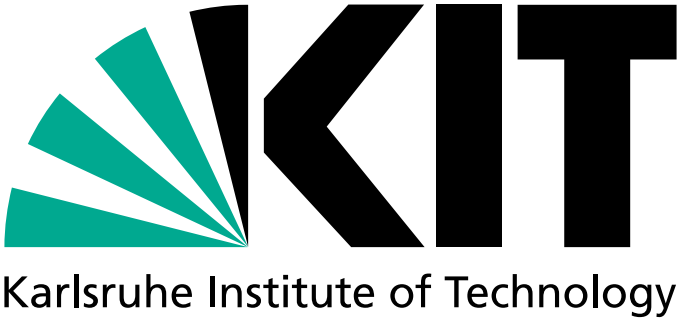
THE INFLUENCE OF ß-BARREL PROTEINS IN THE DISSIMILATORY IRON REDUCTION IN *SHEWANELLA ONEIDENSIS* MR-1



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PUBLICATIONS AND MANUSCRIPTS

1. Schütz B., Schicklberger M., Kürmann J., Spormann A.M., Gescher J.: “Periplasmic electron transfer via the *c*-type cytochromes MtrA and FccA of *Shewanella oneidensis* MR-1”. *Applied and Environmental Microbiology*, 2009.

2. Schicklberger M., Bücking C., Schuetz B., Heide H., Gescher J. “Involvement of the *Shewanella oneidensis* decaheme cytochrome MtrA in the periplasmic stability of the beta-barrel protein MtrB.“ *Applied and Environmental Microbiology*, 2010.

3. Richter K., Bücking C., Schicklberger M., Gescher J.: “A simple and fast method to analyze the orientation of *c*-type cytochromes in the outer membrane of Gram negative bacteria”. *Journal of Microbiological Methods*, 2010.

4. Cordova CD., Schicklberger MF., Yu Y., Spormann AM. “Partial functional replacement of CymA by SirCD in *Shewanella oneidensis* MR-1.” *Journal of Bacteriology*, 2011.

5. Richter K.*, Schicklberger M.*, Gescher J. „Dissimilatory reduction of extracellular electron acceptors in anaerobic respiration.“ *Applied and Environmental Microbiology Applied and Environmental Microbiology*, 2011. (*Shared first author)

6. Bücking C., Schicklberger M., Gescher J. “The biochemistry of dissimilatory ferric iron and manganese reduction in *Shewanella oneidensis*” *Book chapter* (in progress)

7. Schicklberger M., Gescher J. “Genomic plasticity responsible for dissimilatory iron reduction in *Shewanella oneidensis* MR-1”, (in progress)

Inhaltsverzeichnis

ABSTRACT

Microbial dissimilatory iron reduction (DIR) is an important biogeochemical process that has a strong impact on the mineral composition in the earth crust. Bacteria capable of DIR conserve energy by coupling the oxidation of organic substrates to the reduction of metal oxides. Due to their physical and chemical properties many metal oxides are poorly soluble or effectively insoluble under the respective native growth conditions. To overcome the obstacle of inaccessibility microbes have developed various mechanisms to transfer electrons on insoluble metal oxides.

The Gram-negative γ-proteobacterium *Shewanella oneidensis* MR-1 has been adopted as a model organism to elucidate the underlying principles of the DIR due to its wide distribution and genetic tractability. Many studies have underlined that an extended respiratory chain is fundamental to the DIR by *S. oneidensis* MR-1. Electrons are transferred from the cytoplasmic membrane through the periplasm and the outer membrane onto the extracellular terminal electron acceptor. Both the components of a minimal setup of this electron transfer chain and the accompanying processes for protein localization have not been elucidated.

This work focuses on the involvement of outer membrane ß-barrel proteins in DIR. In *S. oneidensis* MR-1 a necessary ß-barrel protein is encoded by the gene *mtrB* (mtr: **m**e**t**al **r**educing) that is part of an operon consisting of two decaheme *c*-type cytochromes (*mtrA* and *mtrC*). A *mtrB* null mutant strain lacks the ability to respire on iron and displays a different distribution of *c*-type cytochromes, in particular MtrC and MtrA, as compared to a wildtype strain. Furthermore, three additional clusters could be identified that are organized similarly to the *mtrCAB* operon and notably contain both a MtrB and a MtrA homolog.

Due to the observed interaction between MtrA and MtrB, as well as the maintained genetic proximity between *mtrA* and *mtrB* homologs, the interdependence of MtrAB was investigated. It was established that a *mtrA* mutant strain exhibits a downstream effect on MtrB resulting in a complete lack of MtrB production. RT-qPCR revealed no transcriptional effect; therefore, we hypothesized a stabilizing effect due to the soluble periplasmic protein MtrA. In order to identify additional potential factors affecting MtrA-dependent stability of MtrB in the periplasmic space, MudPIT (**mu**lti**d**imentional **p**rotein **i**den**t**ification) and BLAST analysis was used and we identified a DegP homolog (SO3942) in *S. oneidensis* MR-1. DegP, a serine

protease, degrades misfolded or mislocated outer membrane proteins upon upregulation by sigma factor σ^E. Via construction of a markerless SO3942 deletion mutant strain in a Δ*mtrA* background and a conditional *degP* mutant in *E. coli*, we could uncouple the dependency of MtrB on MtrA in the absence of DegP in the native and heterologous host, respectively, as visualized through western-blot analysis. Subsequent crosslink experiments were chosen to identify a possible periplasmic interaction of MtrA and MtrB due to a chaperone like function of MtrA. A periplasmic complex of MtrA and MtrB could not be observed. Complementary crosslink experiments showed overlapping hits for two ß-barrel proteins, specifically the gene products of SO2907 and SO3896. Subsequent single and double deletion mutant strains did not affect the stability of MtrB.

Given the abundance of homologous outer membrane ß-barrel proteins encoded for in the genome of *S. oneidensis* MR-1 we isolated and characterized a Δ*mtrB* suppressor strain to determine how a disruption of the integral membrane protein MtrB can be overcome in DIR. The suppression was found to be due to a transcriptional activation of SO4360 and SO4359, which encode for MtrA and MtrB homologous proteins, respectively. The upregulation was caused by an insertion of a mobile genetic element, ISSod1, upstream of the gene cluster encoding for the SO4360-SO4359 module, generating a novel, constitutively active hybrid promoter. Genetic experiments indicated that the *mtrB* homolog, SO4359, was required for the restored growth with ferric citrate as electron acceptor, underlining the importance of a ß-barrel protein in DIR in *S. oneidensis* MR-1. Deletion mutant analysis of SO4360 and *mtrA* in the suppressor strain revealed that MtrA could be excluded from either complex formation or electron transfer reactions whereas SO4360 encodes for a fundamental component for respiration on ferric iron in the suppressor.

Notably, co-expression of SO4360 $SO4359_{STREP}$ *in trans* in a *mtrB* mutant strain could complement for iron reduction. More important, the expression of SO4360 $SO4359_{STREP}$ *in trans* in a strain, which is depleted in all outer membrane *c*-type cytochromes and *mtrA* also could be converted into an ferric citrate reducing strain.

Altogether, these findings revealed the functional interchangeability of the genetic units encoding for the similar protein complexes and suggest a later evolution of a MtrA dependent control of MtrB production linking the establishment of a electron chain through the periplasm to the formation of a integral membrane complex for Fe(III) reduction in *S. oneidensis* MR-1.

ZUSAMMENFASSUNG

Mikrobielle dissimilatorische Eisenreduktion (DER) ist ein wichtiger biogeochemischer Prozess, der einen starken Einfluss auf die Zusammensetzung der Minerale in der Erdkruste ausübt. Bakterien, die der Eisenreduktion fähig sind, konservieren Energie durch die Kopplung der Oxidation von organischen Substanzen an die Reduktion von Eisenoxiden. Aufgrund ihrer physikalischen und chemischen Eigenschaften sind viele Metalloxide, unter den jeweiligen nativen Wachstumsbedingungen, nur schwer löslich. Mikroben haben verschiedene Mechanismen entwickelt, um die Elektronenübertragung auch auf unzugängliches Eisen zu vollziehen.

Das Gram-negative γ-proteobacterium *Shewanella oneidensis* MR-1 hat sich aufgrund der weiten Verbreitung und der leichten Handhabung zu einem Modellorganismus für die Entschlüsselung der unterliegenden Prinzipien der DER entwickelt. Viele Studien haben eine erweiterte Elektronentransportkette als Grundlage der DER in *S. oneidensis* MR-1 hervorgehoben. Die Elektronen werden von der Cytoplasmamembran durch das Periplasma und über die äußere Membran auf den extrazellulären Elektronenakzeptor übertragen. Sowohl die Komponenten für ein minimales Setup der Elektronentransportkette als auch der begleitende Prozess der Proteinlokalisation wurden noch nicht entschlüsselt.

Diese Arbeit fokussiert auf den Einfluss von äußeren Membran ß-Fass Proteinen in der DER. Ein entscheidendes ß-Fass Protein wird in *S. oneidensis* MR-1 vom Gen *mtrB* (**M**e**t**all **r**eduzierend) kodiert, welches Teil eines Operons aus zwei *c*-typ Cytochromen (*mtrA* und *mtrC*) ist. Eine *mtrB* Mutante ist unfähig Eisen zu veratmen und zeigt, im Vergleich zum Wildtyp, eine unterschiedliche Verteilung der *c*-typ Cytochrome MtrC und MtrA. Des Weiteren konnten drei zusätzliche Cluster identifiziert werden, die dem *mtrCAB* Cluster ähnlich organisiert sind und bemerkenswerterweise homologe zu MtrA und MtrB beinhalten.

Aufgrund der beobachteten Interaktion zwischen MtrA und MtrB sowie die beibehaltenen genetische Nähe zwischen den *mtrA* und *mtrB* Homologen wurde die Wechselbeziehung von MtrAB untersucht. Ein Downstream Effekt einer *mtrA* Mutante auf MtrB wurde bewiesen, der als Resultat die Produktion von MtrB unterbindet. RT-qPCR erwies keinen transkriptionellen Effekt auf. Daher nahmen wir einen stabilisierenden Effekt, beruhend auf das lösliche periplasmatische Protein

MtrA, an. Um weitere mögliche Faktoren zu identifizieren, die eine MtrA-abhängige Stabilität auf MtrB im Periplama beeinflussen, wurden MudPIT (**mu**lti**d**imensionale **P**rotein **I**den**t**ifizierung) und BLAST Analysen durchgeführt, in denen ein DegP Homolog (SO3942) in *S. oneidensis* MR-1 identifiziert werden konnte. DegP, eine Serinprotease, baut aufgrund einer Hochregulierung durch den Sigmafaktor σ^E falsch gefaltete oder falsch lokalisierte äußere Membranproteine ab. Durch die Konstruktion einer markerlosen SO3942 Deletion und einer konditionellen *degP* Mutante in *E. coli* konnte, wie durch Westernblot Analyse nachgewiesen wurde, die Abhängigkeit von MtrB auf MtrA in Abwesenheit von DegP sowohl im nativen als auch im heterologen Wirt entkoppeln. Anschließend wurden Crosslink Experimente durchgeführt, um eine mögliche periplasmatische Interaktion zwischen MtrA und MtrB, aufgrund einer Chaperon ähnlichen Funktion von MtrA, aufzuweisen. Ein periplasmatischer Komplex zwischen MtrA und MtrB konnte nicht gezeigt werden. Komplementäre Crosslink Experimente zeigten übereinstimmende Treffer für zwei ß-Fass Proteine, SO2907 und SO3896.

Um zu untersuchen, wie *S. oneidensis* MR-1 den Verlust des integralen Membranproteins MtrB in der DER bewältigt, isolierten und charakterisierten wir einen Δ*mtrB* Suppressor Stamm. Es konnte aufgedeckt werden, dass die Suppression aufgrund der transkriptionellen Aktivierung von SO4360 und SO4359 erfolgt, die jeweils für MtrA und MtrB homologe Proteine kodieren. Die Hochregulierung wurde durch eine Insertion eines mobilen genetischen Elements, ISSod1, vor dem SO4360-SO4359 Modul verursacht, indem ein neuer aktiver konstitutiver Hybridpromoter gebildet wurde. Genetische Experimente deuteten darauf hin, dass das *mtrB* homolog SO4359 für ein wiedererlangtes Wachstum auf Eisenzitrat nötig ist, was wiederum die Bedeutung der ß-Fass Proteine in der DER in *S. oneidensis* MR-1 unterstreicht. Analysen von Deletionsmutanten von SO4360 und *mtrA* konnten einen Einfluss von MtrA in Bezug auf die Komplexbildung oder Elektronentransfer Reaktionen ausschließen, während SO4360 im Suppressor ein essentieller Teil für die Atmung auf Eisenzitrat darstellt. Bemerkenswerterweise konnte eine *in trans* Coexpression von $SO4359_{STREP}$ und SO4360 eine *mtrB* Mutante für die Eisenreduktion komplementieren. Bedeutender ist die Tatsache, dass eine *in trans* Expression von SO4360 und SO4359 in einem *S. oneidensis* MR-1 Stamm, dem äußeren Membran *c*-typ Cytochrome sowie MtrA fehlen, in ein Eisenreduzierenden Stamm konvertiert werden konnte.

Zusammenfassend decken diese Ergebnisse einen funktionellen Austausch der genetischen Einheiten, die für homologe Proteinkomplexe kodieren, auf und schlagen eine spätere Evolution einer MtrA abhängigen MtrB Produktion vor, welche die Etablierung einer Elektronenkette durch das Periplasma mit der Bildung eines integralen äußeren Membrankomplexes für die Eisen (III) Reduktion in *S. oneidensis* MR-1 verbindet.

Symbol	Description
AHDS	Anthrahydroquinone 2,6-disulfonate
AQDS	Anthraquinone 2,6-disulfonate
BLAST	Basic Local Alignment Search Tool
DAP	Diaminopimelic acid
DIR	Dissimilatory iron reduction
DMRB	Dissimilatory metal reducing bacteria
DMSO	Dimethylsulfoxide
EDTA	Ethylene diamine tetra acetate
HEPES	Sodium 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
MFC	Microbial fuel cell
NTA	Nitrilotriacetic acid
OM	Outer membrane
o/n	over night
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween
TMAO	Trimethylamine N-oxide
TEMED	N,N,N',N'-Tetramethylethylendiamine
Tris	Tris-(hydroxymethyl)-aminomethane
ΔOMC	Outer membrane cytochrome-deficient
ΔOMCA	ΔOMC with additional deletion of *mtrA*

INTRODUCTION

1. Dissimilatory iron reduction in the early evolution of microbial respiration

Our understanding of the geochemical conditions at the time when respiratory systems evolved suggests that there was a more limited number of electron acceptors available for microbial respiration than today. Important electron acceptors like oxygen, nitrate or sulfate are considered unlikely to have been present in amounts sufficient to support significant rates of respiration (Kasting, 1993). It is generally presumed that sulfur reduction was one of the earliest forms of microbial respiration, because the known microorganisms that are most closely related to the putative last common ancestor (LCA) are primarily anaerobic sulfur reducing hyperthermophiles (Vargas *et al.*, 1998).

Until recently, speculations about iron(III) (hereinafter referred to as ferric iron or Fe(III)) reduction as one of the earliest forms of microbial respiration has not been considered. Although it was known for many years that Fe(III) could be reduced during anaerobic growth of some microorganisms, these microorganisms had a primarily fermentative metabolism and the formation of iron(II) (herein after referred to as ferrous iron or Fe(II)) was considered to be due to side reactions (or co-metabolism) in their central metabolism (Lovley & Phillips, 1987a, Lovley & Phillips, 1987b). Evidence that Fe(III) reduction is linked to energy conservation was missing and it was often considered that Fe(III) was reduced by non-enzymatic reactions in the culture. The ability of microorganisms to yield energy to support cell growth coupled to the reduction of Fe(III) as electron acceptor was first reported in the 1980s (Balashova & Zavarzin, 1979). Until 1998 it was not recognized that hyperthermophiles could use Fe(III) as final electron acceptor (Vargas *et al.*, 1998). Remarkably, it is known today that most hyperthermophiles can reduce Fe(III) (Lovley *et al.*, 2004).

Several studies have suggested, that in the early earth, a photochemical reaction of Fe(II) released by hydrothermal vents resulted in the formation of Fe(III) (see reviews

(Cairns-Smith, 1992, Russell, 2002)). This ferric iron could have been used as electron acceptor using hydrogen gas (which was part of the atmosphere) as electron donor (Russell, 2002). Since other electron acceptors for hydrogen oxidation such as oxygen, nitrate or sulfate were most probably absent at that time, this geological scenario highly suggests the development of microorganisms conserving energy by the oxidation of hydrogen coupled to the reduction of Fe(III) (Lovley *et al.*, 2004). Additionally, photolithotrophs might have utilized reduced iron as electron donor. Those photolithotrophes were discovered only shortly after the isolation of the first dissimilatory metal reducing bacteria (DMRB) (Straub *et al.*, 2001, Jiao & Newman, 2007). Their activity might have promoted growth of iron reducing bacteria even before substantial amounts of molecular oxygen were present in the atmosphere. Such a microbial community could also have promoted the formation of Banded Iron Formations, depositions of the Precambrian composed of alternating layers of iron-rich and silica-rich material prior to the rise of oxygen (Weber *et al.*, 2006, Konhauser K., 2002). Hence, ferric iron respiration indeed could be considered, next to sulfur respiration, as one of the first forms of energy conservation.

