *et al.*, 2011; Wang *et al.*, 2011). The reduced methanophenazine transfers electrons to the heterodisulfide reductase HdrDE, responsible for the reduction of CoB-S-S-CoM, and so Rnf and HdrDE may be coupled in a redox loop for the generation of a proton gradient for ATP synthesis.

The Rnf complexes are constituted by 6-8 subunits, including three integral membrane proteins, and three flavin and/or Fe-S proteins (Biegel *et al.*, 2011) (**Figure 5.3**), which show similarity to $Na^+$-translocating NADH:quinone oxidoreductases (Nqr) (Steuber, 2001). The comparable subunit organization and the similarity between subunits of Rnf to Nqr led to the suggestion that Rnf is a $Na^+$ pump (Schmehl *et al.*, 1993). There is yet no direct biochemical confirmation that Rnf translocates ions, but indirect evidence was obtained for *A. woodii* Rnf, where activity studies were performed using $NAD^+$ as electron acceptor and ferredoxin and titanium (III) citrate as electron donor, in inverted membrane vesicles (Biegel and Muller, 2010). The activity of

$NAD^+$ reduction from reduced ferredoxin was shown to be coupled to $Na^+$ transport, and $Na^+$ accumulation was prevented by a $Na^+$ ionophore but not by protonophores. Additionally, several inhibitors also abolished $Na^+$ transport coupled to Rnf activity. The results are consistent with the proposal that the Rnf complex catalyzes $Na^+$-translocating ferredoxin:$NAD^+$ oxidoreductase activity (Biegel and Muller, 2010).

Rnf and Nqr complexes are small complexes when compared to the NADH:ubiquinone oxidoreductase (Nuo complex or complex I) with ~14 subunits (Efremov *et al.*, 2010) (**Figure 2.11**). The subunits of complex I are arranged in an "L-shaped" like structure (Efremov *et al.*, 2010; Guenebaut *et al.*, 1998). One arm of the "L" is soluble and is located in the periplasm, while the other arm is embedded in the lipid bilayer. All known redox groups (FMN and Fe-S clusters) are located in the peripheral arm which works as an NADH dehydrogenase, and the integral membrane subunits are involved in quinone reduction and proton translocation (Friedrich and Scheide, 2000). The NuoEFG subunits are considered the electron input module for NADH oxidation and are the less conserved subunits between different organisms, being absent in some cases (*e.g.* cyanobacteria and chloroplasts) (Battchikova *et al.*, 2011; Friedrich and Scheide, 2000). The archaeal complexes work as $F_{420}H_2$ dehydrogenase ($F_{420}H_2$ is the major cytoplasmic electron carrier of methanogens) rather than as NADH dehydrogenases (Kunow *et al.*, 1994).

In SRO our analysis shows that most organisms analyzed contain one, or more, of the Nuo, Rnf and Nqr complexes (except *C.* Dr. audaxviator and *A. degensii*) (**Table 5.1**). A surprisingly high number of SRO contain the *nuo* genes for complex I (15 in a total of 25 genomes). In two *Clostridia* organisms, Nuo is the only complex detected, besides the DsrMK complex. In the case of Archaea the complex I is present as a $F_{420}H_2$:quinone oxidoreductase (Kunow *et al.*, 1994). In most SRO the NuoEFG subunits that form the NADH dehydrogenase module are absent, suggesting that NADH is not the actual electron donor. It is tempting to speculate that these complexes also oxidize Fd. In *Desulfovibrio magneticus* and *Desulfovibrio* sp. FW1012B two clusters of *nuo* genes are present, one of which includes the *nuoEFG* genes.

The Rnf complex is present in most SRO, with the exception of the *Clostridia* and Archaea, suggesting it plays a key role in the energy conservation strategies of many sulfate reducers (**Table 5.1**). In most cases a multiheme cytochrome *c* encoding gene (with 4 to 10 hemes) is found next to the *rnf* genes as reported for *M. acetivorans* (Li *et al.*, 2006). Interestingly, *Desulfobacterium autotrophicum* and *Dc. oleovorans* have two copies of the *rnf* genes, and only one includes the cytochrome *c* gene. The presence of this cytochrome provides an electron input/output module in the periplasm, which may link the cytochrome *c* pool with NAD(H) and/or Fd. In *Deltaproteobacteria* the Rnf complex is always present when the Nuo complex is absent, but in other organisms there are cases

where both complexes are present simultaneously. The Nqr complex has a more limited distribution and is detected in only 5 of the 25 genomes analyzed. Of these, four are marine organisms (*Desulfovibrio salexigens*, *Db. autotrophicum*, *Dc. oleovorans* and *Df. psycrophila*) and the other (*Desulfurivibrio alkaliphilus*) is a haloalkaliphilic bacterium isolated from soda lakes, thus all are likely to have $Na^+$-based bioenergetics. Two of these organisms have copies of the three complexes (Nuo, Rnf and Nqr), namely *D. salexigens* and *S. fumaroxidans*.

## $H^+$-pyrophosphatases and others

The Gram-positive organisms, *C. maquiligensis* and a few *Deltaproteobacteria* contain ion-translocating pyrophosphatases, which are probably involved in energy conservation (**Table 5.1**). This is likely to compensate for the absence of other transmembrane complexes in some of these organisms. A $bc_1$ complex is also present in *C. maquiligensis*, *S. fumaroxidans* and *T. yellowstonii.* A *bd* quinol oxidase is present in 19 of the 25 organisms, and 7 contain a cytochrome *c* oxidase (**Table 5.2**).

**Table 5.2** – Analysis of cytochrome *c* oxidases and *bd* quinol oxidases distribution.

| | Cyt oxid | *bd* oxid |
|---|---|---|
| **Archaea** | | |
| *Archaeoglobus fulgidus* | | |
| *Archaeoglobus profundus* | | |
| *Caldivirga maquilingensis* | | |
| **Deltaproteobacteria** | | |
| ***Desulfovibrionacae*** | | |
| *Desulfovibrio aespoeensis* | | 1 |
| *Desulfovibrio desulfuricans* G20 | 1 | 1 |
| *Desulfovibrio desulfuricans* ATCC 27774 | | 1 |
| *Desulfovibrio magneticus* RS-1 | 1 | 1 |
| *Desulfovibrio piger* | | 1 |
| *Desulfovibrio salexigens* | | 1 |
| *Desulfovibrio* sp. FW1012B | 1 | 1 |
| *Desulfovibrio vulgaris* Hildenborough | 1 | 1 |
| ***Desulfomicrobiacae*** | | |
| *Desulfomicrobium baculatum* | 1 | 1 |
| ***Desulfobacteraceae*** | | |
| *Desulfatibacillum alkenivorans* | | 1 |
| *Desulfobacterium autotrophicum* HRM2 | 1 | 1 |
| *Desulfococcus oleovorans* Hxd3 | | 1 |
| ***Desulfohalobiacae*** | | |
| *Desulfohalobium retbaense* DSM 5692 | | 1 |
| *Desulfonatronospira thiodismutans* ASO3-1 | | 1 |
| ***Desulfobulbaceae*** | | |
| *Desulfotalea psychrophila* | | 1 |
| *Desulfurivibrio alkaliphilus* | 1 | 1 |
| ***Syntrophobacteraceae*** | | |
| *Syntrophobacter fumaroxidans* MPOB | | 1 |
| **Clostridia** | | |
| ***Peptococcaceae*** | | |
| *Desulfotomaculum acetoxidans* DSM 771 | | |
| *Desulfotomaculum reducens* | | 1 |
| *C.* Desulforudis audaxviator MP104C | | |
| ***Thermoanaerobacterales*** | | |
| *Ammonifex degensii* KC4 | | |
| **Nitrospirae** | | |
| *Thermodesulfovibrio yellowstonii* | | 1 |
| **No. of organisms** | 7 | 19 |

## 5.5 Concluding remarks

The comparative genomic analysis reported in this work provides new insights into the energy metabolism of SRO. By comparing phylogenetically distinct organisms capable of sulfate reduction we identified the proteins that can be considered as comprising the minimal set required for this metabolic activity: a sulfate transporter, Sat, a pyrophosphatase, AprAB, DsrAB, DsrC, DsrMK and Fd. The QmoAB proteins are also present in most organisms, being absent only in *C. maquiligensis*. In addition, we identified a higher diversity of possible energy conserving pathways than classically considered to be present in these organisms. The intracellular redox cycling of metabolites (like $H_2$, formate or CO) is not a universal mechanism, but should play a role in bioenergetics of *Deltaproteobacteria* and *T. yellowstonii*, which are characterized by a high number of cytochromes *c* and cytochrome *c*-associated membrane redox complexes. A high number of cytochromes *c* has previously been correlated with increased respiratory versatility in anaerobes (Thomas *et al.*, 2008), and such versatility is also suggested by the apparent redundancy of periplasmic redox proteins and membrane complexes found in many *Deltaproteobacteria*. Redox cycling is associated with energy conservation though charge separation and/or redox loop mechanisms, like the one proposed between Qrc and Qmo complexes (Venceslau *et al.*, 2010). In contrast, the Archaea and *Clostridia* groups contain practically no cytochromes *c* or

associated membrane complexes. The Gram-positive organisms analyzed present some unique traits including the absence of QmoC and DsrJOP proteins.

Overall, this analysis suggests that all SRO use diverse processes for energy conservation involving membrane-based chemiosmotic mechanisms. Many organisms include *nuo* genes for an ion-translocating complex I, which in most cases uses a still unidentified electron donor. Another widespread ion-translocating complex is Rnf, which together with Ech and Coo Hases, provides coupling sites for Fd-associated processes, such as electron bifurcation. The Rnf and Nuo complexes are among those widely distributed among SRO.

In conclusion, this analysis indicates that energy metabolism of SRO is far more versatile than previously considered, involving alternative strategies for energy conservation based on chemiosmotic mechanisms. An interesting aspect of (at least some) SRO is their ability to grow syntrophically in the absence of sulfate. In such situation some modules of this versatile redox machinery may operate in the opposite direction to that of respiratory conditions. Finally, it should be stressed that although drawing theories based on comparative genomic analysis is an attractive and even convincing exercise, no definite conclusions can be drawn until experimental evidence is provided. Thus, much work remains to be carried out to elucidate the bioenergetic mechanisms of SRO.