2. Involvement of microorganisms in the iron cycle

Many reactions relevant to geochemistry are driven or accelerated by the activity of prokaryotes. Those activities include carbon mineralization, nitrogen fixation, sulfate reduction as well as iron transformations (Kappler & Straub, 2005). Bacteria, which gain energy through oxidation of Fe(II) or the reduction of F(III) have a strong influence on the global iron cycle (Kraemer *et al.*, 2005). Iron is the fourth most abundant metal on Earth (Schlegel H.G., 2007) and the most frequently utilized transition metal in the biosphere (Kappler & Straub, 2005). Thus, iron redox reactions have the potential to support a significant amount of microbial biomass. Since the 1980s it has been known that Fe(III) is able to serve as an electron acceptor for the anaerobic oxidation of organic carbon sources (Lovley, 1991, Thamdrup *et al.*, 2000). Today, it is well established that reduction of ferric iron accounts for significant turnover of organic carbon in many environments (Nealson *et al.*, 2002). Although there are Fe(III)-reducing microorganisms which can couple the reduction of ferric iron to the oxidation of complex organic matter, they do not appear to be competitive with fermentative microorganisms in the same habitats (Lovley & Phillips, 1989). It

has been suggested that fermentative microorganisms which are not directly involved in Fe(III) reduction, break down the complex material and Fe(III)-reducing bacteria are using those fermentative end products as carbon sources (Lovley *et al.*, 2004).
In fact, the first identified organism capable of respiring iron seems to follow this observation. *Geobacter metallireducens*, designated *Geological Survey*-15, is a Gram-negative rod shaped bacterium isolated 1988 by Derek Lovley and Elizabeth Phillips from sediments of the Potomac River, USA (Lovley & Phillips, 1988) and this organism couples the oxidation of acetate to CO_2 to the reduction of Fe(III) to Fe(II).

The majority of electron acceptors commonly used by prokaryotes (oxygen, nitrate, sulfate, carbon dioxide) are soluble before and after reduction. In contrast, most of the metals used as microbial electron acceptors show different solubility properties in the oxidized versus the reduced state (DiChristina *et al.*, 2005). Ferrous iron, for example, is stable under neutral or alkaline conditions only in anoxic environments. In contrast, ferric iron is poorly soluble under circaneutral pH and oxic conditions. It precipitates in the form of insoluble iron minerals like ferrihydrite ($Fe_5HOH_8 \cdot 4\ H_2O$), goethite (α-FeOOH), hematite (α-Fe_2O_3) or clay minerals (Roden & Zachara, 1996, Zachara *et al.*, 1998, Zachara *et al.*, 2002, Kostka *et al.*, 1999, Dong *et al.*, 2003). In addition to Fe(III) minerals, Fe(II) and mixed valence Fe(II)-Fe(III) minerals also occur in the environment. The most prominent forms of ferrous iron minerals are vivianite ($Fe_3(PO_4)_2 \cdot 8\ H_2O$) and siderite ($FeCO_3$). Magnetite ($Fe_3O_4$) and green rust are examples for mixed valence iron minerals (Zachara *et al.*, 2002, Cornell & Schwertmann, 2003)

However, at acidic pH, ferrous iron can persist even under oxic conditions and complexation by organic compounds can lead to elevated concentrations of dissolved Fe(III), even at neutral pH (Stumm & Morgan, 1988, Cornell & Schwertmann, 2003) In addition, ferric iron can be reduced chemically by a range of organic and inorganic reductants including hydrogen sulfide, which is a common end product of microbial sulfur and sulfate reduction (Thamdrup *et al.*, 2000, Cornell & Schwertmann, 2003).

Many reactions relevant for the geochemistry are driven by the activity of prokaryotes. The iron minerals described above are used or, in a process called

biomineralisation, produced or transformed by microbial activities. Previous studies using enriched cultures suggested that DMRB only reduce amorphous or poorly crystalline iron oxides in natural sediments. On the contrary, it could be shown that bacteria from the genus *Shewanella* and *Geobacter* are able to reduce crystalline phase ferric iron such as goethite and hematite (Zachara *et al.*, 2002).

Still, low solubility is not the only challenge for an organism that preferentially respires metals at neutral pH. The standard redox potential E° of the Fe(II)/Fe(III) redox couple with +0.77 V (Riedel, 1990) is relatively high and seems to provide a good electron acceptor for the anaerobic respiration coupled to the oxidation of fermentation end products. As mentioned above Fe(III) species predominantly exist as solid metal oxide minerals that are poorly soluble under circumneutral pH conditions. Due to the low solubility the redox potential decreases to much lower values between +0,35 V and -0.3 V at pH 7 (DiChristina *et al.*, 2005). Hence, dissimilatory metal reduction is energetically challenging as well, since the redox potential of the FeIII/II couple can be close to the SO_4^{2-}/H_2S couple (E°`= -0.22 V) (Doong *et al.*, 1996). The produced soluble aqueous Fe(II) can diffuse to the oxic/anoxic interface where it becomes oxidized to Fe(III) again, resulting in insoluble ferric iron minerals establishing a dynamic Fe(III) cycle. It has been estimated that in sediments each iron atom can go through as many as 100 cycles of reduction and oxidation prior to permanent incorporation (Thamdrup *et al.*, 2000).

Next to the chemical oxidation of Fe(II) with molecular oxygen, Fe(II) oxidation is also driven by biological catalyzed processes (Kappler *et al.*, 2010). To gain energy for growth, aerobic, neutrophilic Fe(II) oxidizing microorganisms have to compete successfully with the abiotic oxidation. In addition bacteria also have to cope with encrustation due to ferric iron minerals, which might impair the exchange of metabolites with the environment. In contrast, under acidic conditions Fe(II) persists for a longer period of time and due to the stability of ferrous iron under these conditions aerobic acidophilic Fe(II)-oxidizing bacteria can easily compete with the abiotical oxidation of Fe(II) by O_2. At acidic pH the redox couple Fe(III)/Fe(II) has a redox potential of +0.77 V. Due to the low difference to the corresponding redox couple O_2/H_2O (E_0 equals +1.1 V at pH 2 (Kappler & Straub, 2005)), the produced energy is about -33 kJ/mol iron ($\Delta G°=-nF\Delta E°$). This is just big enough for the

synthesis of 1 mol ATP (~34 kJ/mol) under standard state conditions (Kappler & Straub, 2005). This relationship contributes to the huge amount of iron that is oxidized by aerobic acidophilic bacteria for instance in acid mine drainage (Baker & Banfield, 2003). Fe(II) oxidation also occurs under anoxic conditions. About two decades ago phototrophic bacteria were discovered which use ferrous iron as sole electron donor (Widdel *et al.*, 1993). Since the standard redox potential shifts at neutral conditions to less positive values due to the low solubility of Fe(III), Fe(II) functions as an electron donor for the photosystems of purple or green bacteria ($E^0 \approx$ +0.45 V or +0.3 V respectively) (Clayton & Sistrom, 1978). In addition to the anaerobic Fe(II)-oxidizing phototrophic bacteria, microorganisms capable to couple the oxidation of Fe(II) to the dissimilatory reduction of nitrate have been discovered (Straub *et al.*, 1996).

3. The genus *Shewanella*

Members of the genus *Shewanella* compose a diverse group of Gram-negative facultative anaerobic γ-Proteobacteria widely distributed in marine and freshwater environments. *Shewanella* species have been isolated in the Sargasso-sea, hydrothermal vents in the pacific, as well as in brackish, freshwater and marine sediments. The fact that these bacteria thrive in this assortment of environments confirms their metabolic and respiratory diversity (Hau & Gralnick, 2007).

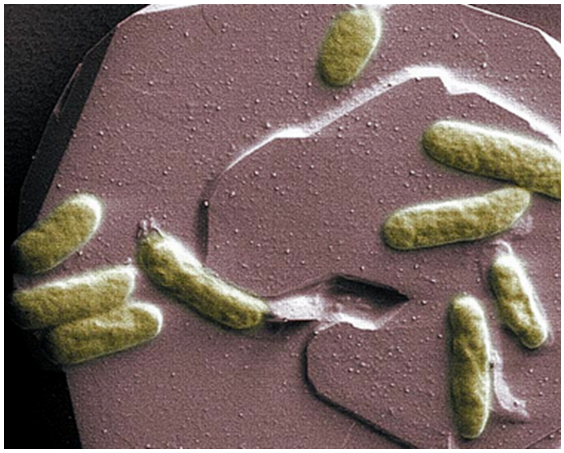


Fig. 1) *S. oneidensis* MR-1 attached to a Hematite surface (picture from PNNL, WA, USA).

The ability to utilize a huge variety of final electron acceptors in the absence of oxygen makes *Shewanella* the most diverse respiratory organisms described so far. The spectrum of usable electron acceptors ranges from fumarate, nitrate, nitrite, dimethylsulfoxide (DMSO), sulfur, sulfite, thiosulfite, trimethylamin-N-oxide (TMAO), ferric iron and manganese oxides to rather toxic transition metals like chromium, uranium, mercury and arsenate (Nealson & Scott, 2006). This respiratory versatility presumably contributes to the ubiquitious distribution of this chemoorganotrophic

organism in the environment. In contrast to their versatility with regard to electron acceptors, *Shewanella* species tend to be rather limited in their ability to use carbon sources. Most *Shewanella* species are nonfermentative and they only can grow on lactate, pyruvate, succinate, formate and *N*-acetylglucosamine (Nealson & Scott, 2006, Scott & Nealson, 1994).

Analysis of the genome revealed that the *Shewanella* chromosome encodes for 41 putative *c*-type cytochromes (five in the outer membrane, 27 in the periplasm and nine in the cytoplasmic membrane). The ability to respire the huge versatility of electron acceptors under anoxic conditions is presumably due to the large number of *c*-type cytochromes. Due to the ability to use oxygen, the sequenced genome and the short generation time *S. oneidensis* MR-1 was established as a model organism for the investigation of the DIR (Gescher, 2010).

Tab. 1) Redox potentials of electron donors/acceptors *S. oneidensis* MR-1 can utilize.

Redox couple	**E°' (mV)**	**Reference**
CO_2/formate	-432	(Thauer *et al.*, 1977)
$H+/H_2$	-414	(Thauer *et al.*, 1977)
$S_2O_3^{2-}/HS^-+HSO_3^-$	-402	(Thauer *et al.*, 1977)
NAD/NADH	-320	(Thauer *et al.*, 1977)
$S°/HS^-$	-270	(Thauer *et al.*, 1977)
Pyruvate/lactate	-190	(Thauer *et al.*, 1977)
2,6-AQDS/2,6-AHDS	-184	(Hernandez & Newman, 2001)
(Hematite) 1/2 $\alpha Fe_2O_3/Fe^{2+}$	-159	(Hernandez & Newman, 2001)
HSO_3^-/HS^-	-116	(Thauer *et al.*, 1977)
(Goethite) $\alpha FeOOH/Fe^{2+}$	-88	(Hernandez & Newman, 2001)
Menaquinone ox/red	-74	(Thauer *et al.*, 1977)
Fumarate/succinate	+33	(Thauer *et al.*, 1977)
Ubiquinone ox/red	+113	(Thauer *et al.*, 1977)
TMAO/TMA	+130	(Styrvold & Strom, 1984)
DMSO/DMS	+160	(Styrvold & Strom, 1984)
NO^{2-}/NH^{4+}	+344	(Thauer *et al.*, 1977)
NO^{3-}/NO^{2-}	+420	(Thauer *et al.*, 1977)
1/2 O_2/H_20	+818	(Thauer *et al.*, 1977)

4. Current scientific knowledge

4.1 Proteins involved in DIR in *S. oneidensis* MR-1

Currently, efforts to define the minimal protein set for dissimilatory iron reduction and the dissembling mechanism for correct protein incorporation have yielded the most information using *S. oneidensis* MR-1 as a model organism. The genetic tractability and comparable physiology to *E. coli* has enabled studies using a wide variety of genetic and biochemical techniques including heterologous expression in *E. coli* as a major strategy (Schicklberger *et al.*, 2011, Schuetz *et al.*, 2009, Gescher *et al.*, 2008, Pitts *et al.*, 2003, Clarke *et al.*, 2008a). Here the recent discoveries are highlighted, including the use of a synthetic biology approach, to identify factors required for extracellular electron transfer in *S. oneidensis* MR-1.

In order to transfer electrons to extracellular insoluble metals in addition to other electron acceptors, *S. oneidensis* MR-1 utilizes an extensive electron transfer network that spans to the outer membrane (Meyer *et al.*, 2004). Certainly, not all putative electron transfer proteins are required simultaneously. The so called ‘minimal setup’ of proteins which are believed to be responsible for the ability to respire on ferric iron as well as important protein-protein or protein-electron shuttle interactions will be discussed in the following sections.

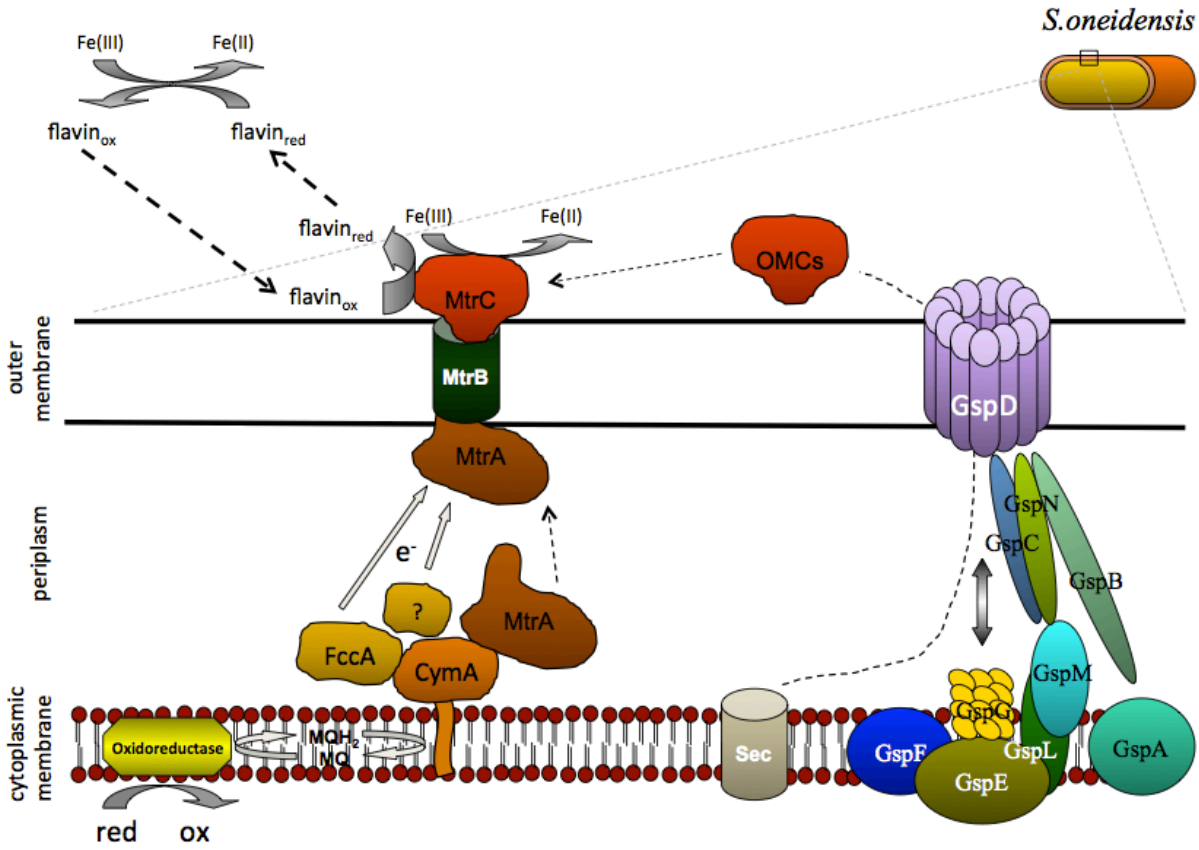


Fig. 2) Overview of components involved in the dissimilartoy iron reduction in *S. oneidensis* MR-1. Upper right: cross section of a *S. oneidensis* MR-1 cell. Solid unidirectional arrows represent electron transfer, dashed arrows indicate protein or shuttle movements. Bidirectional arrow indicates polymerization and degradation of GspG proteins.

4.2 Components for electron transfer in the cytoplasmic membrane

Due to the low redox potential of ferric (hydr)oxides under circa neutral pH conditions ($E^{0'}$= -0.3 to 0.35 V), ubiquinone with a redox potential of +66 mV is thermodynamically unfavorable as electron carrier. Instead, menaquinone with a redox potential of -74 mV is required for anaerobic respiration (Firer-Sherwood *et al.*, 2008). Mutations in genes involved in the biosynthesis of menaquinone as well as incubation with electron transfer inhibitors (e.g. 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) and antimycin A) revealed the importance of menaquinone in the respiration of iron (Saffarini *et al.*, 2002, Carpentier *et al.*, 2005).

Today, it is well established that electrons are transferred across the cytoplasmic membrane via the menaquinol pool by the catalytic activity of a tetraheme *c*-type cytochrome protein called CymA (**cy**toplasmic **m**embrane protein **A**). CymA, a member of the NapC/NirT family, is bound to the cytoplasmic membrane by a α-helical anchor and functions as the central branching point that couples electron transfer from the menaquinone pool to downstream electron transport components, in general soluble periplasmic decaheme *c*-type cytochromes. Except for the use of thiosulfate and TMAO, mutants deficient in *cymA* are impaired in the ability to use a wide array of soluble and insoluble electron acceptors including fumarate, nitrate, nitrite, DMSO, Mn(VI), and Fe(III) (Myers & Myers, 1997, Myers & Myers, 2000). The apparent midpoint potential of CymA (−200 mV; revealed using protein film voltammetry) is about 130 mV below the potential of the menaquinol-menaquinone couple ($E^{\circ\prime}$ = −74 mV) (Firer-Sherwood *et al.*, 2008). Thus, electron transfer from menaquinone seems to be thermodynamically unfavorable, but once the electrons enter the heme network, electron transfer could be driven by the exogenous metal oxide (Firer-Sherwood *et al.*, 2008). No further energy-conserving step is conducted by the cell downstream of CymA, since all additional electron transfer reactions are localized to the periplasm or the outer membrane. In other words, all further reactions do not contribute to the formation of a proton gradient at the cytoplasmic membrane (Richter *et al.*, 2012). Hence, the only function of CymA and all further *c*-type cytochromes, which are involved in the formation of an electron transport chain to ferric iron, is to recycle menaquinone. Consequently, the observed minor differences in the redox potentials of further cytochromes involved in the electron transport to ferric iron might be sufficient for establishing an extended respiratory chain (Richter *et al.*, 2012).