## 5.6 Acknowledgment

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## 5.7 Supplementary Material

The locus tags of all genes mentioned in Tables 5.1 and 5.2 can be found in Appendix C.

## 5.8 References

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# Chapter 6

# Concluding remarks

Chemiosmotic energy-generation mechanisms of sulfate reducing organisms are still a puzzle. This group of prokaryotes plays important roles in anaerobic ecosystems, where fermentation products abound, such as formate and hydrogen, which can function as carbon and energy sources to SRO. Oxidation of formate and $H_2$ is performed by periplasmic enzymes, and the electrons have to be transported across the membrane for the reduction of sulfate, that occurs in the cytoplasm. The pathways of electron flow, the participation of the quinone pool, and the coupling mechanisms for generation of a proton motive force are still not fully demonstrated and understood in SRO. Progresses have been made, namely with the isolation and characterization of membrane bound complexes. Two membrane redox complexes, Quinone-interacting membrane-bound oxidoreductase (QmoABC) and Dissimilatory sulfite reductase complex (DsrMKJOP), conserved in all sulfate reducers[1], have been suggested as possible electron donors for the adenosine phosphosulfate reductase or sulfite reductase, respectively. However, the electron entry point from the periplasmic oxidation of $H_2$ and formate into the membrane was still missing. This gap in the electron transfer chain was fulfilled with the isolation of a new membrane-bound complex, named Quinone-reductase complex (Qrc), from *Desulfovibrio vulgaris* Hildenborough.

Qrc receives electrons from hydrogen and formate oxidation, *via* the Type I cytochrome $c_3$ (TpI$c_3$), which are transferred to the menaquinone pool. Qrc is the first example of membrane

[1] There is one single exception, the *qmo* genes are not found in the archaeon *Caldivirga maquilingensis*.

bound complex in sulfate reducers able to reduce the menaquinone pool. In sulfate reducers, hydrogenases (Hase) and formate dehydrogenases (Fdhs) are soluble enzymes, while in other organisms they are usually linked to the membrane by a third subunit responsible for quinone interaction. Since this subunit is missing in SRO there is the need for a link to the membrane quinone pool. The genomic analysis of SRO showed that *qrc* genes are only present in *Deltaproteobacteria* sulfate reducers. Moreover, the organisms that have Qrc also have soluble Hase and/or Fdh enzymes, and the Tpl$c_3$.

We propose that a redox loop mechanism involving Qrc and Qmo acts to transfer electrons to the sulfate reduction pathway, coupled to proton release in the periplasm. Further studies have to be carried out to investigate in detail the electron transfer partnerships involving membrane-bound complexes, and to verify the electrogenic involvement of Qrc. Experiments with lipossomes containing both Qrc and Qmo complexes are planned for the near future.

The Qrc complex proteins have homology to the subunits of the complex iron-sulfur molybdoenzyme (CISM) family, including enzymes such as formate dehydrogenase or nitrate-, dimethyl sulfoxide-, thiosulfate-, polysulfide and tetrathionate reductases. However, the molybdopterin catalytic cofactor responsible for chemical catalysis is absent in Qrc, as it was found in two other bacterial respiratory complexes, the alternative complex III (ActB subunit) and complex I (Nqo3/NuoG subunit). The absence of the catalytic site of the CISM enzymes in these complexes implies that the respective subunits are not involved in chemical catalysis. This raises the question of the phylogenetic

relationship between Qrc and CISM complexes. Did Qrc evolve from CISM enzymes and lost the capacity to bind a molybdopterin cofactor (while gaining a cytochrome *c* subunit) or did both proteins evolve from an ancient precursor that did not have any catalytic cofactor and this was inherited later in CISM proteins? In early times the availability of molybdenum was low because the ocean was predominantly sulfidic, so it might be speculated that the ancestral protein lacked molybdenum. To address this issue studies using phylogenetic analysis of this family of enzymes should be carried out to look for evolutionary and ancestral relationships.

Qrc is rich in redox centers, since it contains six hemes *c*, one [3Fe-4S] cluster and at least three [4Fe-4S] clusters, that were characterized in detail by UV-visible and EPR spectroscopies. The midpoint redox potentials of redox centers in Qrc are in agreement with TpI$c_3$ as the electron donor, and menaquinol the electron acceptor. The Qrc complex was found to also form a supercomplex with TpI$c_3$ and [NiFe]-hydrogenase. Such supramolecular organizations have been frequently reported in aerobic respiratory chains.

The molecular mechanism of sulfite reduction has long been under debate. The complete reduction of sulfite to sulfide is a six-electron reaction. A new mechanism for this process was recently proposed based on the X-ray structure of *D. vulgaris* DsrAB bound to DsrC. The process includes DsrAB, DsrC and the DsrMKJOP membrane bound complex, in which DsrC plays a central role. The model demands experimental support, which was addressed in this study. The new model has already "inspired" geobiologists to revise the metabolic network fluxes

used in mathematical models of sulfur fractionations in the biological reduction of sulfate, which are used to date ancient sulfur metabolism in geological samples (see for example (Bradley *et al.*, 2011)[2]).