Accordingly, heterologous gene expression for the reconstruction of the putative electron transport chain of *S. oneidensis* MR-1 in *E. coli*, could show that expression of the menaquinone dehydrogenase *cymA* was necessary and sufficient to convert *E. coli* into a dissimilatory iron reducing bacteria when nitrilotriacetic acid (NTA)-chelated Fe(III) was used as the electron acceptor (Gescher *et al.*, 2008). Further experiments showed that this result is due to the ability of ferric NTA to pass the outer membrane of *E. coli* cells (Gescher *et al.*, 2008). Hence, a respiratory chain to the cell surface was not established.

4.3 Periplasmic electron carrier

MtrA is so far the only soluble periplasmic *c*-type cytochrome that was shown to be necessary under ferric iron-reducing conditions (Beliaev *et al.*, 2001). It is localized in the periplasm as a soluble cytochrome and also is associated with the outer membrane of *S. oneidensis* MR-1 (Schuetz *et al.*, 2009). MtrA was first identified in the soluble fraction of whole cell preparations (Beliaev *et al.*, 2001). Pitts *et al.* showed that MtrA not only is a periplasmic electron transfer protein but also has a reductase function for chelated ferric iron when overexpressed in *E. coli* (Pitts *et al.*, 2003). *In vitro* assays as well as heterologous expression experiments revealed that MtrA can be directly reduced by CymA (Schuetz *et al.*, 2009). MtrA is associated to the outer membrane by an integral membrane protein complex, which is composed of three proteins. The outer membrane ß-barrel protein MtrB seems to interact with the MtrA on the periplasmic side and the outer membrane cytochrome MtrC on the outer surface of the outer membrane. It was demonstrated *in vitro* that this complex has the capability to transfer electrons over a liposomal membrane (Hartshorne *et al.*, 2009, Ross *et al.*, 2007). Hence, the *in vivo* function of this complex is most likely outer membrane-spanning electron transfer.

Today it remains unknown if soluble MtrA serve as electron donor for MtrA that is associated within the MtrABC complex or if the complex itself is dynamic and re-reduced MtrA displace the oxidized MtrA from the complex. Using small-angle X-ray scattering and analytical ultracentrifugation it was suggested recently for MtrA to be shaped like an extended molecular “wire” with a maximum protein dimension of 104 Å and a rod like aspect ratio of 2.2 to 2.5 (Firer-Sherwood *et al.*, 2011). According to them, one MtrA molecule contacting CymA span much of the 130-250 Å distance of periplasmic space and thus probably might be sufficient to engage in redox shuttling. However, Schuetz *et al.* also could show that MtrA is able to receive electrons from the periplasmic fumarate reductase FccA *in vitro*, which suggests a *c*-type cytochrome network in the periplasm of *S. oneidensis* MR-1 (Schuetz *et al.*, 2009). All together, it is strong evidence for the involvement of MtrA in the periplasmic electron transfer during ferric iron reducing conditions.

Interestingly, the respiratory fumarate reductase FccA is the most prominent cytochrome in the periplasm of *S. oneidensis* MR-1 cells, which have been grown

anaerobically under ferric iron-reducing conditions. FccA is a 63.8 kDa tetraheme *c*-type flavocytochrome which is located in the periplasm and functions as the terminal fumarate reductase. This enzyme is unique in comparison to other fumarate reductases, since it is a soluble, monomeric periplasmic protein. FccA contains an N-terminal tetraheme domain and a flavin domain with a noncovalently bound flavin adenine dinucleotide (FAD) close to the active site (Leys *et al.*, 1999, Pessanha *et al.*, 2009). The crystal structure has been solved and it indicates a direct electron transfer through all four hemes to fumarate via the FAD cofactor (Leys *et al.*, 1999). The midpoint redox potentials for the four heme sites of the FccA ortholog in *S. frigidimarina* cover a region between -73 mV and -243 mV (Dobbin *et al.*, 1999). Several studies showed *in vitro* and *in vivo* an electron transfer of CymA to FccA (Schwalb *et al.*, 2002, Morris *et al.*, 1994, Schuetz *et al.*, 2009). Due to the high abundance and its connection to CymA and MtrA, FccA was suggested as being a transient electron storage protein under anaerobic conditions, which would allow *S. oneidensis* MR-1 for a short amount of time to use a carbon and electron source even if no terminal electron acceptor is present (Schuetz *et al.*, 2009).

4.4 Outer membrane *c*-type cytochromes

Analysis of the *S. oneidensis* MR-1 genome revealed five genes encoding for putative outer membrane *c*-type cytochromes (Myers & Myers, 2004). All of them are most likely acylated with a lipoprotein anchor and are exposed to the extracellular surface (Myers & Myers, 2003, Bücking *et al.*, 2010, Richter *et al.*, 2010). *S. oneidensis* MR-1 expresses the two outer membrane cytochromes MtrC and OmcA (**o**uter **m**embrane **c**ytochrome **A**) under ferric iron-reducing conditions (Bücking *et al.*, 2010, Beliaev *et al.*, 2001, Borloo *et al.*, 2007). Based on the high similarity of the nucleotide sequence and the close proximity in the genome, MtrC, MtrF and OmcA most likely arose through gene duplication events (Coursolle & Gralnick, 2010). The function of SO2931 and SO1659 is still unknown (Bücking *et al.*, 2010). Disruption of the genes encoding OmcA or MtrC did not affect *S. oneidensis* MR-1 in its growth on TMAO, DMSO, nitrate, nitrite, fumarate, thiosulfate and AQDS (anthroquinone-2,6-disulfonic acid) (Myers & Myers, 2001). In contrast, reduction of insoluble iron species was severely impaired (Borloo *et al.*, 2007, Coursolle & Gralnick, 2012). Additionally, complementation of a strain deficient in all outer membrane

cytochromes with MtrC or MtrF recovered the strain for iron reduction, whereas complementation with OmcA did not rescue the phenotype for ferric iron reduction at all (Bücking *et al.*, 2010). Still, *omcA* expression could partly restore the capability of an outer membrane cytochrome deficient strain to respire on manganese oxides. It was suggested that MtrC and OmcA form a high affinity complex in the outer membrane (Shi *et al.*, 2006). Treatment by proteinase K significantly degraded OmcA and MtrC by 71 and 31%, respectively, and immunofluorescence confirmed a more prominent cell surface exposure of OmcA and a partial exposure of MtrC (Myers & Myers, 2003). This is in line with recent findings of a trans membrane protein complex in which MtrC may protrude into the outer membrane ß-barrel protein MtrB and thus is less exposed to the surface (Clarke *et al.*, 2011).

A recent study revealed the crystal structure of the outer membrane decaheme *c*-type cytochrome MtrF (Clarke *et al.*, 2011). The ten hemes are organized across four domains in a crossed formation in which a staggered 65 Å octaheme chain transects the length of the protein and is bisected by a 45 Å tetraheme chain. MtrF shows surface exposure of the terminal heme V and X suggesting heme X accepting electrons from MtrA (or the MtrA homolog MtrD) and heme V to be solvent exposed for electron output to solid substrates (Clarke *et al.*, 2011). The more buried hemes II and VII are possible sited for electron exchange with soluble substrate or electron shuttles. The ability of MtrF to rapidly exchange electrons with solid surfaces was confirmed by protein film voltammetry (PFV) (Clarke *et al.*, 2011). In addition, direct electron transfer on solid graphite electrodes probed by PFV could also be shown with the outer membrane decaheme *c*-type cytochromes MtrC and OmcA from *S. oneidensis* MR-1, which are considered to be the final reductases for dissimilatory iron and manganese reduction (Hartshorne *et al.*, 2007, Firer-Sherwood *et al.*, 2008, Wang *et al.*, 2008).

4.5 ß-barrel proteins involved in electron transfer

Essential for the extension of the electron transfer chain from MtrA to the outer membrane in *S. oneidensis* MR-1 is the ß-barrel protein MtrB. MtrB is part of a seven gene cluster (*mtrDEFomcAmtrCAB)* and is translated together with *mtrC* and *mtrA* from a polycistronic mRNA. MtrB consists of 697 amino acids and its calculated

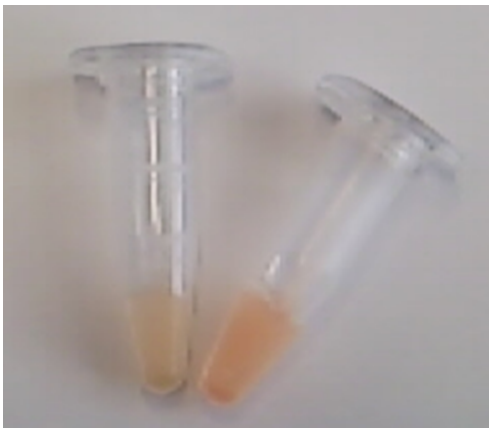


Fig. 3) Outer membrane fractions of Δ*mtrB* (left) and wildtype strain (right) grown under anoxic conditions with fumarate as electron acceptor. The typical reddish color in the wildtype is due to the *c*-type cytochromes.

molecular weight is about 77.5 kDa. MtrB is the connecting link of the periplasmic oxidoreductase MtrA and the outer membrane decaheme *c*-type cytochrome MtrC (Beliaev & Saffarini, 1998, Myers & Myers, 2002, Hartshorne *et al.*, 2009). Membrane fractions form cells grown anaerobically with Fe(III)-citrate as electron acceptor could demonstrate that MtrA is only associated to the membrane (insoluble fraction) when MtrB is present (Hartshorne *et al.*, 2009). This result shows the requirement of MtrB for the association of MtrA to the membrane and is consistent with the bioinformatic predictions of MtrA to be a soluble decaheme protein, which is localized in the periplasm. Furthermore, a *mtrB* null mutant in *S. oneidensis* MR-1 shows mislocalization of the final reductases MtrC and OmcA to the cytoplasmic membrane, the periplasmic space and the supernatant respectively (Myers & Myers, 2002) (Fig. 3).

Protein purification, analytical ultracentrifugation and cross-linking methods revealed a MtrCAB complex with the stoichiometry of 1:1:1 (Ross *et al.*, 2007). Using protein film voltammetry (PFV) Hartshorne *et al.* proposed a novel trans-outer membrane electron transfer system, in which the MtrB porin serves as a sheath within which MtrA and MtrC can embed sufficiently at the inner and outer faces of the membrane, respectively. Electron transfer then can take place between MtrA and MtrC. Further, PFV and spectropotentiometric results of MtrA, MtrC and MtrABC, respectively, revealed that the redox properties of MtrC and MtrA are modulated on formation of the MtrABC complex (Hartshorne *et al.*, 2009). Upon formation of a MtrABC complex, MtrC has a more negative redox potential due to MtrA which might be necessary for extracellular electron transfer. MtrABC complex stability is dependent on an additional function attributed to MtrA. Knock out mutations in either of the three proteins and subsequent monitoring of complex assembly revealed that a stable MtrAB subcomplex is formed in the absence of MtrC whereas a MtrBC complex is

not assembled when MtrA is absent. However, the reason of this interdependence still is not known.

Modules similar to MtrAB have been identified three additional times in *S. oneidensis* MR-1. So far, only two of the pathways have been reported to show physiological expression during anaerobic growth with ferric iron and DMSO as electron acceptors. One pathway is expressed by the gene cluster SO1774-SO1777 which upregulated during anaerobic metal reduction (Beliaev *et al.*, 2001). This gene cluster encodes for *omcA* and *mtrCAB*, respectively. The second cluster, SO1424-SO1432, shows activity in the respiration of DMSO under anoxic conditions and encodes for *dmsEFABGH* (**d**i**m**ethyl **s**ulfoxide reducing protein EFABGH) (Gralnick *et al.*, 2006). In addition, analysis of the *S. oneidensis* MR-1 chromosome revealed a higher similarity of genes SO1771-SO1773 (encoding for *mtrDEF*) to the *mtrCAB* gene cluster whereas the genes SO4362-SO4357 reflect a higher homology to the *dms* gene cluster (Gralnick *et al.*, 2006). Genome analysis of the chromosomes from all sequenced *Shewanella* species that have multiple copies of *mtrCAB* and *dmsABEFGH* revealed that *mtrCAB* homologs as well as *dmsABEFGH* homologs are clustered together. The conditions under which the *mtrDEF* and SO4359-62 gene clusters are expressed in *S. oneidensis* MR-1 have not yet been identified (Coursolle & Gralnick, 2012). Therefore the specific functions of the proteins encoded by these genes are still unknown.

All of the four pathways share MtrA (SO1777) and MtrB (SO1776) homologs, which are periplasmic *c*-type cytochromes and integral outer membrane ß-barrel proteins, respectively (Coursolle & Gralnick, 2010). Homologous proteins for MtrA are MtrD, DmsE and the gene product of SO4360 with an amino acid identity of 68 %, 64 % and 52 %, respectively (respective e-values: $1e^{-160}$, $2e^{-147}$, $2e^{-103}$). MtrE, DmsF and the gene product of SO4359 are homologs of MtrB and show each an amino acid identitiy of 36 % 35 % 25 % compared to MtrB (respective e-values are: $2e^{-134}$, $1e^{-120}$, $1e^{-39}$). All of the genes encoding for homologous proteins seem to derive from gene duplication events (Gralnick *et al.*, 2006). Notably, homologous gene clusters encoding for similar MtrAB modules are phylogenetically distributed among a range of bacteria phyla, suggesting that they are widely used in electron exchange with the extracellular environment (Tab.2). Using cyclic voltammetry, and deletion mutations

Ross *et al.* demonstrated that the Mtr pathway from *S. oneidensis* MR-1 is functionally reversible, and Jiao and Newman could show that a module in *Rhodopseudomonas palustris* TIE-1 homologous to MtrAB is necessary for photoautotrophic iron oxidation, which emphasizes the hypothesis of the widely used MtrAB module in extracellular electron exchange (Jiao & Newman, 2007, Ross *et al.*, 2011).

Tab. 2) Distribution of MtrAB like modules in proteobacteria (adopted from Habil. Prof. Gescher) (Gescher, 2010).

Class of Proteobacteria	Strain	Number of AB moduls	Number of moduls similar to *S. oneidensis mtrCAB* cluster	Number of moduls similar to *S. oneidensis dmsEFAB* cluster	Number of moduls similar to *S. oneidensis mtrDEF* cluster	Number of moduls similar to *R. palustris pioABC*	Number of moduls with unknown function
α	*Magnetospirillum magnetotacticum*	1	0	0	0	0	1
	Rhodopseudomonas palustris BisA53	1	0	0	0	1	0
	Rhodopseudomonas palustris CGA009	1	0	0	0	1	0
	Rhodopseudomonas palustris TIE-1	1	0	0	0	1	0
β	*Sideroxydans lithotrophicus*	1	0	0	0	0	1
	Rhodoferax ferrireducens	1	1	0	0	0	0
	Dechloromonas aromatica	1	0	0	0	0	1
γ	*S. oneidensis*	4	1	2	1	0	
	S sp. MR4	3	1	1	1	0	0
	S. sp. MR7	3	1	1	1	0	0
	S. frigidimarina	3	1	2	0	0	0
	S. sp. ANA3	2	1	0	1	0	0
	S. baltica OS155	2	1	0	1	0	0
	S. loihica	2	1	0	1	0	0
	S. putrefaciens 200	3	1	2	0	0	0
	S. putrefaciens CN32	2	1	1	0	0	0
	S. baltica OS185	2	1	0	1	0	0
	S. sediminis HAW-EB3	9	1	6	1	0	1

γ	*S. pealeana ATCC700345*	4	1	2	1	0	0
	S. baltica OS199	3	1	1	1	0	0
	S. benthica	1	1	0	0	0	0
	S. halifaxensis	3	1	1	1	0	0
	S. woodyi	2	1	1	0	0	0
	S. piezotolerans	3	1	1	1	0	0
	S. baltica OS223	2	1	1	0	0	0
	V. parahaemolyticus Pery4-66	1	1	0	0	0	0
	V. parahaemolyticus AQ4037	1	1	0	0	0	0
	V. parahaemolyticus AN5034	1	1	0	0	0	0
	V. parahaemolyticus K5030	1	1	0	0	0	0
	V. parahaemolyticus AQ3810	1	1	0	0	0	0
	V. parahaemolyticus RIMD	1	1	0	0	0	0
	V. sp. EX25	1	1	0	0	0	0
	V. vulnificus YJ016	1	1	0	0	0	0
	V. vulnificus CNCP6	1	1	0	0	0	0
	Aeromonas hydrophila	1	1	0	0	0	0
	Halorhdospira halophila	1	0	0	0	1	0
δ	*Geobacter sp. M21*	1	0	0	0	0	1
	Geobacter uraniumreducens	1	0	0	0	0	1
	Nitrosococcus oceani	1	0	0	0	0	1

4.6 Type-II secretion system (T2SS)

Additional factors have been identified that facilitate the formation of the extracellular electron transport chain in *S. oneidensis* MR-1. Proteins, which are not directly part of the minimal set of electron transfer enzymes but considered to be necessary were also found using the synthetic biology approach of heterologous protein expression in *E. coli*. Donald *et al.* could show a functional production of the outer membrane *c*-type cytochrome OmcA from *S. oneidensis* MR-1 in an *E. coli* B strain but not in a K-12 derivate suggesting that a functional type II secretion system (T2SS) is required for surface localization (Donald *et al.*, 2008). An independent study at the same time could also show the necessity of the T2SS for the proper localization of the cell surface lipoproteins MtrC and OmcA in *S. oneidensis* MR-1 (Shi *et al.*, 2008). *S. oneidensis* MR-1 mutants in *gspD* and *gspG*, key components of the T2SS, had impaired ability to reduce Fe(III)- or Mn(IV)-oxides (Shi *et al.*, 2008). Supporting work also has shown an involvement of the T2SS in the establishment of the outer membrane complex during anerobic growth with DMSO (Gralnick *et al.*, 2006). The deletion of the same key components of the T2SS impaired *S. oneidensis* MR-1 in the capacity to anaerobically reduce of DMSO, although not entirely (Gralnick *et al.*, 2006). Consistent with the previous findings, the proper integration of the final reductase DmsA has been shown to be insufficient in the absence of the T2SS (Gralnick *et al.*, 2006). It seems most likely the T2SS is necessary for the translocation of the decaheme *c*-type cytochromes MtrC and OmcA as well as the molybdenum and iron sulfur cluster containing proteins DmsAB across the outer membrane to the surface of bacterial cells where they form a protein complex which is required for outer membrane electron transfer. Further supporting this hypothesis is a study by Shi *et al.*, where the authors observed that proteinase K treatment on *S. oneidensis* MR-1 and western-blot analysis, revealed a resistance of the final reductases to the enzyme in cells which have a non-functional version of the T2SS (Shi *et al.*, 2008). Richter *et al.* developed an *in vivo* method to detect the orientation of outer membrane *c*-type cytochromes in Gram-negative bacteria and could nicely show the interplay of T2SS, the final reductase MtrC of *S. oneidensis* MR-1 and riboflavin, which underlines the findings mentioned above (Richter *et al.*, 2010).

5. Strategies for extracellular electron transfer in *S. oneidensis* MR-1

Prokaryotes can use a wide variety of soluble electron acceptors, such as oxygen, nitrate, nitrite, sulfate and fumarate, which are accessible to their intracellular enzymes. However, many substrates, due to their physical and chemical properties, are either poorly soluble under the respective native growths conditions for the bacteria, toxic when incorporated or simply too large to enter the cell. These extracellular substrates that serve as terminal electron acceptors present a challenge to microorganisms. To overcome the inaccessibility microbes developed various mechanisms to transfer electrons on those insoluble terminal electron acceptors. Several strategies for extracellular electron transport have been reported (Richter *et al.*, 2012, Gralnick & Newman, 2007, Weber *et al.*, 2006, Tebo *et al.*, 2005, Croal *et al.*, 2004). Common to all these mechanisms are multiheme *c*-type cytochromes, proteins with FeS-clusters, quinones and multicopper oxidases, which seem to be fundamental components in the capability of transferring electrons derived from a reduced carbon source across (two) membranes and the periplasmic space to the exogenous electron acceptor.

Probably the most thoroughly investigated strategy is the direct transfer of electrons to insoluble electron acceptors through proteins, which are localized at the outer surface of the cell (Beliaev *et al.*, 2001, DiChristina *et al.*, 2002, Shi *et al.*, 2007, Hartshorne *et al.*, 2009). Here, the interface between electron-transfer proteins and the extracellular electron acceptor is crucial for the activity of reducing bacteria. For direct electron transfer from a catalytic heme group to insoluble metal oxide a distance of <15 Å is required (Kerisit, 2007). Recent structural data has elucidated the actual electron transfer mechanism of heme to metal. Molecular dynamics simulations and *ab initio* electronic structure calculations of an isolated periplasmic STC (small tetraheme cytochrome) molecule from *S. oneidensis* MR-1 showed a direct contact of the hemes I and IV to the iron oxide mineral hematite (Fe_2O_3) (Kerisit, 2007). The crystal structure of STC revealed a surface exposure for heme I and IV of about 290 and 250 $Å^2$ respectively. A recent study revealed the crystal structure of MtrF. It shows surface exposure of the terminal heme V and X of 250 and 300 $Å^2$ respectively. Based on the previous report using STC, the authors suggested

that those terminal hemes have sufficient surface exposure such that they could be involved in direct contact with the iron surface (Clarke *et al.*, 2011). Furthermore, the ability of MtrF, MtrC, and OmcA to rapidly exchange electrons with solid surfaces was confirmed by protein film voltammetry (PFV) (Clarke *et al.*, 2011, Hartshorne *et al.*, 2007, Eggleston *et al.*, 2008, Firer-Sherwood *et al.*, 2008). Taken together, these studies suggest that terminal reductases located at the outer periphery of *S. oneidensis* MR-1 may transfer electrons directly to metal oxides.

In addition, the physiological relevance of an indirect electron transfer mechanism has been emphasized in recent studies through the identified role of non-enzymatic factors. Using iron oxides trapped within nanoporous glass beads Lies *et al.* demonstrated the ability of *S. oneidensis* MR-1 to transfer electrons onto inaccessible electron acceptors which are localized more than 50 µm away from the cell surface (Lies *et al.*, 2005). They suggested that next to direct electron transfer *S. oneidensis* MR-1 has the ability to respire insoluble electron acceptors from a distance using electron shuttles. Those shuttles may be soluble, yet reduced outside of the cell e.g. humic substances or shuttles derived from bacteria (for further discussion see (Lovley & Blunt-Harris, 1999, Lovley *et al.*, 1996)).

Flavins have been shown to be excreted by *S. oneidensis* MR-1 and facilitate the reduction of insoluble substances and accelerate the reduction of electrodes (Marsili *et al.*, 2008, Coursolle *et al.*, 2010, von Canstein *et al.*, 2008). The production of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and riboflavin, by cultures of *S. oneidensis* MR-1 and their intra- and extracellular concentrations were assessed under aerobic and anaerobic growth conditions with FAD being the predominant flavin within cells. In the supernatant FMN was the predominant extracellular flavin, followed by riboflavin, which has lead to a focus on FMN and riboflavin involvement in metal oxide respiration (Canstein *et al.*, 2008). Supporting this idea are several reports indicating that both riboflavin and FMN bind to forms of smectite-clay, forming specific 1:1 interactions with Fe(III) in clays (Mortland, 1984, Mortland, 1983). In *S. oneidensis* MR-1 several studies have linked reduction of flavins to terminal reductases involved in metal oxide respiration. The requirement of the outer membrane decaheme *c*-type cytochromes MtrC and OmcA for the reduction of flavins was shown using mutants lacking various Mtr-associated proteins

(Coursolle *et al.*, 2010). Additionally, the involvement of the outer membrane reductases in the reduction of flavins was also indicated by measuring the oxidation kinetics of reduced MtrF by FMN (Clarke *et al.*, 2011). Notable is the fact, that only 40 % of the MtrF heme groups were oxidized even at a molar ration of 20 FMN:1 heme, suggesting that only a subgroup of the MtrF hemes participate in FMN reduction. In contrast addition of Fe(III) citrate, Fe(III)-EDTA, or Fe(III)-NTA to reduced MtrF fully oxidized all 10 hemes groups (Clarke *et al.*, 2011). A recent study by Gralnick on the mechanism of flavin release using mutagenesis screens on *S. oneidensis* MR-1 revealed that the gene product of *ushA*, encoding a predicted 5′-nucleotidase, resulted in accumulation of FAD in culture supernatants with a corresponding decrease in FMN and riboflavin (Covington *et al.*, 2010). Fractionation experiments show that UshA activity is periplasmic and it is hypothesized that *S. oneidensis* MR-1 secretes FAD into the periplasmic space, where it is hydrolysed by UshA to FMN and adenosine monophosphate (AMP). FMN could then diffuse through outer membrane porins where it accelerates extracellular electron transfer.
Currently, two hypotheses have been offered for the role of flavins in metal oxide respiration. Since soluble iron forms become reduced faster than insoluble Fe(III)-oxides (Beliaev *et al.*, 2001) a possible explanation of the accelerated reduction of insoluble iron by flavins is increased available soluble iron due to chelation (Albert, 1953, Marsili *et al.*, 2008).

In another hypothesis flavins are predicted to have electron shuttle activity, which was found to be the primary mechanism for the electron transfer over a distance (Ross *et al.*, 2009, Baron *et al.*, 2009). Chemical reduced FMN and/or riboflavin was shown to be able to transfer electrons on poorly soluble Fe(III)-oxides, which resulted in the formation of Fe(II) at a ratio of one molecule FMN to two molecules Fe(II) (von Canstein *et al.*, 2008). Nevertheless, the influence of flavins in the respiration of exogenous electron acceptors seems to be not only restricted to iron species since Marsili *et al.* also observed binding of riboflavin to Mn(IV)-oxide surfaces which are commonly used as substrates for *S. oneidensis* MR-1 (Marsili *et al.*, 2008).

When cultivated under conditions of electron acceptor limitation another strategy to transfer electrons on exogenous substances is the use of extracellular filamentous conductive appendages called “nanowires” (Reguera *et al.*, 2005). Using

nanofabricated electrodes patterned on top of individual nanowires and conductive atomic force microscopy, nanowires of *S. oneidensis* MR-1 were found to be electrically conductive along micrometer-length scales (Gorby *et al.*, 2006). Mutants deficient in genes encoding for the *c*-type cytochromes MtrC and OmcA produced those extracellular appendages, but were found to be nonconductive.

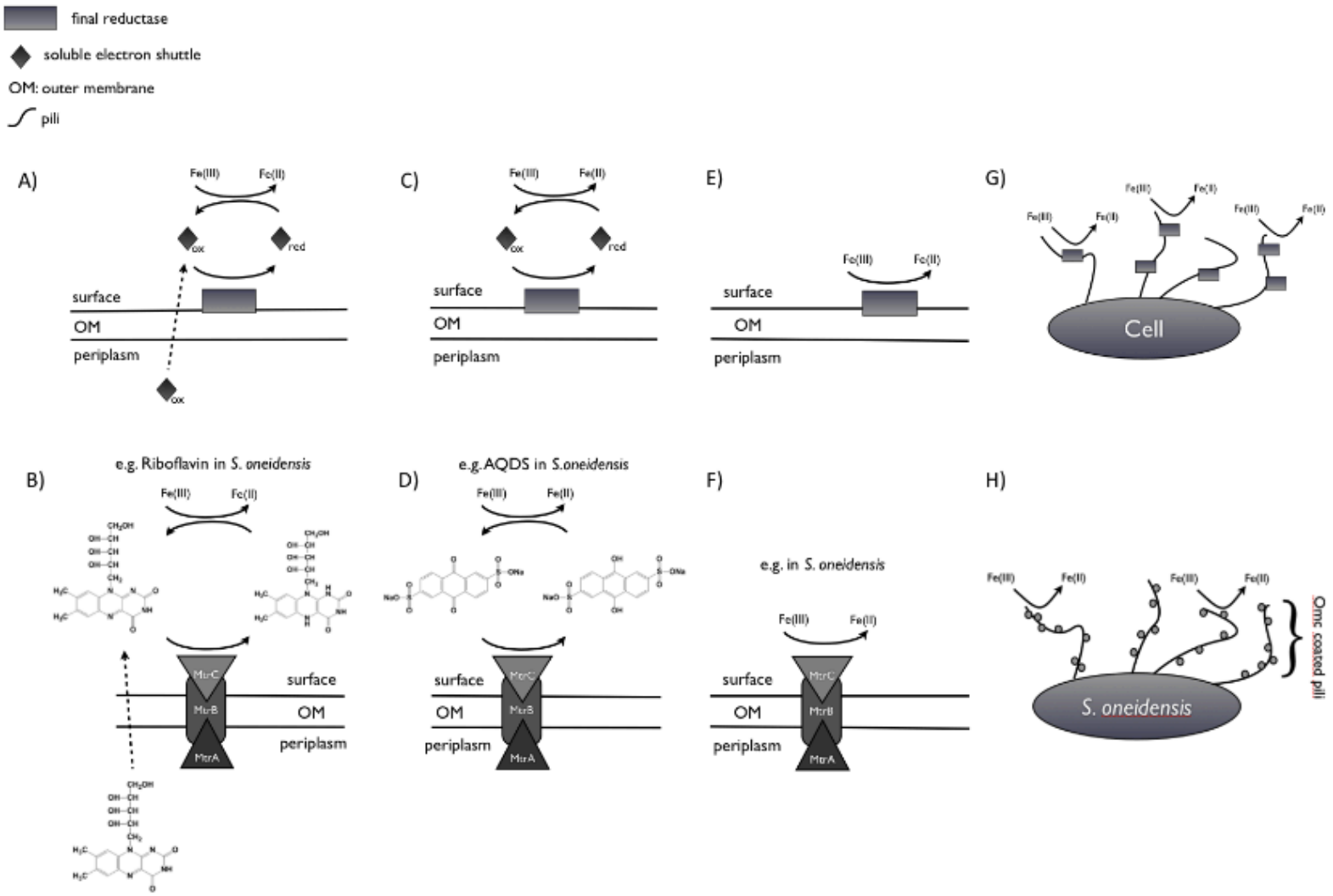


Fig. 4) Strategies of *S. oneidensis* MR-1 for elelctron transfer onto insoluble extracellular elctron acceptors. A) C) E) G) Overview of possible Strategies in general B) FAD as an example for endogenous electron shuttles produced by *S. oneidensis* MR-1 D) AQDS as an exogenous electron shuttle F) electron transfer through direct contact H) electron transfer through direct contact with outer membrane coated pili termed “nanowires”.

MATERIALS AND METHODS

1. Chemicals and materials

If not stated otherwise, all chemicals and materials were purchased from AppliChem (Darmstadt), Fluka (Neu-Ulm), Merck (Darmstadt), Roche Diagnostics (Mannheim), Serva (Heidelberg), Sigma (Deisenhofen), Roth (Karlsruhe) or Roth (Nürnberg). Enzymes were obtained from New England Biolabs (Schwalbach) or MBI Fermentas (St. Leon-Rot).

2. Microorganisms and plasmids

Bacterial strains used in this study are listed in Table 3a and 3b. Plasmids that were used or constructed in this study are listed in Table 4.

Tab. 3a) *S. oneidensis* MR-1 strains used in this study.

Species and strain	**Relevant genotype**	**Reference or source**
JG 7*	wildtype strain	(Myers & Nealson, 1988)
JG 55	Δ*mtrB*	(Schuetz *et al.* 2009)
JG 53	Δ*mtrA*	(Schuetz *et al.* 2009)
JG 69	Δ*mtrB* pBAD$mtrB_{strep}$	(Schuetz *et al.* 2009)
JG132 (ΔOMC)	Δ*mtrD-F* Δ*omcA* Δ*mtrC* ΔSO1659 ΔSO2931 *Para mtrAB*	(Buecking *et al.* 2010)
JG 171 (ΔOMCA)	Δ*mtrD-F* Δ*omcA* Δ*mtrC* ΔSO1659 ΔSO2931Δ*mtrA Para mtrB*	This study
JG198 (ΔOMC $mtrB_{STREP}$)	Δ*mtrD-F* Δ*omcA* Δ*mtrC* ΔSO1659 ΔSO2931 *Para* $mtrAB_{STREP}$	This study
JG199 (ΔOMCA $mtrB_{STREP}$)	Δ*mtrD-F* Δ*omcA* Δ*mtrC* ΔSO1659 ΔSO2931 Δ*mtrA Para* $mtrB_{STREP}$	This study
JG 296	Δ*mtrD-F* Δ*omcA* Δ*mtrC* ΔSO1659 ΔSO2931 Δ*mtrA Para* $mtrB_{STREP}$ Δ*degP*	This study
JG 308	Δ*mtrD-F* Δ*omcA* Δ*mtrC* ΔSO1659 ΔSO2931 Δ*mtrA Para* $mtrB_{STREP}$ Δ*degP* pBAD$degP_{HIS}$	This study
JG 447	Δ*mtrA* $mtrB_{V5}$	This study
JG 486	Δ*mtrA* $mtrB_{V5}$ pBAD $mtrA_{STREP/HIS}$	This study
JG 523	$mtrB_{STREP}$	This study
JG 509	$mtrB_{STREP}$ ΔSO2907	This study
JG 533	$mtrB_{STREP}$ ΔSO3896	This study
JG 591	$mtrB_{STREP}$ ΔSO2907 ΔSO3896	This study
JG 240	Δ*mtrB* suppressor strain	This study
JG 363	Δ$mtrB^{S}$ ΔSO4359	This study

JG 536	Δ*mtrB*^{S} ΔSO4359 pBAD SO4359$_{STREP}$	This study
JG 592	Δ*mtrB*^{S} ΔSO4359 ΔSO4360 pBAD SO4359$_{STREP}$	This study
JG 593	Δ*mtrB*^{S} ΔSO4359 Δ*mtrA* pBAD SO4359$_{STREP}$	This study
JG 560	ΔmtrB pBAD SO4360/59$_{STREP}$	This study
JG 594	Δ*mtrD-F* Δ*omcA* Δ*mtrC* ΔSO1659 ΔSO2931 Δ*mtrA Para mtrB*$_{STREP}$	This study

* JG abbreviation for Johannes Gescher, Number equals position in strain collection.

Tab. 3b) *E. coli* strains used in this study.

Species and strain	**Relevant genotype**	**Reference or source**
JG 22 (DH5αZ1)	*aci*^{q}*, PN25-tetR, Sp*^{R}*, deoR, supE44, Δ(lacZYA-argFV169), Phi80 lacZΔM15*	(Lutz & Bujard, 1997)
JG 63	JG22 pKD46 *recA*	This study
JG 89	JG22 pKD46	This study
JG 98 (WM3064)	*thrB1004 pro thi rpsL hsdS lacZΔM15RP4–1360 Δ(araBAD)567 ΔdapA1341::[erm pir(wt)]*	W. Metcalf, University of Illinois
JG 143 (BL21 (DE3))	*fhuA2 [lon] ompT gal (λ DE3) [dcm]* Δ*hsdS*	(Studier & Moffatt, 1986)
JG 146	JG22 Δ*(napC–F)* Δ*(frdA–D)4380508::(P*$_{tet}$ *cymA)* pEC86	(Schuetz *et al.* 2009)
JG 197	JG146 pMAL *mtrB*$_{STREP}$	This study
JG 264	JG143 pRSF *mtrAB*$_{STREP}$	This study
JG 265	JG143 pRSF *mtrB*$_{STREP}$	This study
JG 271	JG143 pKD46 *recA*	This study

JG 282	JG290 pRSF *mtrB*$_{STREP}$	This study
JG 283	JG290 pRSF *mtrAB*$_{STREP}$	This study
JG 290	JG143 *Δ(972210-972243) 972210::(tetAR)*	This study
JG 446	JG98 *mtrBV5*-epitope	This study
JG 304	JG98 pMQ ΔSO4359	This study
JG 305	JG98 pMQ ΔSO4357 ΔSO4358	This study
JG 306	JG98 pMQ Δ*mtrC*	This study
JG 526	JG98 pSB Δ*mtrA*	This study
JG 557	JG98 pSB ΔSO4360	This study
JG 525	JG98 pSB ΔSO3896	This study
JG 558	JG22 pBAD SO4359$_{STREP}$	This study
JG 559	JG22 pBAD SO4360 SO4359$_{STREP}$	This study
JG 555	JG22 pBAD *wt::mCherry*	This study
JG 556	JG22 pBAD *ISSod1::mCherry*	This study

Tab. 4) Plasmids used in this study.