The role of DsrC in sulfite reduction involves its two conserved cysteines, and two aspects are key to the process: 1) the involvement of a DsrC persulfide as an intermediate species responsible for $H_2S$ release; and 2) the intramolecular disulfide bond in oxidized DsrC as a substrate for the DsrK subunit of the Dsr complex. The preliminary results presented herein agree with the proposed mechanism: i) most DsrC in the cell is not associated with DsrAB, showing that is available to interact with other proteins; ii) the Dsr complex co-purifies with DsrAB and DsrC; and iii) the DsrC protein, namely its oxidized form, interacts with the Dsr complex through DsrK. Nevertheless, further work has to be carried out to test the proposed mechanism of sulfite reduction.

The data described here points to electron transfer from the menaquinol pool to the hemes *b* of DsrM, and then to the catalytic $[4Fe\text{-}4S]^{3+}$ cluster in DsrK. It will be fundamental to show that DsrK can reduce oxidized DsrC, and whether this can be coupled to proton transfer. The transfer of two electrons from the quinone pool to sulfite reduction *via* DsrC implies that DsrAB needs four electrons from its donor, and not six. The electron donor of DsrAB is not known, but is very tempting to consider a ferredoxin-reducing protein, or even a ferredoxin, as

[2] **Bradley, A. S., Leavitt, W. D. and Johnston, D. T.** (2011). Revisiting the dissimilatory sulfate reduction pathway. *Geobiology* **9**, 446-457.

an hypothesis, because DsrAB has a ferredoxin domain placed to transfer electrons to the active site. In the case of assimilatory sulfite reductases (which do not have the ferredoxin domain) ferredoxin is the electron donor. The genomic analyses of SRO showed that in some cases a ferredoxin gene is located in the vicinity of the *dsr* genes. Unravelling the electron donor to DsrAB is a fundamental issue towards the understanding of the sulfite reduction pathway.

The genomic survey also showed that only the *dsrMK* genes are strictly conserved. The DsrMK proteins are related to HdrED of methanogens, and seem to be the minimum module of the Dsr complex necessary for sulfate reduction. This fact gives more support to the idea that DsrMK is involved in electron transfer from the menaquinol pool, as suggested by our data. In the case of methanogens, the electron donor to HdrED is the reduced methanophenazine pool. In *Methanosarcina mazei* and *Methanosarcina barkeri* HdrED is connected to a membrane bound [NiFe]-hydrogenase (Vht) that reduces methanophenazine through an electrogenic redox loop mechanism. Interestingly, sulfate reducers from the *Clostridia* genus that contain only the DsrMK module, are also the only ones with a membrane-associated [FeFe]-hydrogenase. This indicates that perhaps an analogous redox loop to VhtACG and HdrED is operational in *Clostridial* sulfate reducers.

In the case of *Desulfovibrio desulfuricans* ATCC 27774 the Dsr complex was isolated as DsrMKJOP, so the DsrJOP module is intrinsically associated with the DsrMK module. Our results indicate that this complex is not an electron acceptor of cytochrome TpI$c_3$, in contrast to the Qrc complex. It is still not

clear what is the physiological role of DsrJOP, namely whether DsrJ is an electron entry or exit point, or a catalytic subunit. Considering a putative catalytic activity, due to the comparison with SoxAX cytochrome that also has an unusual His/Cys heme coordination, the question is what could be a possible substrate for DsrJ, since all sulphur chemistry in SRO is assumed to take place in the cytoplasm.

Our genomic analysis indicates that the Rnf complex is present in several SRO and absent in all Archaea, *Clostridia* and *Nitrospira* sulfate reducers, but in some of those the Nuo complex is present. The intriguing question about the function of Nuo complexes in SRO is that in most cases the NADH electron input model (NuoEFG) is missing, so the electron donor to the complex is not known. The roles of Rnf and complex I homologs in the metabolism of SRO have yet to be investigated.

In summary, the work of this thesis contributed to the clarification of the electron transfer chains in sulfate respiration, namely by the isolation of a new respiratory complex – Qrc – and also by studies involving DsrC and the DsrMKJOP complex. Nevertheless, many questions still remain to be answered.