Plasmids	Relevant genotype	Reference or source
pBAD202	*Para neo*	Invitrogen (Karlsruhe, Germany)
pMS2	pMAL $mtrB_{STREP}$	This study
pMS3	pBAD $mtrB_{STREP}$	This study
pMS7	pBAD *recA*	This study
pMS8	pKD46 *recA*	This study
pRSF-Duett	*T7lac-1 T7lac-2 lacI neo*	Merk (Darmstadt, Germany)
pMS9	pRSF $mtrB_{STREP}$	This study
pMS10	pRSF $mtrAB_{STREP}$	This study
pMS11	pBAD $degP_{HIS}$	This study
pMQ150	*cen6, r6k, ura3, neo, bla*	(Shanks *et al.*, 2006)
pMALp2E	$lacI^q$ *Ptac malE lacZa bla*	New England Biolabs (Schwalbach, Germany)
pMS12	pMQ150 Δ*mtrB*	(Schuetz *et al.* 2009)
pMS13	pMQ150 Δ*mtrA* in ΔOMC	This study
pMS14	pMQ150 genomic $mtrB_{STREP}$	This study
pMS15	pMQ150 genomic $mtrB_{V5}$	This study
pMS16	pMQ150 ΔSO4359	This study
pMS17	pBAD $SO4359_{STREP}$	This study
pMS18	pBAD *wt::mCherry*	This study
pMS19	pBAD *ISSod1::mCherry*	This study
pSB377	$r6k\ tet^R$	(Kaniga *et al.*, 1994)
pMS20	pSB377 Δ*mtrA*	This study
pMS21	pSB377 ΔSO4360	This study
pMS22	pMQ150 Δ*mtrC*	This study
pMS23	pMQ150 ΔSO4357 ΔSO4358	This study
pMS24	pMQ150 ΔSO2907	(Stephan, 2011)
pMS25	pSB377 ΔSO3896	This study

3. Growth conditions and media

3.1 Aerobic growth of *S. oneidensis* MR-1 and *E. coli*

For aerobic growth *E. coli* and *S. oneidensis* MR-1 strains were grown as batch cultures in Luria-Broth (LB) medium consisting of 1% (w/v) Bactotryptone, 1% (w/v) NaCl and 0.5% (w/v) yeast extract. All flasks were continuously shaken at 180 rpm at 30°C and 37°C, respectively.

If necessary, 2,6-diaminopimelic acid (DAP) (100 µg ml^{-1}), kanamycin (kan) (50 µg ml^{-1}), ampicillin (amp) (100 µg ml^{-1}), tetracycline (tet) (15 µg ml^{-1}) or chloramphenicol

(chl) (15 µg ml^{-1}) was added to the media. Growth was determined by optical density measurements at 600 nm.

For growth on agar plates 2% agar was added to the medium prior to autoclaving. For preparation of agar plates containing 10% (w/v) sucrose (suc), one volume of filter-sterilized, threefold concentrated sucrose solution was heated to 60°C and mixed with two volumes of autoclaved, 1.5x concentrated LB medium.

3.2 Anaerobic growth of *S. oneidensis* MR-1

S. oneidensis MR-1 strains are described in Tab. 3a, and were grown aerobically at 30°C in LB medium or anaerobically in minimal medium (4 M) [1.27 mM K_2HPO_4, 0.73 mM KH_2PO_4, 5 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 485 µM $CaCl_2$, 9 mM $(NH_4)_2SO_4$, 5 µM $CoCl_2$, 0.2 µM $CuSO_4$, 57 µM H_3BO_3, 5.4 µM $FeCl_2$, 1.0 mM $MgSO_4$, 1.3 µM $MnSO_4$, 67.2 µM Na_2EDTA, 3.9 µM Na_2MoO_4, 1.5 µM Na_2SeO_4, 2 mM $NaHCO_3$, 5 µM $NiCl_2$, and 1 µM $ZnSO_4$, pH 7.4] supplemented with 50 mM lactate as carbon and electron source and 50 mM ferric citrate or 50 mM fumarate as electron acceptor. Anaerobic media bottles were sealed with rubber stoppers. Oxygen was removed from the medium by repeatedly flushing the headspace of each bottle for 2 min with nitrogen followed by a 2 min application of vacuum. Nitrogen gas and vacuum cycles were repeated 25 times before bottles were autoclaved. Anaerobic 150 ml serum bottles were inoculated with a *S. oneidensis* MR-1 o/n culture to an initial OD_{600} of 0.01. The optical density during anaerobic growth on ferric citrate was measured at a wavelength of 655 nm to avoid scattering caused by ferric iron.

4. Molecular biological methods

4.1 Induction conditions and cell harvest

Induction of an arabinose promoter (P_{ara}) was achieved by 0.15 mM to 1 mM sterile-filtered L-arabinose solution. Anaerobic cultures were induced at an OD_{600} of 0.2-0.3. After induction, cultivation was continued for additional 4 hours. Aerobic cultures in LB were induced at an OD_{600} of 0.4-0.6 and grown for additional 4 hours. At a final OD of 0.5 to 0.8 cells were harvested at 7000 rpm (Sorvall GSA rotor) or 14000 rpm

(Eppendorf Microcentrifuge) at 4°C for 10 min. Induction of the tetracycline promoter was achieved by supplementing the media with 100 µl l^{-1} media of a 2 mg ml^{-1} anhydrous tetracycline stock solution. The IPTG promoter was induced by the addition of 50 µM Isopropyl-β-D-thio-galactoside.

4.2 Isolation of plasmid DNA

Plasmid-DNA was isolated from 3 ml o/n cultures of transformed *E. coli* or *S. oneidensis* MR-1 cells using the Wizard Plus SV Miniprep DNA purification System (Promega, Mannheim) according to manufacturers' instructions.

4.3 Isolation of chromosomal DNA

Isolation of chromosomal DNA was conducted according to Marmur *et al.* (Marmur, 1961) Briefly: roughly 3 g cells were resuspended in 25 ml resuspension buffer (10 mM Tris/HCl, 1 mM EDTA, 0.35 M Sucrose, pH 8). Cell lysis is achieved by addition of 1 g lysozyme with subsequent incubation for 20 min at 37°C. For full lysis of cells, lysis solution (100 mM Tris/HCl, 0.3 M NaCl, 20 mM EDTA, 2% SDS) supplemented with 12.5 ml 5 M Na_2ClO_4, 2 ml ß-mercaptoethanol and 125 µl (20 mg ml^{-1}) proteinase K (possible precipitation was solubilized at 50°C) was combined with cell suspension and incubated for 1-3 hours at 55°C. Subsequently 15 ml Phenol:Chlorophorm (v/v) was added and gently mixed for 30 min at RT. After incubation cell debris was spun down by centrifugation at 4°C and 12000 rpm (GSA rotor) for 10 min. Supernatant was transferred into a DNase free GSA container and 12 ml of Phenol:Chlorophorm solution was added. The suspension was incubated at 37°C for 20 min and subsequently centrifuged (4°C, 12000 rpm, 10 min; GSA rotor). The supernatant was transferred into a DNase free Erlenmeyer flask. Addition of 0.6x Isopropanol (w/v) precipitates genomic DNA. DNA was collected and transferred into a DNase free redcap tube. Subsequently, DNA was gently washed twice with 76% ethanol and supplemented with 25 µl DNase free RNase (10 mg ml^{-1}). RNA degradation was achieved by incubation at 37°C for one hour. After incubation 5 ml of Chloroform:Isoamyl alcohol (25:1, v/v) was added and gently mixed. Solution was centrifuged at 4°C at 9000 rpm for 10 min. Supernatant was transferred into a DNase free Erlenmeyer flask and supplemented with 0.1x (w/v) 3 M Na-Acetate solution and

2 volumes 95% ethanol (DNA get's visible again). Finally DNA was washed twice as described above, dried and resuspended in 1 ml TE (10 mM Tris/HCl, 1 mM EDTA, pH 8) buffer.

4.4 RNA extraction and cDNA synthesis

Total RNA was isolated from three independently grown cultures of *S. oneidensis* MR-1 wildtype and Δ*mtrA* mutant, using the enzymatic lysis and mechanical disruption protocol of the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was stabilized with RNAprotect Bacteria reagent (Qiagen, Hilden, Germany). Cells were enzymatically treated using lysozyme (Sigma-Aldrich, St. Louis, MO), and mechanically disrupted with acid-washed glass beads (Sigma-Aldrich, St. Louis, MO). Isolated RNA samples were treated with RNase free *DNaseI Amplification grade* (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions to remove genomic DNA. To exclude false positive signals in subsequent qPCR reactions due to genomic DNA contamination, the isolated total RNA was used as template for a PCR with primers 19 and 20 that would amplify a 100 bp region that would only be detectable in the presence of genomic DNA. Using *SuperScript III reverse transcriptase* (Invitrogen, Karlsruhe, Germany) with random hexamers, cDNA synthesis from total RNA was carried out according to the manufacturer's instructions. Reverse transcription control reactions were performed on triplicate samples with and without reverse transcriptase enzyme.

4.5 Polymerase chain reaction (PCR)

For amplification of DNA fragments polymerase chain reaction (PCR) was implemented in a S1000^{TM} termal cycler (Bio-rad, Munich). This method was chosen for preparative amplification of DNA fragments for subsequent cloning and for analytical control of previous cloning experiments. For analytical control the ready to use MangoMix^{TM} (Bioline, Luckenwalde) was used. MangoMix is a complete ready-to-use 2x pre-optimized reaction mix containing Mango*Taq*^{TM} DNA Polymerase, dNTPs, red and orange reference dyes and 3 mM MgCl. A standard reaction of 20 µl contained:

- MangoMix (2x) 10 µl
- Primer forward (2pmol μl^{-1}) 5 µl
- Primer reverse (2pmol μl^{-1}) 5 µl
- Template DNA (0.1-0.5 µg μl^{-1}) 1 µl

For preparative amplification of genes or DNA fragments Phusion High fidelity Polymerase (NEB) was used. Phusion DNA Polymerase possesses 5' → 3' polymerase activity and a 3' → 5' exonuclease activity and will generate blunt-ended products. This enzyme was used when high fidelity for cloning was necessary. A standard reaction of 50 µl contained:

- 5 x Phusion GC buffer 10 µl
- 10 mM dNTPs (200 µM each) 1 µl
- Primer forward (2pmol μl^{-1}) 12.5 µl
- Primer reverse (2pmol μl^{-1}) 12.5 µl
- DMSO 1.5 µl
- Phusion DNA Polymerase 0.5 µl
- Template DNA (0.1-0.5 µg μl^{-1}) 1 µl
- ddH_2O 11µl

The following standard program was used:

95°C (98°C)	5 min	initial denaturation
95°C (98°C)	0.5 min	denaturation
40°C-60°C	0.5 min	annealing step
72°C	1-5 min	elongation
72°C	5 min	final elongation
6°C	∞	pause

Steps two through four were repeated 30 times. As denaturing temperature for MangoMix 95°C and for Phusion polymerase 98°C was chosen. Annealing temperature was depending on the respective primer pair. The elongation time was set according to the size of the expected DNA fragment (MangoMix (Taq polymerase): 1 kb min^{-1}; Phusion polymerase: 2 kb min^{-1}). A single colony also could

be used as DNA template. The colony was patched on a master plate prior to addition to the reaction.

4.6 Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

To monitor mRNA levels in cells, RT-qPCR was performed with primers designed using the program primer3 (http://frodo.wi.mit.edu/primer3/) (Rozen & Skaletsky, 2000). The qPCR was performed using primer pairs 19 and 20, 23 and 24, 25 and 26, as well as 27 and 28 which were designed to amplify fragments of *mtrB*, *dnaK, gyrB*, and *recA,* respectively (Tab.1). Equal amounts of cDNA were added in a 25 µl reaction for amplification of each gene region. Triplicates were run on a single qPCR experiment using SSoFAST EvaGreen Supermix (Bio-Rad, Munich, Germany). The final primer concentration was 500 nM. All reactions were performed with the following program: 95.0°C for 3 min, 40 cycles of 95.0°C for 20 s, 64.0°C for 30 s with additional plate reading, and 72.0°C for 30 s and a melt curve to determine primer specificity: 95.0°C for 15 s, 60.0°C for 10 s and a stepwise increase by 0.5°C from 60.0°C to 95.0°C for 5 s. PCR amplification and detection were conducted with a C1000 Thermal Cycler upgraded with a CFX96 Optical Reaction Module (Bio-Rad, Munich, Germany). Each real-time PCR was performed in triplicates based on three independent RNA extractions. A 100 bp fragment from the *dnaK* gene (amplified from genomic DNA using primers 21 and 22) was amplified using primers 23 and 24 and used for derivation of the standard curve (Tab.1). 10-fold dilution steps of the *dnaK* amplificate from $1*10^{8}$ to $1*10^{-2}$ copies μl^{-1} were used to generate the standard curve. *RecA*, *gyrB* and *dnaK* were selected as the internal reference genes for normalization of the expression ratio of *mtrB* from all samples. The normalization factor for the Δ*mtrA* mutant of 0.7545 was calculated using the program geNORM (http://medgen.ugent.be/~jvdesomp/genorm) (Vandesompele *et al.*, 2002). The standard deviation of the normalization factor of 0.0654 was calculated using the formula:

$$SD\ NF_n = \mathrm{NF_n} * \sqrt{\left(\frac{\mathrm{SD\ REF_1}}{\mathrm{n} * \mathrm{REF_1}}\right)^2 + \left(\frac{\mathrm{SD\ REF_2}}{\mathrm{n} * \mathrm{REF_2}}\right)^2 + \left(\frac{\mathrm{SD\ REF_3}}{\mathrm{n} * \mathrm{REF_3}}\right)^2}$$

The standard deviation of the normalized gene of interest (GOI) *mtrB* of 0.059 was calculated using the formula:

$$SD\ GOI_{norm} = \mathrm{GOI_{norm}} * \sqrt{\left(\frac{\mathrm{SD\ NF_n}}{\mathrm{NF_n}}\right)^2 + \left(\frac{\mathrm{SD\ GOI}}{\mathrm{GOI}}\right)^2}$$

The standard error for the GOI of 0.034 was calculated using the formula:

$$SE = \frac{SD}{\sqrt{m}}$$

(m=number of measurements, i.e. triplicates) (Vandesompele *et al.*, 2002)

4.7 Purification and buffer change of plasmid DNA and DNA fragments

Plasmids and DNA-fragments were purified and concentrated using the Wizard DNA purification system (Promega) according to manufacturers instructions.

4.8 Restriction of DNA

Digestion of DNA was done using restriction enzymes from NEB or Fermentas according to manufacturers instruction. Inactivation was achieved by heat or by purification of the DNA fragments.

4.9 Dephosphorylation

To prevent a religation of digested Plasmid DNA, linearized DNA was treated with alkaline phosphatase (Roche) following manufacturers instructions.

4.10 Ligation

DNA fragments were incubated with T4-DNA ligase (NEB) or Quick-DNA ligase (NEB) in a final volume of 20 µl o/n at 8°C or for 30 min at RT, respectively. Plasmid and insert DNA were used in a 1:3 ratio. After incubation the DNA was transformed directly in chemical competent cells or dialyzed on a Millipore Express (Millipore, Schwalbach) membrane with double distilled water for 30 min prior transformation in electro-competent cells.

4.11 One-step isothermal *in vitro* recombination

In vitro recombination of DNA fragments was done as described by Gibson *et al.* (Gibson *et al.*, 2009). Two adjacent DNA fragments sharing terminal sequence overlaps were joined into a covalently sealed molecule in a one-step isothermal reaction. T5 exonuclease removed nucleotides from the 5' ends of double-stranded DNA fragments.

Tab. 5) 5x reaction buffer.

Substance	**Stock conc. [M]**	**Final conc. [mM]**	**Amount added [µl]**
Tris/HCl pH 7.5	1	500	125
$MgCl_2$	1	50	12.5
dNTPs	0.01	1	25
DTT	1	50	12.5
NAD	0.1	5	12.5
PEG-8000		25%	62.5 mg
H_2O			62.5
		Final volume	**250**

Tab. 6) T5 exonuclease storage buffer.

Substance	**Stock conc. [mM]**	**Final conc. [mM]**	**Amount added [µl]**
Tris/HCl pH 7.5	1	50	500
NaCl	1	100	1000
DTT	1	1	10
EDTA	0.05	0.1	20
Triton X-100		0.1%	10 mg
H_2O			8470
		Final volume	**10000**

Tab. 7) Reaction Mastermix.

Substance	**Amount added [µl]**
5x isothermal reaction buffer	80
Phusion polymerase 2 U μl^{-1}	5
NEB Taq DNA-Ligase 40 U μl^{-1}	40
Epicentre T5 Exonuclease 0.1 U μl^{-1}	16
H_2O	239
Final volume	**400**

Complementary single stranded DNA overhangs annealed and Phusion DNA polymerase filled the gaps. In a final step, the Taq DNA ligase sealed the nicks. The T5 exonuclease is heat-labile and is inactivated during the 50°C incubation. The Reaction Mastermix was aliquoted to 15 µl each. The reaction was performed in a final volume of 20 µl for 1.5 hours at 50°C. The DNA fragments were used in equimolar concentrations.