# Appendixes

# APPENDIX A

## Supplementary Material of Chapter 3

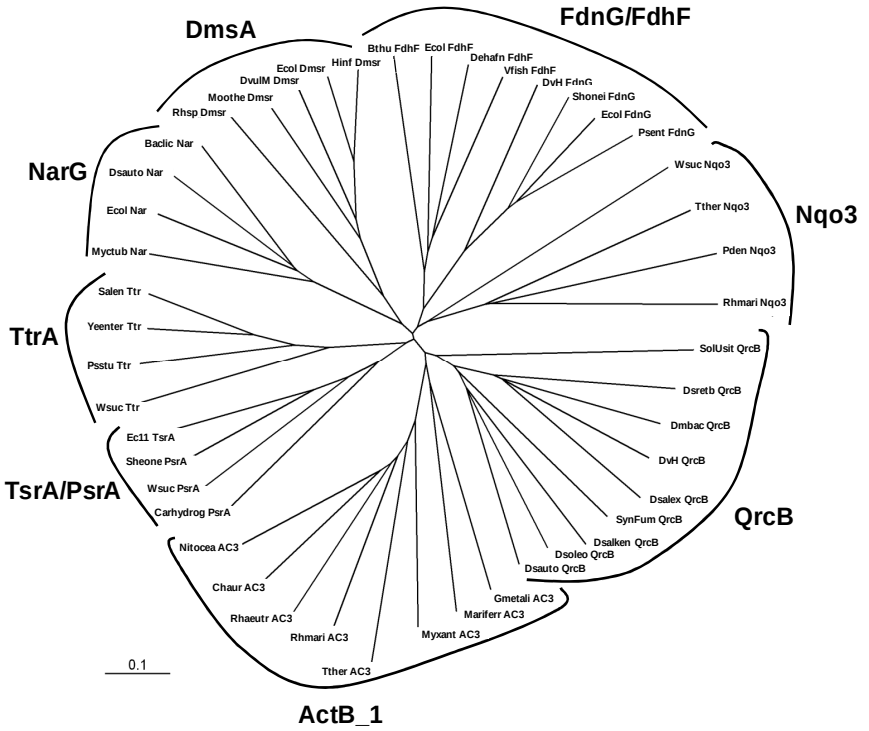


**Figure A.1** – Dendogram for QrcB proteins and related subunits. Abbreviations: QrcB, Quinone reductase complex, subunit B; ActB_1, Alternative Complex III, domain 1 of subunit B; PsrA, Polysulfide reductase, subunit A; TtrA, Tetrathionate reductase, subunit A; TsrA, Thiosulfate reductase, subunit A; NarG, Nitrate reductase, alpha subunit; DmsA, Dimethyl sulfoxide reductase, alpha subunit; FdhF, Formate dehydrogenase F, alpha subunit; FdnG, Formate dehydrogenase G, alpha subunit; Nqo3, NADH-quinone oxidoreductase, subunit 3.

Organisms: DvH, *D. vulgaris* Hildenborough; DvulM, *Desulfovibrio vulgaris* Miyazaki; SynFum, *Syntrophobacter fumaroxidans* MPOB; Dsoleo, *Desulfococcus oleovorans* Hxd3; Dsalken *Desulfatibacillum alkenivorans* AK-01; SolUsit, *Solibacter usitatus* Ellin6076; Dmbac, *Desulfomicrobium baculatum* DSM 4028; Dsalex, *Desulfovibrio salexigens* DSM 2638; Dsretb, *Desulfohalobium retbaense* DSM 5692; Dsauto, *Desulfobacterium autotrophicum* HRM2; Gmetali, *Geobacter metallireducens* GS-15; Mariferr, *Mariprofundus ferrooxydans* PV-1;

Myxant, *Myxococcus xanthus* DK 1622; Rhmari, *Rhodothermus marinus* DSM 4252; Chaur, *Chloroflexus aurantiacus* J-10-fl; Nitocea, *Nitrosococcus oceani*; Rhaeutr, *Ralstonia eutropha* JMP134; Tther, *Thermus thermophilus*; Carhydrog, *Carboxydothermus hydrogenoformans* Z-2901; Wsuc, *Wolinella succinogenes*; Sheone, *Shewanella oneidensis*; Yeenter, *Yersinia enterocolitica* 8081; Psstu, *Pseudomonas stutzeri* A1501; Salen, *Salmonella enteritidis* PT4; Ec1l, *Escherichia coli* SE11; Ecol, *Escherichia coli* K12; Myctub, *Mycobacterium tuberculosis*; Baclic, *Bacillus licheniformis*; Rhsp, *Rhodobacter sphaeroid*es; Hinf, *Haemophilus influenzae*; Moothe, *Moorella thermoacetica* ATCC 39073; Bthu, *Bacillus thuringiensis*; Dehafn, *Desulfitobacterium hafniense*; Vfish, *Vibrio fischeri*; Shonei, *Shewanella oneidensis*; Psent, *Pseudomonas entomophila* L48; Pden, *Paracoccus denitrificans* PD1222.

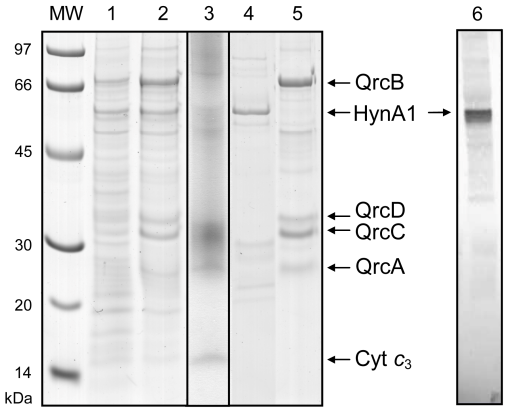


**Figure A.2** – Tricine-SDS-PAGE of the Qrc fraction from the first chromatographic step (lane 1), of the Qrc fraction from the second chromatographic step (lane 2, 3 and 6), of the first fraction from the IMAC column (lane 4); and of the purified Qrc (lane 5). Lanes 1, 2, 4 and 5 are stained with Coomassie; lane 3 is stained for hemes and lane 6 corresponds to a Western blot with antibodies against [NiFe] Hase from *D. vulgaris.* The primary antibody was used at 1:500 dilution for 1 h at room temperature followed by incubation with anti-mouse IgG antibody conjugated to alkaline phosphatase at 1:7500 dilution. The detection was performed with a colorimetric method using a solution of nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). MW, molecular weight markers; HynA1, large subunit of $[NiFe]_1$ Hase.

**Table A.1** – Identification by mass spectrometry of proteins present in the 360 kDa band from hr CN-PAGE.

| Protein name | NCBI entry[a] | Gene locus tag | Gene | Coverage (%)[b] | Peptides[c] | Mass (kDa)[d] |
|---|---|---|---|---|---|---|
| QrcB | 46579108 | DVU0694 | *qrcB* | 30 | 3 | 72.2 |
| [NiFe] Hase, large sub | 46580331 | DVU1922 | *hynA1* | 28 | 2 | 62.6 |
| ATP synthase B chain | 46579188 | DVU0775 | *atpD* | 28 | 1 | 50.7 |

[a] NCBI entry is the protein accession number.
[b] Coverage is the protein sequence coverage by the matching peptides.
[c] Peptides indicate the number of different peptides matching the protein sequence.
[d] Mass corresponds to the theoretical molecular mass of the identified protein only from its amino acid sequence.

# Appendix B

## Supplementary Material of Chapter 4

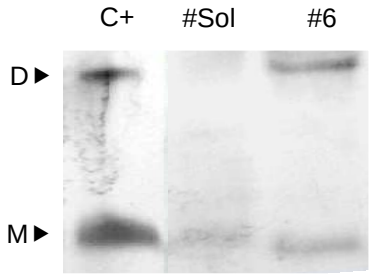


**Figure B.1** – Western blot analysis of freshly prepared *D. vulgaris* soluble fraction (#Sol) and the fraction from the first ion-exchange purification containing DsrC not associated with DsrAB (#6, the same as in Figure 4.1.1), using DsrC antibodies. The fractions were resolved on a 15 % SDS-PAGE gel in non-reducing conditions; C+, positive control with recombinant DsrC; M, monomer; D, dimer.

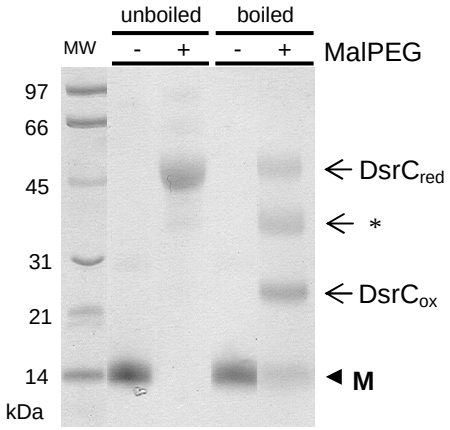


**Figure B.2** – Formation of disulfide bond in DsrC in boiling conditions (as in (Cort *et al.*, 2008)), followed by MalPEG labelling. Samples were subjected to a 10 % Tricine-SDS-PAGE (5 μg/lane). DsrC labelled with two MalPEG moieties is indicated with (*).