4.12 Construction of an *E. coli* BL21 conditional *degP* mutant

Primers 35 and 36 were used to amplify the *tetAR* genes from transposon Tn10 (Tab. 15). Following the method described by Datsenko and Wanner, the resulting PCR fragment was used to delete the native promoter of the *E. coli degP* protease gene (Lipinska *et al.*, 1988) from the chromosome and subsequently set *degP* under tet-promoter control (Datsenko & Wanner, 2000). For better recombination efficiency plasmid pMS8 was chosen. Clones were screened for their tetracycline resistant phenotype. *DegP* was induced using 100 µl l^{-1} media of a 2 mg ml^{-1} anhydrous tetracycline stock solution.

4.13 Preparation of electro-competent cells

Bacterial cells were grown in SOB medium (0.2% tryptone, 0.5% yeast extract, 10 mM NaCl and 3 mM KCl). A flask containing 25 ml SOB medium was inoculated with 1% (v/v) of an o/n culture of the appropriate strain. At an OD_{600} of 0.5-0.7, cells were chilled on ice for 30 min. Cell suspensions were pelleted in a pre-cooled centrifuge at 6000 g at 4°C for 10 min (Hettich centrifuge) and washed twice with ice-cold water and resuspended in a small amount of water prior electroporation. For storage at -70°C 10% (v/v) glycerol solution was used for the last washing step.

4.14 Electroporation

Electro-competent cells can take up DNA when subjected to a strong voltage pulse. As a rule of thumb, 100 µl competent cells and 2-10 µl DNA solution were pipetted into a precooled electroporation cuvette with a distance of 1 mm between electrodes (Bio-rad). Electroporation was achieved by applying a high voltage pulse of 1.75 kV

for 5 ms using a MicroPulser^{TM} (Bio-Rad). Subsequently 450 µl SOC medium (Tab. 8) devoid of antibiotics was added to the cells. Cells were transferred into a sterile culture tube and incubated at 30° (for *S. oneidensis* MR-1) or 37°C (for *E. coli*) for one hour under continuous shaking. If working with strain WM3064 the SOC medium was supplemented with DAP. After incubation, cells were spread onto agar plates supplemented with the antibiotic that the plasmid, which had been transformed into the cells, conferred resistance to. Increased transformation efficiency was achieved when transformants stayed in SOC medium overnight at room temperature and were plated the next morning.

Tab. 8) Composition of SOC medium.

Substance	Stock conc. [M]	Final conc [mM]	Amount added [µl]
KCl	1	2.5	25
NaCl	5	10	20
MgSO4	1	10	100
MgCl	1	10	100
Glusose	1	20	200
SOB			9555

4.15 Construction of markerless deletion mutants and fusion of STREP- or V5-epitopes to *mtrB* in the *S. oneidensis* MR-1 genome

All primers used for deletion or knock in mutant strains are described in Tab. 15 (Tab. 15). The pMQ150 plasmid was linearized using BamHI and SalI (Tab. 4). The pSB377 plasmid was linearized using NotI (Tab. 4). For markerless deletion mutant strains 500 bp PCR fragments flanking the corresponding genes were amplified and cloned into the linearized pMQ150 plasmid. For deletion mutants due to gene disruption, a 500 bp fragment within the gene of interest was amplified and cloned into the pSB377 plasmid. Cloning was achieved using the method according to Gibson *et al.* (Gibson *et al.*, 2009). WM3064 was used as the donor strain for mating with *S. oneidensis* MR-1.

E. coli strain WM3064 cells harboring pMQ150 or pSB377 plasmid versions were grown overnight at 37°C in LB medium supplemented with 100 µg µl^{-1} DAP and the appropriate antibiotic (Tab. 3b, 4). *S. oneidensis* MR-1 was grown in LB medium o/n at 30°C. *E. coli* and *S. oneidensis* MR-1 cultures (1 ml of each) were harvested and

washed twice with LB medium. After washing steps, the cells were resuspended, combined and spun down at 14000 rpm for 2 min (Eppendorf Microcentrifuge). The supernatant was removed, and the combined cells were resuspended in 500 µl LB. For mating, cells were spot plated on LB+DAP plates and incubated o/n at 30°C. After incubation the spots were recovered in 1 ml of LB medium and diluted in a ratio of 1:5, 1:25 and 1:125. Subsequently, the cells were plated on LB medium plates supplemented with kanamycin or chloramphenicol. After two days, colonies were picked and patched onto LB+kanamycin or LB+chloramphenicol selective, sucrose and LB plates.

E. coli WM3064 cells were not able to grow on LB plates as DAP was omitted. pMQ and pSB have an R6K-type origin of replication and is only replicated in cells expressing the π protein which is conferred by the *pir* gene. π is essential for the stabilization of the R6K ori. *S. oneidensis* MR-1 does not contain *pir*, therefore only transconjugants which integrated the plasmid into the chromosome through a cross-over event were able to grow on antibiotic selective plates. Single colonies were picked and streaked out (diluted) once on LB+kanamycin or LB+chloramphenicol plates to avoid contamination with *E. coli* cells. Additionally, integration of the pMQ plasmid was checked by transferring colonies onto LB plates supplemented with 10% sucrose. This was done by picking single colonies with a sterile pipette tip, transferring them onto a fresh LB+kanamycin plate first, followed by an LB+sucrose plate and an LB plate using the same tip (‘Pick & Patch’ technique). Growth on LB ensured a sufficient number of cells adhered to the tip. In contrast, growth deficiency on sucrose confirmed the successful integration of pMQ into the genome. pMQ contains the *sacB* gene which renders cells sensitive to sucrose due to the activity of the enzyme levansucrase. This enzyme catalyses the synthesis of sugar polymers leading eventually to cell death (pSB does not bare a gene which encodes for levansucrase).

In order to facilitate the second cross-over event selected colonies were cultured overnight in LB in the absence of antibiotic. After diluting 1:10 in fresh LB medium and additional growth for 3-4 hours, several dilutions were prepared in LB (1:10, 1:100, 1:1000). 50 µl from each suspension were plated on LB+sucrose plates to screen for cells having lost the deletion plasmid. A second recombination event had supposedly taken place in cells that were able to form colonies on sucrose-containing plates. Colonies were streak-purified once, and finally picked and re-streaked in

parallel on LB+kanamycin and LB+sucrose. Cells not growing on kanamycin-containing plates but growing on LB+sucrose were PCR tested for occurrence of a mutation event or reversion to the wildtype (Tab. 15). Colonies having the same band pattern as the wildtype had apparently restored the original state, whereas mutant colonies displayed a truncated ~1 kb DNA fragment. Positive colonies were checked by PCR and sequencing of PCR products was carried out by GATC Biotech (Konstanz).

4.16 Promoter prediction

BPROM bacterial promoter predictor was used to identify entire (-35/-10) putative promoter regions. (SoftBerry,Mt. Kisco, NY; http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). *E. coli* based predictions were deemed suitable due to the recent study on single molecule characterization of the Sigma 70 transcription factor of *S. oneidensis* MR-1 indicating that it recognizes -35/-10 regions with a similar motif to that of *E. coli* (Gassman *et al.*, 2008).

5. Electrophoresis

5.1 Agarose gel electrophoresis

Agarose gel electrophoresis was employed to separate and visualize DNA samples due to their negative charge (Sambrook *et al.*, 1989). Agarose gels were prepared by suspending 1% (w/v) dry agarose in 1x TAE buffer (Tab. 9) and subsequent boiling of the suspension until complete dissolving of the agarose was achieved. The suspension was supplemented with 0.5 µg/ml Midori Green (Nippon Genetics, Düren) for subsequent detection of DNA molecules using UV light at a wavelength of 312 nm and cast in a gel tray.
DNA samples were mixed with 6x loading buffer (Tab. 10). As length standard ‘1kb Gene ladder’ (Fermentas) was used in parallel to the other samples to determine length of DNA fragments. Gels were run submerged in 1x TAE buffer at a constant voltage of 120 V.

Tab. 9) 50x TAE buffer.

Substance	**Concentration**
Tris/HCl pH 8	2 M
Glacial acetic acid	1 M
EDTA	50 mM

Tab. 10) 6x loading dye.

Substance	**Amount**
Bromophenol blue	0.05 g
Xylene cyanol	0.05 g
Glycerol	10 ml
1M Tris/HCl pH 8	0.2 ml
H_2O	9.8 ml
Final volume	**20 ml**

5.2 Polyacrylamide gel electrophoresis

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for separation of proteins due to their respective molecular weight (MW) (Laemmli, 1970). The discontinuous gels consist of a stacking gel (Tab. 11), with a bigger pore size due to a lower amount of acrylamide, and a separating gel (Tab. 12) with a higher amount of acrylamide. Samples were supplemented with loading buffer (Tab. 13) and subsequently boiled at 95°C for 5 min to denature the protein. SDS masks the native charge and ß–mercaptoethanol reduces potential disulfide bonds of the proteins. Proteins focus in the stacking gel due to the pH and isotachophoresis. Separation according to the size is achieved in the separating gel. A volume of up to 50 µl was loaded into each well and PageRuler^{TM} Prestained Protein Ladder (Fermentas) was included as a length marker on every gel. Gels were run at room temperature in electrophoresis buffer (3 g l^{-1} Tris, 14.4 g l^{-1} SDS, 1 g l^{-1} glycerine) at a constant voltage of 120 V.

Tab. 11) Stacking gel (4%); Mixture for 6 mini-gels.

Substance	**Composition**	**Volume**
H2O		26 ml
Stacking gel buffer	2 M Tris/HCl pH 6.8	4.5 ml
Acylamide solution	30% (w/v) acrylamide/0.8% (w/v) bisacrylamide	4.95 ml
SDS	10% (w/v)	360 µl
TEMED		45 µl
Ammoniumpersulfate (APS)	10% (w/v)	180 µl

Tab. 12) Separating gel 10%; Mixture for 6 mini-gels.

Substance	**Composition**	**Volume**
H2O		25.6 ml
Separating gel buffer	2 M Tris/HCl pH 8.8	15.75 ml
Acylamide solution	30% (w/v) acrylamide/0.8% (w/v) bisacrylamide	21 ml
SDS	10% (w/v)	630 µl
TEMED		63 µl
Ammoniumpersulfate (APS)	10% (w/v)	315 µl

Tab. 13) Composition of 2x loading dye.

Substance	Composition	Volume
Glycerine		150 µl
Stacking gel buffer	2 M Tris/HCl pH 6.8	120 µl
ß-mercaptoethanol		50 µl
SDS	10% (w/v)	200 µl
Bromophenol blue	0.5% (w/v)	50 µl
H2O		400 µl

6. Staining methods

6.1 Protein determination

Protein concentration was determined according to the method described by Bradford (Bradford, 1976). **B**ovine **s**erum **a**lbumin (BSA) served as protein standard: 0, 2, 4, 6, 8 and 10 µg of BSA were used for the calculation of a standard curve. Samples were diluted 1:10, 1:50 and 1:100. 1 ml of Bradford reagent (0.01% (w/v)

Coomassie G 250, 5% (v/v) Ethanol, 8.5% phosphoric acid) was pipetted to 100 µl of the standard and samples, which were prepared in duplicates. After 15 min of incubation at room temperature 200 µl of each reaction were transferred on a 96-well microtiter plate (NUNC type F plates; Apogent, Roskilde, Denmark). Absorption was measured at 595 nm using the Infinite M200pro plate reader (Tecan, Mainz).

6.2 Heme staining

The heme staining procedure makes use of the *c*-type cytochromes characteristic covalently bound heme groups, which possess a peroxidase activity. Heme containing bands on polyacrylamide gels are stained with 3,3',5,5'-**t**etra**m**ethyl**b**enzidine (TMB) in the presence of hydrogen peroxide (Thomas *et al.*, 1976).

After completion of an SDS-PAGE run, gels were immediately placed in a 12.5% (w/v) trichloroacetic acid (TCA) bath for 30 min to fix proteins on the gel matrix and prevent diffusion. After 30 min incubation the TCA-solution was decanted and gels were washed twice with water. Following another 30 min of incubation with water, the liquid was discarded and gels were covered with TMB solution supplemented with citrate buffer. The staining reaction was catalyzed by the addition of 30 µl 25% H_2O_2.

6.3 Coomassie stain

The Coomassie dyes (R-250 and G-250) bind to proteins through ionic interactions between dye sulfonic acid groups and positive protein amine groups. To visualize proteins through unspecific staining with Coomassie R250, gels were covered with fixing solution (0.25% (w/v) Coomassie blue R250, 30% (v/v) methanol, 20% (v/v) acetic acid) and incubated for 1 hour or o/n at RT. Subsequently gels were destained with destaining solution I (30% (v/v) methanol, 20% (v/v) acetic acid) until protein bands were visible. To lighten up the background, gels were washed additional 30 min in destaining solution II (10% (v/v) acetic acid).

7. Western blotting

Western blotting was performed following the standard procedure (Towbin *et al.*, 1979). Proteins subjected to SDS-PAGE were transferred to nitrocellulose membranes by semi-dry Western blotting. For that purpose six Whatman filter papers and one sheet of nitrocellulose paper (Schleicher und Schüll, Dassel) were cut to the size of the separating gel that was to be blotted. Three filter papers soaked in blotting transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) were placed onto the cathode, followed by polyacrylamide gel, nitrocellulose membrane, and three filter papers also soaked in blotting buffer. Any air bubbles were removed. Finally, the anode was placed on top of the sandwich. Transfer of proteins onto the nitrocellulose membrane was achieved with a current of 1.5 mA cm^{-2} for 1.5 hours. Successful transfer was controlled by shortly staining the blot with Ponceau S solution (0.1% w/v in 1% acetic acid).

8. Immunodetection of STREP and V5 tagged proteins

For visualization of STREP or V5 recombinant proteins through immunodetection, fractions were run on 10% SDS polyacrylamide gels and blotted onto nitrocellulose membrane (Roth, Karlsruhe, Germany) using a semi-dry transfer blot (Biorad, Munich, Germany). Western blotting was performed following the standard procedure (Towbin *et al.*, 1979). The membrane was incubated in 0.3% BSA/Tris buffered saline (TBS) (10 mM Tris/HCl; 150 mM NaCl, pH 7.5) for 1 hour at room temperature or at 4°C overnight with shaking to block unspecific binding sites. The membrane was washed twice with TBS/Tween/Triton buffer (20 mM Tris/HCl; 500 mM NaCl; 0.05% (v/v) Tween; 0.2% (v/v) Triton, pH 7.5) for 10 min at room temperature. Subsequently the membrane was incubated for one hour in 0.3% BSA/Tris solution with a primary antibody specific for either the STREP- or V5-epitope in a 1:1000 dilution (Qiagen, Hilden, Germany). After incubation, the membrane was washed twice with TBS/Tween/Triton buffer for 10 min. The appropriate secondary anti-mouse or anti-rabbit antibody conjugated to alkaline phosphatase (Sigma-Aldrich, Munich, Germany) was added in a 1:17500 dilution in 0.3% BSA/Tris buffer. The membrane was washed for one hour. The buffer was discarded and the membrane was washed four times with TBST buffer (20 mM Tris/HCl; 500 mM NaCl; 0.05% (v/v)

Tween, pH 7.5) for 5 min and additional five times with ddH_2O for 2 min. For signal production the AP-conjugate Substrate Kit (Bio-rad) was used accordingly to manufacturers instructions. Imaging of the blot was performed using the ChemiDoc XRS+ (Bio-rad, Munich, Germany).

9. Ferrous iron measurements and determination of growth on ferric citrate

From anaerobic grown *S. oneidensis* MR-1 cultures in 4 M minimal media supplemented with 50 mM lactate as carbon and electron source and 50 mM ferric citrate as electron acceptor, three parallel one milliliter samples were taken at the corresponding time points. For Fe(II) fixation under aerobic conditions 100 µl of each sample were acidified and diluted with 900 µl of 2 M HCl. Subsequently 20 µl of the acidified samples and different ammonium iron-(II)-sulfate dilutions (0, 0.2, 0.4, 0.6, 0.8 and 1 mM) of a 1 mM stock solution as standard were supplemented with 180 µl of a 1 mg ml^{-1} ferrozine [3-(2-pyridyl)-5,6-bis(4-phe- nylsulfonic acid)-1,2,4-triazine] in 50% ammonium acetate solution. If necessary, samples were diluted further 1:10 or 1:100. Absorption was determined at 562 nm in the Infinite M200 pro plate reader. Cell growth was determined by measuring the OD at 655 nm to avoid scattering due to iron.

10. Fractionation of cells

The periplasmic fraction of anaerobically grown *S. oneidensis* MR-1 cells was isolated as described by Pitts *et al.* with polymyxin B (Pitts *et al.* 2003). After polymyxin B treatment, the cells were harvested at 15,000 g at 4°C for 10 min and resuspended in 100 mM HEPES (pH 7.4) containing 10% glycerol and 0.1 mg/ml DNaseI and passed through a French pressure cell at 137 MPa. Unbroken cells were removed by a centrifugation step at 3000 g and 4°C for 10 min. The supernatant was centrifuged at 208,000 g and 4°C for 60 min. The pellet contains the cell membranes. Seperation of outer and cytoplasmic membranes was conducted according to Leisman *et al.* (Leisman *et al.*, 1995). The pellet, containing the cell membranes, was resuspended in 20 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.4) containing 0.5% N-lauroylsarcosine, protease inhibitor (Complete Protease Inhibitor Cocktail Tablets,

Roche) and 10% glycerol. Subsequently, the membrane suspension was incubated for 60 min on ice and thereafter centrifuged at 208,000 g and 4°C for 60 min. The supernatant comprised the cytoplasmic membranes. The pellet, containing the outer membrane, was resuspended with 100 mM HEPES (pH 7.4) supplemented with protease inhibitor and 10 % glycerol.

11. Amino acid sequence analyses

Genome sequence data for *S. oneidensis* MR-1, *S. baltica*, *S. putrefaciens*, *S. amazonensis*, *S. denitrificans*, *S. frigidimarina*, *S. pealeana*, *S. woodyi*, *S.* sp. ANA-3, *S.* sp. MR-4, *S.* sp. MR-7, *S*. sp. W3-18-1, *S*. *violacea*, *S. loihica*, *S. halifaxensis*, *S. piezotolerans*, *S. sediminis*, and *S. benthica* were obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). *S. oneidensis* MR-1 MtrB homologs in other *Shewanella* species were identified via BLAST analysis. Multiple alignments of MtrB homologs from *Shewanella* sp. were generated using ClustalW application of GeneiousPro 5.5.2 (Biomatters, Auckland, New Zealand). LOGO diagrams were generated from http://weblogo.berkeley.edu using ClustalW alignment files.

12. Genome sequencing

Genomic DNA was isolated using the Illustra bacteria genomic Prep kit (GE Healthcare) according to the manufacturer's instructions. Sequencing was performed on an Illumina HiSeq 2000 with a read length of 46 base pairs by GATC Biotech. The reads were assembled using the software BWA (Li & Durbin, 2009) identification of single nucleotide polymorphisms as well as insertions and deletions was done with SAMtools (Li & Durbin, 2009) and the assembly viewer Tablet (Milne *et al.*, 2010).

13. Fluorescence microscopy

Prior to microscopy, cells were immobilized on pads composed of PBS solidified with 1% agarose. Microscopy was performed with a Leica DM 5500 B Upright research microscope (Wetzlar, Germany) equipped with Leica DFC360FX camera (Wetzlar, Germany) and a HCX PL Fluotar 100.0x1.30 Oil for fluorescense imaging and a N

PLAN 100.0x1.25 Oil objective for phase contrast microscopy. Image processing was carried out using Leica Application Suite (LAS AF lite) Version 2.6.0 build 7002.

14. *In vivo* crosslink

In vivo crosslink was performed according to manufacturers instructions (Thermo Fischer, Walldorf). DSP (Lomant's Reagent) is a water insoluble thiol-cleavable crosslinker. For intracellular crosslinking experiments, cells were washed twice with Reaction buffer (0.1 M HEPES, 0.15 M NaCl, pH 7.2) and subsequently subjected to DSP (solved in DMSO) with a final concentration of 1-2 mM. The reaction mixture was incubated for 30 min at RT or 2 hours on ice. After incubation stop-solution (1 M Tris, pH 7.5) was added to a final concentration of 20 mM and incubated for 15 min. Subsequently cells were fractionated as described above. Samples were separated by SDS-PAGE without ß-mercaptoethanol.

15. Protein purification

15.1 Affinity purification

Purification of 6xHIS-tagged recombinant proteins was achieved using a Ni-NTA Superflow column (Qiagen) with a BioLogic DuoFlow system (Bio-rad) according to manufacturers instructions. $MtrA_{STREP/HIS}$ and $MtrB_{V5}$ were expressed with 1 mM arabinose in *S. oneidensis* MR-1 Δ*mtrA* under anaerobic conditions with 50 mM lactate as electron donor and carbon source and 50 mM fumarate as electron acceptor. Periplasm was isolated as stated above. Samples were supplemented with Imidazole to a final concentration of 20 mM and loaded on the column with constant flow of 1 ml min^{-1} with washing buffer (50 mM HEPES, 300 mM NaCl and 20 mM Imidazole; pH 8). Column was washed with 10 column volumes of washing buffer and subsequently eluted with 50 mM HEPES, 300 mM NaCl, 250 mM Imidazole; pH 8. Fractions were collected and pooled prior subjection to SDS-PAGE.

15.2 Ion exchange chromatography

S. oneidensis MR-1 cells bearing a V5-epitope extension at the 3' end of the *mtrB* gene were grown anaerobically on 4 M minimal medium and fractionated as described above. Total membrane fractions were solubilized in 5% Triton / 50 mM HEPES buffer pH 7.4 and loaded onto a Q-Sepharose anion exchange column. The column was washed with 4 column volumes of 5% Triton / 50 mM HEPES buffer pH 7.4. Membrane complexes were isolated using a NaCl gradient from 0-0.5 M NaCl in 5% Triton / 50 mM HEPES buffer pH 7.4. Several fractions were collected and pure fractions were pooled upon analysis via SDS-PAGE.

15.3 Gel filtration

Equilibration of a Superdex^{200} 10/300 column (GE-Healthcare) or Superdex^{200} (3 cm x 1 m) was achieved with 5 column volumes with 50 mM HEPES, 250 mM NaCl, 0.02% DDM, pH 8. Affinity or ion exchange chromatographically purified samples were loaded, separated and eluted applying a constant flow rate of 1 ml min^{-1}. Elution of proteins was monitored by the absorption of 280 nm for proteins in general or 410 nm for *c*-type cytochromes.

16. Mass spectrometry

16.1 MudPIT analysis

Tryptic digestion: A periplasmic protein fraction (30 µg) was diluted 1:1 with 8 M Urea in 100 mM Tris-HCl, pH 8.7, reduced with 5 mM DTT for 20 min at 55°C and carboxyamidomethylated with 25 mM iodoacetamide for 30 min at 37°C in the dark. The procedure followed a digestion with 0.5 µg lysyl endopeptidase (Wako, Germany) for 3 hr at 37°C. Then the digest was diluted with 100 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.7, to 1 M urea and digested with 0.5 µg trypsin (Promega, Germany) for 5 h at 37°C. The resulting peptide mixture was adjusted to pH ~3 with formic acid and peptides were solid phase-extracted using batch C_{18} reversed phase. Thereafter, the peptides were lyophilized in a speedvac. The extracted peptides were dissolved

in 10 µl 5% HPLC grade acetonitrile, acidified with 0.5 µl formic acid and applied to a MudPIT column.

LC-MS/MS: An automated 17-step, 34-h MudPIT analysis (**Mu**lti**d**imensional **P**rotein **I**dentification **T**echnology) was set up essentially as described earlier (Kislinger & Emili, 2003, Washburn *et al.*, 2001, Wolters *et al.*, 2001). Briefly, a quaternary HPLC-pump (Ultimate, Dionex) was interfaced with a linear ion-trap mass spectrometer (LTQ-FT-ICR, Thermo Scientific,) equipped with a nanoelectrospray source (Thermo Scientific). A 100 µm inner diameter fused silica capillary (Optronis, Germany) was pulled to a fine tip and packed with 8 cm of 5 µm Zorbax Eclipse XDB-C_{18} reversed phase, followed upstream by 8 cm of 5 µm Partisphere strong cation exchange resin (SCX) (Phenomenex, Germany). The samples were manually loaded on the column using a pressure vessel (Proxeon Biosystems, Odense, Denmark). The MS was operated by cycles of one survey scan (400–1800 *m*/*z*), followed by a data-dependent FT-SIM-scan on the precursor ion and by one CID fragmentation at 35% normalized collision energy, which were continuously repeated throughout the entire MudPIT analysis. Singly charged precursor ions were rejected.

Peak lists were generated using extract_msn (BioWorks-Package, Thermo Scientific) and MS/MS spectra searched by an in-house installation of the Open Mass Spectrometry Search Algorithm [OMSSA, version 2.1] against *S. oneidensis* MR-1 protein database (July 2007). Peptide hits were considered significant if the precursor and product ion masses matched within 5 ppm and 0.5 Da, respectively, and if the E-value was below 0.01. Two missed cleavages were allowed. Modifications of amino acids taken into account were: fixed carbamidomethylation of cysteine and optional oxidation of methionine.

16.2 LC-MS/MS (liquid chromatography – tandem mass spectrometry)

For in-gel digestion the excised gel bands were destained with 30 % ACN, shrunk with 100 % ACN, and dried in a Vacuum Concentrator (Concentrator 5301, Eppendorf, Hamburg, Germany). Digest with trypsin was performed overnight at 37 °C in 0.05 M NH_4HCO_3 (pH 8). About 0.1 µg of protease was used for one gel band. Peptides were extracted from the gel slices with 5 % formic acid.

All LC-MS/MS analyses were performed on an ion trap mass spectrometer (Agilent 6340, Agilent Technologies) coupled to a 1200 Agilent nanoflow system via a HPLC-

Chip cube ESI interface. Peptides were separated on a HPLC-Chip with an analytical column of 75 µm in diameter and 150 mm length and a 40-nL trap column, both packed with Zorbax 300SB C-18 (5 µm particle size). Peptides were eluted with a linear acetonitrile gradient with 1 %/min at a flow rate of 300 nL/min (starting with 3% acetonitrile).

Tab. 14) Gradient for HPLC with solvent a: 0.1% formic acid and solvent B: 80% Acetonitril 0.1% formic acid.

Time in min	Percentage B
0	3
30	30
32	80
45	80
50	3

MS/MS analyses were performed using data-dependent acquisition mode. After a MS scan (standard enhanced mode), a maximum of three peptides were selected for MS/MS (CID, standard enhanced mode). Singly charged precursor ions were excluded from selection. The automated gain control was set to 350000. The maximum accumulation time was set to 300 ms.

Mascot Distiller 2.3 was used for raw data processing and for generating peak lists, essentially with standard settings for the Agilent Ion Trap. Mascot Server 2.3 was used for database searching with the following parameters: peptide mass tolerance: 1.1 Da, MS/MS mass tolerance: 0.3 Da, enzyme: “trypsin” with 2 uncleaved sites allowed for trypsin; variable modifications: Carbamidomethyl (C), Gln->pyroGlu (N-term. Q), Propionamide (C) and oxidation (M). For protein and peptide identification the databases *Shewanella* and NCBI were used.

17. Primers used in this work

Tab. 15) Primers used in this study.

No.	Name	Sequence	Purpose
1	BspHI_*recA*_for	CGGCGGTCATGAGTATCGACGAAAACAAACAG	pKD46*recA*
2	HindIII_*recA*_rev	GCCAAGCTTTTAAAAATCTTCGTTAGTTTC	pKD46*recA*
3	pKD46_*exo*_int_for	GAGGCACTGGCTGAAATTGG	pKD46*recA*
4	*recA*_int_rev	GTTTACGTGCGTAGATTGGG	pKD46*recA*
5	*mtrB*_NcoI_for	CATGCCATGGATGAAATTTAAACTCAATTT	pBAD$mtrB_{STREP}$
6	*mtrB*_strep_HindIII_rev	GGGAAGCTTTTATTTTTCGAACTGCGGGTGGCTCCAGGCGCCGAGTTTGTAACTC ATGCT	pBAD$mtrB_{STREP}$
7	Δ*mtrA*_up_for	GATCCCCGGGTACCGAGCTCGAATTCGTAACATTCCCAGCGGTCGGTC	Δ*mtrA*
8	Δ*mtrA*_up_rev	AATAGGCTTCCCAATTTGTCCC	Δ*mtrA*
9	Δ*mtrA*_down_for	CGAATTCTGGGACAAATTGGGAAGCCTATTCAGCGCTAAGGAGACGAG	Δ*mtrA*
10	Δ*mtrA*_down_rev	AGCTTGCATGCCTGCAGGTCGACTCTAGAGGTTCGAGGGCATTGAGGC	Δ*mtrA*
11	*mtrBgen*_up_for	ATGATTACGAATTCGAGCTCGGTACCCGGGGGGGTGGCGGATGAACTGTC	*mtrB* STREP-epitope fusion
12	*mtrBgen*_up_rev	GGCGCCTGGAGCCACCCGCAGTTCGAAAAATAATCCATTTGCCTCATATGCTC	*mtrB* STREP-epitope fusion
13	*mtrBgen*_down_for	TTATTTTTCGAACTGCGGGTGGCTCCAGGCGCCGAGTTTGTAACTCATGCT	*mtrB* STREP-epitope fusion
14	*mtrBgen*_down_rev	CGGCCAGTGCCAAGCTTGCATGCCTGCAGGCAAAAGACACCAGTTATGATG	*mtrB* STREP-epitope fusion
15	Δ*degP*_up_for	ATGATTACGAATTCGAGCTCGGTACCCGGGCCATCTCGAGTAAGATCTTTTTG	Δ*degP*
16	Δ*degP*_up_rev	CTATTCATAACTCCAAATAAGGG	Δ*degP*
17	Δ*degP*_down_for	CTTATTTGGAGTTATGAATAGTCAATTGGCGAATCTGATC	Δ*degP*
18	Δ*degP*_down_rev	CGGCCAGTGCCAAGCTTGCATGCCTGCAGGCACTTCAGAGGTGAACTTGC	Δ*degP*
19	RT_*mtrB*_for	CGGCTTAAAACAAGCCTCTG	RTq-PCR

20	RT_*mtrB*_rev	CCAAAGGTGGGGTTAAAAGC	RTq-PCR
21	*dnaK*_for	ATGGGTAAAATTATTGGTATC	RTq-PCR
22	*dnaK*_rev	TTATTTCTTGTCGTCTTTCAC	RTq-PCR
23	RT_*dnaK*_for	CGTGACGTGAACATCATGC	RTq-PCR
24	RT_*dnaK*_rev	CAGAAACCTGTGGTGGAGC	RTq-PCR
25	RT_*gyrB*_for	GCTTGATTGAAGTCGGTGGT	RTq-PCR
26	RT_*gyrB*_rev	CGTTTCGCTTCAGAAATGGT	RTq-PCR
27	RT_*recA*_for	AGCTATAGCCGCTGAAATCG	RTq-PCR
28	RT_*recA*_rev	CCTCGACATTGTCATCATCG	RTq-PCR
29	HindIII_*mtrA*_rev	GGGAAGCTTTTAGCGCTGTAATAGCTTGC	*mtrA* for pRSF
30	BspHI_*mtrA*_for	GAAATATCATGAAGAACTGCCTAAAAATG	*mtrA* for pRSF
31	NdeI_*mtrB*_for	GGAATTCCATATGAAATTTAAACTCAATTTGATC	*mtrB* for pRSF
32	KpnI_*mtrBstrep*_rev	CGGGGTACCTTATTTTTCGAACTGCGGGTGGCTCCAGGCGCCGAGTTTGTAACTC ATGCT	*mtrB* for pRSF
33	pRSF_MCS1_for	GGATCTCGACGCTCTCCCT	pRSF test for MCS1 insertion
34	pRSF_MCS2_for	TTGTACACGGCCGCATAATC	pRSF test for MCS2 insertion
35	BL21_Δ*degP*_ rev	CAGATTGTAAGGAGAACCCCTTCCCGTTTTCAGGAAGGGGTTGAGGGAGACTAA GCACTTGTCTCCTGTTT	constitutive *degP E. coli*
36	E.coli_*degPtet*_for	TTTGTAAAGACGAACAATAAATTTTTACCTTTTGCAGAAACTTTAGTTCGTTTAAG ACCCACTTTCACATTTAA	constitutive *degP E. coli*
37	SO4359_up_for	ATGATTACGAATTCGAGCTCGGTACCCGGGGGTTGCTACGGGTAAATAAATG	ΔSO4359
38	SO4359_up_rev	ATTTAGGGGATGATTCTAATG	ΔSO4359
39	SO4359_down_for	CATTAGAATCATCCCCTAAATTACTCACTCCATTACTTCAG	ΔSO4359
40	SO4359_down_rev	CGGCCAGTGCCAAGCTTGCATGCCTGCAGGCATAATATTCACGCAGAGGTG	ΔSO4359

41	BspHI_SO4359_for	CATGTCATGAAGTTAAGTAAAACGACAATTGC	pBAD_SO4359$_{STREP}$
42	SO4359 Strep_down_for	TTATTTTTCGAACTGCGGGTGGCTCCAGGCGCCAAAGCTTTTCTTATATAAGAAA CTG	pBAD_SO4359$_{STREP}$
43	SO4362_seq_rev	GCTTGTGCAAACTCATCTGC	Δ*mtrB*^{S} determination
44	*recG*_seq_rev	GTGCGGTCTTCGTAGCGCAG	Δ*mtrB*^{S} determination
45	*mCherry*_natprom_for	CAATATTGGGATTGTATTTTAATATGGTTTCCAAAGGGGAAG	promoter fusion to *mCherry*
46	*mCherry*_pBAD_rev	CCGCCAAAACAGCCAAGCTGGAGACCGTTTTTATTTGTATAACTCATCCATAC	promoter fusion to *mCherry*
47	natprom_*mCherry* rev	CTTCCCCTTTGGAAACCATGGACCCAGCACCTGCATATAG	promoter fusion to *mCherry*
48	pBAD_*mtrBs*_prom_for	GATCAATTCGCGCGCGAAGGCGAAGCGGCATTGCTGCAAGTCGTTAGCAC	promoter fusion to *mCherry*
49	pSB_*mtrA*_for	CTTAACGGCTGACATGGGAATTCCTGCAGCCCGGGCCATCACAATGGCAATGTCT	Δ*mtrA* in Δ*mtrB*^{S}
50	pSB_*mtrA*_rev	CAAGCTCAATAAAAAGCCCCACCGCGGTGGCGGCCGGCAAGTGACACAATTCTC	Δ*mtrA* in Δ*mtrB*^{S}
51	pSB_SO4360_for	CAAGCTCAATAAAAAGCCCCACCGCGGTGGCGGCCACCGTTATGGCATTGCTGAC	Δ*mtrA* in Δ*mtrB*^{S}
52	pSB_SO4360_rev	CTTAACGGCTGACATGGGAATTCCTGCAGCCCGGGCCTGTCATCGCTTACCATTG	Δ*mtrA* in Δ*mtrB*^{S}
53	pSB377_for	CTGACATGGGAATTCCTGCAGC	pSB377 test primer
54	pSB377_rev	CTGCTATCGATGACCTTCATGTTAAC	pSB377 test primer
55	*mtrC*_up_for	ATGATTACGAATTCGAGCTCGGTACCCGGGGCTTATCGTCTTGGTGACAGC	Δ*mtrC* in Δ*mtrB*^{S}
56	*mtrC*_up_rev	TTTGCCCAAGCAGGGGGAGC	Δ*mtrC* in Δ*mtrB*^{S}
57	*mtrC*_down_for	GCTCCCCCTGCTTGGGCAAATTTTTTCCCTGCATAGGTTTGGC	Δ*mtrC* in Δ*mtrB*^{S}
58	*mtrC*_down_rev	CGGCCAGTGCCAAGCTTGCATGCCTGCAGGGCATGCTTAAGTTGCCACCAG	Δ*mtrC* in Δ*mtrB*^{S}
59	SO4357/58_up_for	ATGATTACGAATTCGAGCTCGGTACCCGGGGGCCTTTGTAGGGTGCAAATTC	ΔSO4357/58 in Δ*mtrB*^{S}
60	SO4357/58_up_rev	TAGTTGTAATAAATATGGATAGCGC	ΔSO4357/58 in Δ*mtrB*^{S}
61	SO4357/58_down_for	GCGCTATCCATATTTATTACAACTATAGAATCATCCCCTAAATTTAAAAGC	ΔSO4357/58 in Δ*mtrB*^{S}
62	SO4357/58_down_rev	CGGCCAGTGCCAAGCTTGCATGCCTGCAGGATCGGGGTGGATGTATATTC	ΔSO4357/58 in Δ*mtrB*^{S}
63	pSB377_SO3896_for	CTTAACGGCTGACATGGGAATTCCTGCAGCCCGGGCATCTCTGCATCAGTCGCAT	ΔSO3896