# APPENDIX C

## Supplementary Material of Chapter 5

| | locus | $H^+$-PPi | DsrK | QmoA | Peripl. Sol. Hase [NiFe] | | Peripl. Sol. Hase [FeFe] | | Peripl. Sol. FDH | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | HynBA | HysBA | HydAB | | FdhAB | FdhABC3 |
| **Archaea** | | | | | | | | | | |
| *Archaeoglobus fulgidus* | AF | | 0502; 0547; 0543 | 0663 | | | | | | |
| *Archaeoglobus profundus* | Arcpr_ | | 1730 | 1260 | | | | | | |
| *Caldivirga maquilingensis* | Cmaq_ | 0949 | 0851 | | | | | | | |
| **Deltaproteobacteria** | | | | | | | | | | |
| ***Desulfovibrionacae*** | | | | | | | | | | |
| *Desulfovibrio aespoeensis* | DaesDRAFT_ | | 0513 | 2028 | 1307 | | 3275 | | 2455; 2841 | |
| *Desulfovibrio desulfuricans* G20 | Dde_ | | 2272 | 1111 | 2138 | 2135 | 2281 | | 0813; 0717; 3513 | |
| *Desulfovibrio desulfuricans* ATCC 27774 | Ddes_ | | 0450 | 2127 | 1038 | | 1503 | | | 0555 |
| *Desulfovibrio magneticus* RS-1 | DMR_ | | 03710 | 05410 | 15600 | | 12960 | | 37460; 24450; 15750 | |
| *Desulfovibrio piger* | DESPIG_ | | 02236 | 02771 | 02534 | 00296 | | | | 00975 |
| *Desulfovibrio salexigens* | Desal_ | | 0185 | 0231 | 1916 | 2049 | 1332 | | 2659 | 1924 |
| *Desulfovibrio* sp. FW1012B | DFW101DRAFT_ | | 3642 | 1163 | 0356 | | 3123 | | 0146; 0343 | |
| *Desulfovibrio vulgaris* Hildenborough | DVU | | 1289 | 0848 | 1922 | 1918 | 1769 | | 0587 | 2812 |
| ***Desulfomicrobiacae*** | | | | | | | | | | |
| *Desulfomicrobium baculatum* | Dbac_ | | 0271 | 3199 | 0766 | 1969 | | | 0971; 1570 | |
| ***Desulfobacteraceae*** | | | | | | | | | | |
| *Desulfatibacillum alkenivorans* | Dalk_ | 3373 | 0606 | 1568 | 2276 | | | | | |
| *Desulfobacterium autotrophicum* HRM2 | HRM2_ | | 31650 | 04500 | 22360 | 11680 | | | 03430; 40980 | 47140 |
| *Desulfococcus oleovorans* Hxd3 | Dole_ | 2820 | 0451 | 0999 | | | | | 1334 | |
| ***Desulfohalobiacae*** | | | | | | | | | | |
| *Desulfohalobium retbaense* DSM 5692 | Dret_ | | 0236 | 1965 | 0266 | | | | 0226 | |
| *Desulfonatronospira thiodismutans* ASO3-1 | DthioDRAFT_ | | 2263 | 1406 | | | | | 1315; 1367 | |
| ***Desulfobulbaceae*** | | | | | | | | | | |
| *Desulfotalea psychrophila* | DP | | 3074 | 1106 | 0159 | | | | | |
| *Desulfurivibrio alkaliphilus* | DaAHT2_ | | 2299 | 1470 | 0520 | | | | | |
| ***Syntrophobacteraceae*** | | | | | | | | | | |
| *Syntrophobacter fumaroxidans* MPOB | Sfum_ | 3037 | 1147 | 1287 | 2952 | | 0848 | | 3510; 1274; 0036 | |
| **Clostridia** | | | | | | | | | | |
| ***Peptococcaceae*** | | | | | | | | | | |
| *Desulfotomaculum acetoxidans* DSM 771 | Dtox_ | 3931 | 0076 | 3576 | | | | | | |
| *Desulfotomaculum reducens* | Dred_ | 2985 | 0635 | 0638 | | | | | | |
| *C.* Desulforudis audaxviator MP104C | Daud_ | 0308 | 2191 | 1884 | | | | | | |
| ***Thermoanaerobacterales*** | | | | | | | | | | |
| *Ammonifex degensii* KC4 | Adeg_ | | 0034 | 1079 | | 1787 | | | | 0283 |
| **Nitrospirae** | | | | | | | | | | |
| *Thermodesulfovibrio yellowstonii* | THEYE_ | | A0006 | A1831 | | A1365 | | | A0891 | |

*(continued on next page)*

| | locus | Cyt $c_3$ | QrcA | TmcA | HmcA | NhcB | OhcA |
|---|---|---|---|---|---|---|---|
| **Archaea** | | | | | | | |
| *Archaeoglobus fulgidus* | AF | | | | | | |
| *Archaeoglobus profundus* | Arcpr_ | | | | | | |
| *Caldivirga maquilingensis* | Cmaq_ | | | | | | |
| **Deltaproteobacteria** | | | | | | | |
| ***Desulfovibrionacae*** | | | | | | | |
| *Desulfovibrio aespoeensis* | DaesDRAFT_ | 1104; 2527 | 1214 | 1365 | 1617 | 1137 | |
| *Desulfovibrio desulfuricans* G20 | Dde_ | 3182 | 2932 | 3710 | 0653 | | |
| *Desulfovibrio desulfuricans* ATCC 27774 | Ddes_ | 2013 | | 0847 | | 2039 | |
| *Desulfovibrio magneticus* RS-1 | DMR_ | 02560; 21540 | 18010 | 42490 | 12830 | | 33620 |
| *Desulfovibrio piger* | DESPIG_ | 02928 | | | | 01442 | |
| *Desulfovibrio salexigens* | Desal_ | 0756; 1385; 2447 | 1042 | 3040 | 1378 | | 0831 |
| *Desulfovibrio* sp. FW1012B | DFW101DRAFT_ | 0515; 1530 | 2229 | 1322 | 1417 | | |
| *Desulfovibrio vulgaris* Hildenborough | DVU | 3171 | 0695* | 0263 | 0536 | | 3144 |
| ***Desulfomicrobiacae*** | | | | | | | |
| *Desulfomicrobium baculatum* | Dbac_ | 1057; 3004 | 3390 | 0567 | 0568 | | 1392 |
| ***Desulfobacteraceae*** | | | | | | | |
| *Desulfatibacillum alkenivorans* | Dalk_ | 2817; 4187 | 1267 | | | 1818 | 1086 |
| *Desulfobacterium autotrophicum* HRM2 | HRM2_ | 42360 | 18930; 15120 | 25810; 44980 | | | |
| *Desulfococcus oleovorans* Hxd3 | Dole_ | 3170; 0267 | 2546 | 0305; 0462 | 2250 | | 3005 |
| ***Desulfohalobiacae*** | | | | | | | |
| *Desulfohalobium retbaense* DSM 5692 | Dret_ | 0413; 1266; 1800; 2420 | 0270 | 1027 | 0877 | | 0167 |
| *Desulfonatronospira thiodismutans* ASO3-1 | DthioDRAFT_ | 1352; 2183; 2529 | | 1590; 2239 | | 1143 | |
| ***Desulfobulbaceae*** | | | | | | | |
| *Desulfotalea psychrophila* | DP | | | | | | |
| *Desulfurivibrio alkaliphilus* | DaAHT2_ | | | | | | |
| ***Syntrophobacteraceae*** | | | | | | | |
| *Syntrophobacter fumaroxidans* MPOB | Sfum_ | 4047 | 0611 | | | | 0090 |
| **Clostridia** | | | | | | | |
| ***Peptococcaceae*** | | | | | | | |
| *Desulfotomaculum acetoxidans* DSM 771 | Dtox_ | | | | | | |
| *Desulfotomaculum reducens* | Dred_ | | | | | | |
| *C.* Desulforudis audaxviator MP104C | Daud_ | | | | | | |
| ***Thermoanaerobacterales*** | | | | | | | |
| *Ammonifex degensii* KC4 | Adeg_ | | | | | | |
| **Nitrospirae** | | | | | | | |
| *Thermodesulfovibrio yellowstonii* | THEYE_ | A1056 | | | A1283 | | |

*(continued on next page)*

| | locus | RnfC | Complex I NuoA | Complex I NuoE | NqrA | $bc_1$ complex | Cyt *c* oxidase SubI $Caa_3$ | Cyt *c* oxidase FixN $Cbb_3$ | *bd* oxidase CydA |
|---|---|---|---|---|---|---|---|---|---|
| **Archaea** | | | | | | | | | |
| *Archaeoglobus fulgidus* | AF | | 1828 | | | | | | |
| *Archaeoglobus profundus* | Arcpr_ | | 1579 | | | | | | |
| *Caldivirga maquilingensis* | Cmaq_ | | | | | 1911 | | | |
| **Deltaproteobacteria** | | | | | | | | | |
| ***Desulfovibrionacae*** | | | | | | | | | |
| *Desulfovibrio aespoeensis* | DaesDRAFT_ | 0784 | 2249 | | | | | | 0525 |
| *Desulfovibrio desulfuricans* G20 | Dde_ | 0581 | | | | | 1823 | | 3204 |
| *Desulfovibrio desulfuricans* ATCC 27774 | Ddes_ | 1238 | 1665 | | | | | | 2196 |
| *Desulfovibrio magneticus* RS-1 | DMR_ | | 27810; 13420 | 13400 | | | 14880 | | 28300 |
| *Desulfovibrio piger* | DESPIG_ | 01995 | | | | | | | 02209 |
| *Desulfovibrio salexigens* | Desal_ | 0920; 1740 | 1489 | | 0329 | | | | 0083 |
| *Desulfovibrio* sp. FW1012B | DFW101DRAFT_ | | 2043; 0761 | 0759 | | | 0413 | | 0013 |
| *Desulfovibrio vulgaris* Hildenborough | DVU | 2792 | | | | | 1815 | | 3271 |
| ***Desulfomicrobiacae*** | | | | | | | | | |
| *Desulfomicrobium baculatum* | Dbac_ | 1960 | | | | | 1541 | | 2518 |
| ***Desulfobacteraceae*** | | | | | | | | | |
| *Desulfatibacillum alkenivorans* | Dalk_ | 4354 | | | | | | | 2357 |
| *Desulfobacterium autotrophicum* HRM2 | HRM2_ | 32070; 23770 | | | 06920 | | | 15110 | 40720 |
| *Desulfococcus oleovorans* Hxd3 | Dole_ | 0306; 2830 | | | 2536 | | | | 2649 |
| ***Desulfohalobiacae*** | | | | | | | | | |
| *Desulfohalobium retbaense* DSM 5692 | Dret_ | 0528 | | | | | | | 0136 |
| *Desulfonatronospira thiodismutans* ASO3-1 | DthioDRAFT_ | | 1977 | | | | | | 0042 |
| ***Desulfobulbaceae*** | | | | | | | | | |
| *Desulfotalea psychrophila* | DP | 0770 | 1321 | | 1802 | | | | 0926 |
| *Desulfurivibrio alkaliphilus* | DaAHT2_ | | 1930 | | 0042 | | | 0175 | 0790 |
| ***Syntrophobacteraceae*** | | | | | | | | | |
| *Syntrophobacter fumaroxidans* MPOB | Sfum_ | 2699 | 0199 | 1956 | | 0422 | | | 0339 |
| **Clostridia** | | | | | | | | | |
| ***Peptococcaceae*** | | | | | | | | | |
| *Desulfotomaculum acetoxidans* DSM 771 | Dtox_ | | 1205 | | | | | | |
| *Desulfotomaculum reducens* | Dred_ | | 2046 | | | | | | 1639 |
| *C.* Desulforudis audaxviator MP104C | Daud_ | | | | | | | | |
| ***Thermoanaerobacterales*** | | | | | | | | | |
| *Ammonifex degensii* KC4 | Adeg_ | | | | | | | | |
| **Nitrospirae** | | | | | | | | | |
| *Thermodesulfovibrio yellowstonii* | THEYE_ | | A0911 | | | A1158 | | | A1307 |