64	pSB377_SO3896_rev	CAAGCTCAATAAAAAGCCCCACCGCGGTGGCGGCCAGGCAACCGCTAAATAGTA	ΔSO3896
65	*mtrB*_up_for	GTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGGTCAATGGGGTGGCGG ATGAAC	*mtrB* V5-epitope fusion
66	*mtrBV5*_up_rev	GGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGTAA TCCATTTGCCTCATATGCTC	*mtrB* V5-epitope fusion
67	*mtrBV5*_down_for	TTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACC GAGTTTGTAACTCATGCT	*mtrB* V5-epitope fusion
68	*mtrB*_down_rev	GAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTACGATCCTGTGTTATCT AAAAACA	*mtrB* V5-epitope fusion
69	pMQ150for	CTGGCGAAAGGGGGATGTG	pMQ150 test primer
70	pMQ150rev	CATTAGGCACCCCAGGCTTTAC	pMQ150 test primer
71	pBAD_test_for	GATTAGCGGATCCTACCTG	pBAD202 test primer
72	pBAD_test_rev	GGACCACCGCGCTACTGC	pBAD202 test primer
73	SO_4359_seq_rev	GGAGCTCGACTCAAAAGCAC	Sequencing/Test primer
74	*mtrB*_inside_rev	CGGTATTTTCATCCGTGGTTTCAC	Sequencing/Test primer
75	Strep_for	GGCGCCTGGAGCCACCCGCAGTTCGAAAAA	Sequencing/Test primer
76	Strep_rev	CGAACTGCGGGTGGCTCC	Sequencing/Test primer
77	*E.coli_degP*_seq_rev	GTCGTTGCTGAAGAAGTCTC	Sequencing/Test primer
78	cond_*deg*_for	CGCCCTTAATTTTTGATTGC	Sequencing/Test primer
79	SO_3896_test_rev	CAAGCGAACGCGCTAAAAAC	Sequencing/Test primer
80	SO_3896_test_for	CAACTTAAGGGATGATTTTAG	Sequencing/Test primer

RESULTS

1. Involvement of the *S. oneidensis* MR-1 decaheme cytochrome MtrA in the periplasmic stability of the beta-barrel protein MtrB

The object of this study was to identify mechanisms, which contribute to the formation of an outer membrane complex responsible for the respiration on ferric iron. We and others identified a puzzling phenotype for an *mtrA* deletion mutant, in which it was shown that MtrA is necessary for the production of the outer membrane ß-barrel protein MtrB (Schicklberger *et al.*, 2011, Hartshorne *et al.*, 2009). MtrB is transcribed and translated in the cytoplasm, transported via the sec-system over the cytoplasmic membrane, and potentially guided by periplasmic chaperons through the periplasm for correct localization in the outer membrane. It is unknown where and when MtrA might be involved and it was the aim of this study to identify at which step MtrA has to be present and to elucidate the specific effects on MtrB production and/or localization.

1.1 Involvement of outer membrane cytochromes in MtrB stability in *S. oneidensis* MR-1

MtrB forms a detectable membrane-spanning complex with MtrC and MtrA (Ross *et al.*, 2007). In a mutant lacking the cytochromes MtrC and OmcA the assembly of a MtrA/MtrB subcomplex was detected and it was therefore suggested that MtrB stability is not dependent on MtrC or OmcA expression (Hartshorne *et al.*, 2009). Bücking *et al.* could show that the outer membrane *c*-type cytochrome MtrF is also a potent reductase of ferric iron species that seems to be able to interact with the heterodimer MtrAB (Bücking *et al.*, 2010). MtrF is highly similar to MtrC (E-value: $3e^{-63}$). Hence, it was tested whether a strain devoid of any outer membrane cytochromes (herein after referred to as ΔOMC) was still able to correctly localize MtrB. In this strain *mtrA* and *mtrB* are under arabinose promoter control. The ΔOMC strain was modified via the subsequent deletion of *mtrA* (resulting in strain ΔOMCA). The *mtrB* gene in strains ΔOMC and ΔOMCA was modified by a 3` extension coding

for a STREP-tag epitope to allow for immunodetection (resulting in strains ΔOMC *mtrB*$_{STREP}$ and ΔOMCA *mtrB*$_{STREP}$, respectively (Fig. 5A). Complementation assays demonstrated that the STREP-tag epitope did not interfere with the function of MtrB (data not shown). Strains ΔOMC *mtrB*$_{STREP}$ and ΔOMCA *mtrB*$_{STREP}$ were grown in 4 M minimal media supplemented with 50 mM lactate as carbon and electron source and 50 mM fumarate as terminal electron acceptor. For expression of *mtrB* or *mtrAB*, respectively, 1 mM arabinose was added to the media (Fig. 5A). Western-Blot analysis of outer membrane protein fractions of ΔOMC *mtrB*$_{STREP}$ and ΔOMCA *mtrB*$_{STREP}$ (Fig. 5B) revealed that in the absence of all outer membrane cytochromes MtrB is only detectable when MtrA gets coexpressed. Therefore, outer membrane cytochromes do not seem to be involved in MtrB production.

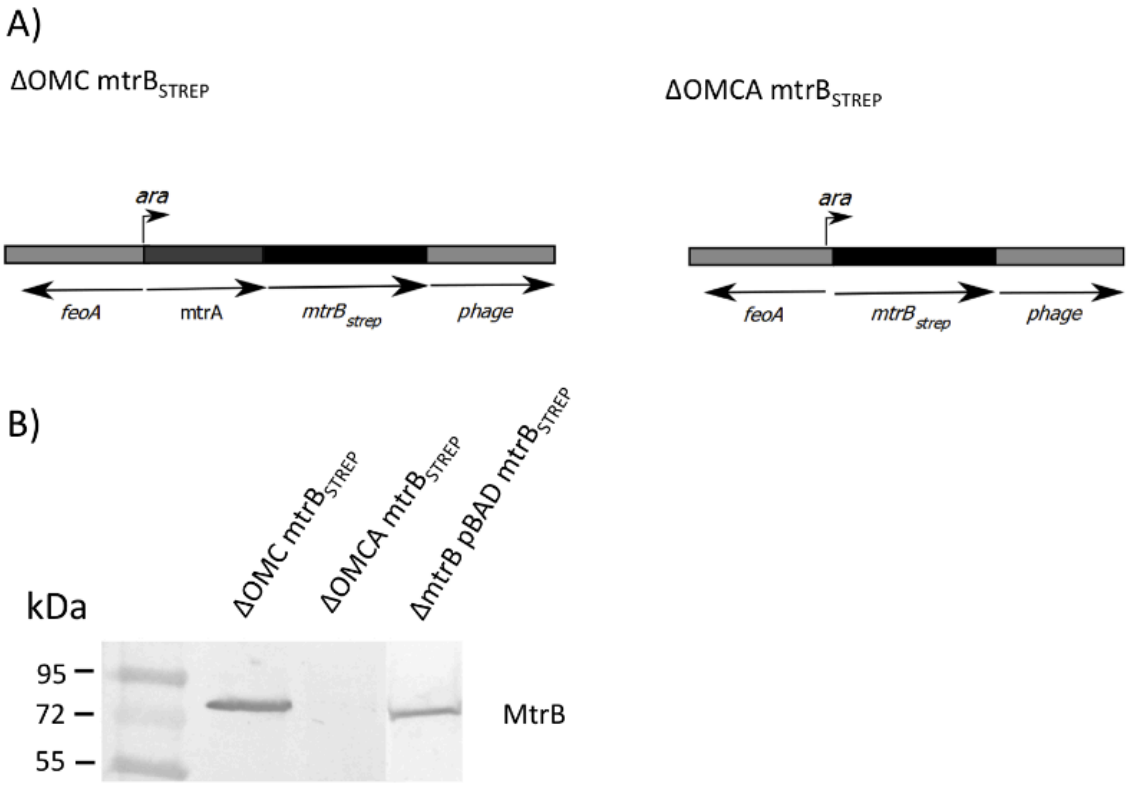


Fig. 5) A) Relevant genotype of ΔOMC *mtrB*$_{STREP}$ and ΔOMCA *mtrB*$_{STREP}$. Arrows indicate gene orientation. Both *mtrAB*$_{STREP}$ *and mtrB*$_{STREP}$ are under arabinose inducible promoter control. B) Corresponding Western-Blot of OM fractions of ΔOMC *mtrB*$_{STREP}$ and ΔOMCA *mtrB*$_{STREP}$. Whole cells were grown anaerobically and fractionated and 50 µg samples of OM proteins were loaded on a 10% SDS-PAGE. The positions of molecular mass markers (in kilodaltons) are indicated on the left of the gel. A membrane fraction of a complemented Δ*mtrB* mutant strain was used as positive control.

1.2 Effect of *mtrA* expression on *mtrB* transcription

The genes *mtrA* and *mtrB* are adjacent and encoded in the same operon. One hypothesis how *mtrA* expression could affect *mtrB* expression would be via regulatory elements within the *mtrA* gene. Hence, we tested whether a deletion of *mtrA* has a downstream effect on the quantity of *mtrB* transcripts. This, in turn, would imply that the absence of MtrB in a *mtrA* deletion mutant could be due to either a drastically reduced number of *mtrB* transcripts, or that the deletion could influence the stability of the transcript.

Independent triplicates of *S. oneidensis* MR-1 wild type and a markerless *mtrA* deletion mutant were grown under fumarate reducing conditions with lactate as electron donor and carbon source to subsequently quantify and compare the number of *mtrB* transcripts in these strains. Fumarate was chosen as electron acceptor instead of ferric iron since *mtrA* mutants are unable to grow under ferric iron reducing conditions. Ruebush *et al.* indicated that the protein expression pattern of cells growing on either chelated iron or fumarate as electron acceptor is similar (Ruebush *et al.*, 2006). Therefore, the necessary enzyme set for ferric iron reduction seems to be present even under fumarate reducing conditions and fumarate was a suitable electron acceptor for our experiments. The conducted experiments revealed that wild type cells contain a 1.24±0.059 fold higher number of *mtrB* transcripts compared to the Δ*mtrA* mutant. Previously, it has been reported that only a more than 1.5-fold change in gene expression ratio is biologically significant (Gao *et al.*, 2004). This shows that *mtrB* is transcribed in almost equal amounts in both strains and that MtrA does not seem to be necessary for *mtrB* transcription or mRNA stability.

1.3 Heterologous *mtrB* and *mtrAB* expression in *E. coli*

Since we could not exclude that, in addition to MtrA and outer membrane cytochromes, other *S. oneidensis* MR-1 specific proteins might influence the production of MtrB, we used *E. coli* for heterologous expression of either *mtrB* or *mtrA and mtrB* to determine whether the same pattern of MtrA dependent MtrB stability would be detectable. *E. coli* DH5αZ1 P_{tet} *cymAmtrA* pEC86 was used as a host for heterologous expression of either $mtrB_{STREP}$ from a plasmid alone or together

with the genome encoded copy of *mtrA*. Interestingly, MtrB was not detectable when produced without concurrent expression of *mtrA* even when expressed in *E. coli* (Fig. 6). In contrast, but in agreement with the previous results in a *S. oneidensis* MR-1 background, MtrA co-expression resulted in a strongly detectable signal of MtrB. This suggests that ubiquitously distributed components for export, maturation and localization of ß-barrel proteins in Gram-negative bacteria are involved in the observed MtrA dependent MtrB stability.

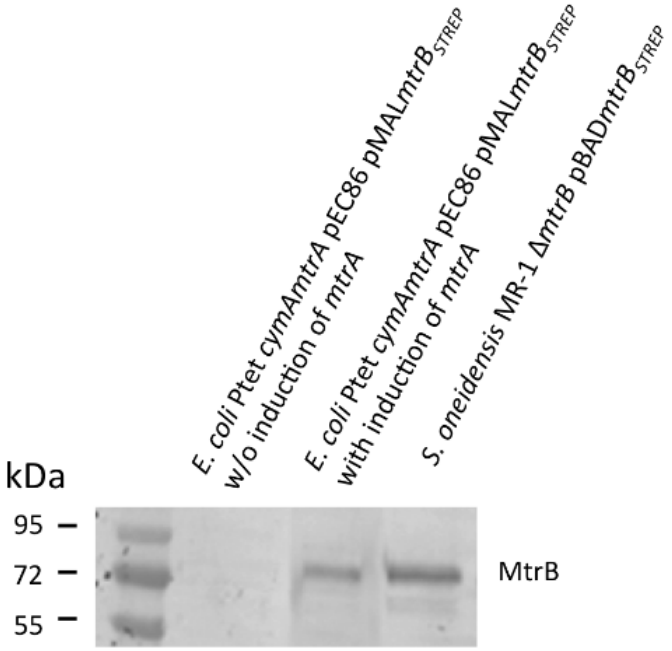


Fig. 6) Western-Blot of *E. coli* P_{tet} cymAmtrA pEC86 pMAL*mtrB*$_{STREP}$ membrane fractions either supplemented with or without 0.43 mM anhydrous tetracycline. Whole cells were grown anaerobically and fractionated and 50 µg samples of OM proteins were loaded on a 10% SDS-PAGE. The positions of molecular mass markers (in kilodaltons) are indicated on the left of the gel. A membrane fraction of a complemented Δ*mtrB* mutant strain was used as positive control.

1.4 MudPIT analysis of periplasmic protein fractions from *S. oneidensis* MR-1 grown under ferric iron reducing conditions

Given our results, that transcription of *mtrB* is not affected by a deletion of *mtrA* and that other *S. oneidensis* MR-1 specific proteins are not involved in MtrB stability we

hypothesized that MtrB could be degraded in the periplasm prior to incorporation into the outer membrane. To investigate this possibility, a MudPIT mass spectrometry screen for periplasmic proteases of *S. oneidensis* MR-1 expressed under dissimilatory iron reducing conditions was conducted. From all detected soluble proteases, we identified – via bioinformatic analysis using the *SignalP* program – two candidates with a typical leader sequence for sec-dependent export into the periplasm (data not shown). One of these (SO3942, score: 93.13, coverage: 37.6%, nine peptides detected) is annotated as a serine protease of the HtrA/DegQ/DegS family while the other (SO3411, score: 15.03, coverage: 3.9%, two peptides detected) is annotated as a putative protease. DegP protease dependent protein hydrolysis is ubiquitously distributed in Gram-negative bacteria. Via its PDZ-domain, DegS detects unfolded proteins or protein aggregates in the periplasm. This detection initiates a cascade of proteolytic events that results in an upregulation of *degP* expression. Alternatively, the Cpx regulon can activate *degP* expression in the event of the production of aberrant outer membrane ß-barrel proteins (Gerken *et al.*, 2010). DegP itself is then responsible for degradation of the unfolded proteins (Bos *et al.*, 2007). In the genome of *S. oneidensis* MR-1 SO3942 is encoded directly upstream of *degS*. BLAST analysis revealed that this protein is highly similar to DegP from *E. coli* (Score 447, E-value $7e^{-127}$). Due to this similarity and clustering we refer to *S. oneidensis* MR-1 SO3942 as *degP*. The above mentioned importance of DegP for the degradation of incorrectly folded proteins, specifically ß-barrel proteins, in the periplasm was the reason for a further investigation of the potential influence of DegP on the degradation of MtrB in the absence of MtrA.

1.5 Influence of *E. coli degP* expression on MtrB stability

If DegP degrades MtrB in the absence of MtrA it should be possible to uncouple this MtrA dependence in the absence of DegP. Therefore, we constructed, a conditional *degP* mutant in *E. coli* BL21 via the integration of a tetracycline resistance cassette. In this mutant *degP* was under tet-promoter control (herein after referred to as *E. coli* BL21 P_{tet} *degP*) (Fig. 7A). *E. coli* BL21 P_{tet} *degP* was used as a host for T7 polymerase dependent expression of either $mtrB_{STREP}$ alone or *mtrA* and $mtrB_{STREP}$ co-expression from independent T7 promoters. Both strains were grown in LB media and at an OD_{600} of 0.3 the media was supplemented with 50 µM IPTG for T7

polymerase induction for the expression of *mtrB* and *mtrAB,* respectively. The samples were split into duplicates and *degP* expression was induced via addition of anhydrous tetracycline to only one duplicate from the original two samples. When *degP* expression was induced, MtrB was again only detectable in strain *E. coli* BL21 P_{tet} *degP* $mtrAB_{STREP}$ producing also MtrA. In contrast, MtrB was detectable even without concomitant MtrA production, when the addition of anhydrous tetracycline was omitted in strain *E. coli* BL21 P_{tet} *degP* $mtrB_{STREP}$ (Fig. 7B). Moreover, MtrB was for the most part correctly localized to the outer membrane and only to a minor extend detectable in the cytoplasmic membrane fraction or the soluble protein pool. Therefore, the presence of MtrA seems to be crucial for periplasmic stability of MtrB. Notably, the requirement of MtrA can be mitigated by turning off *degP* expression in *E. coli*.

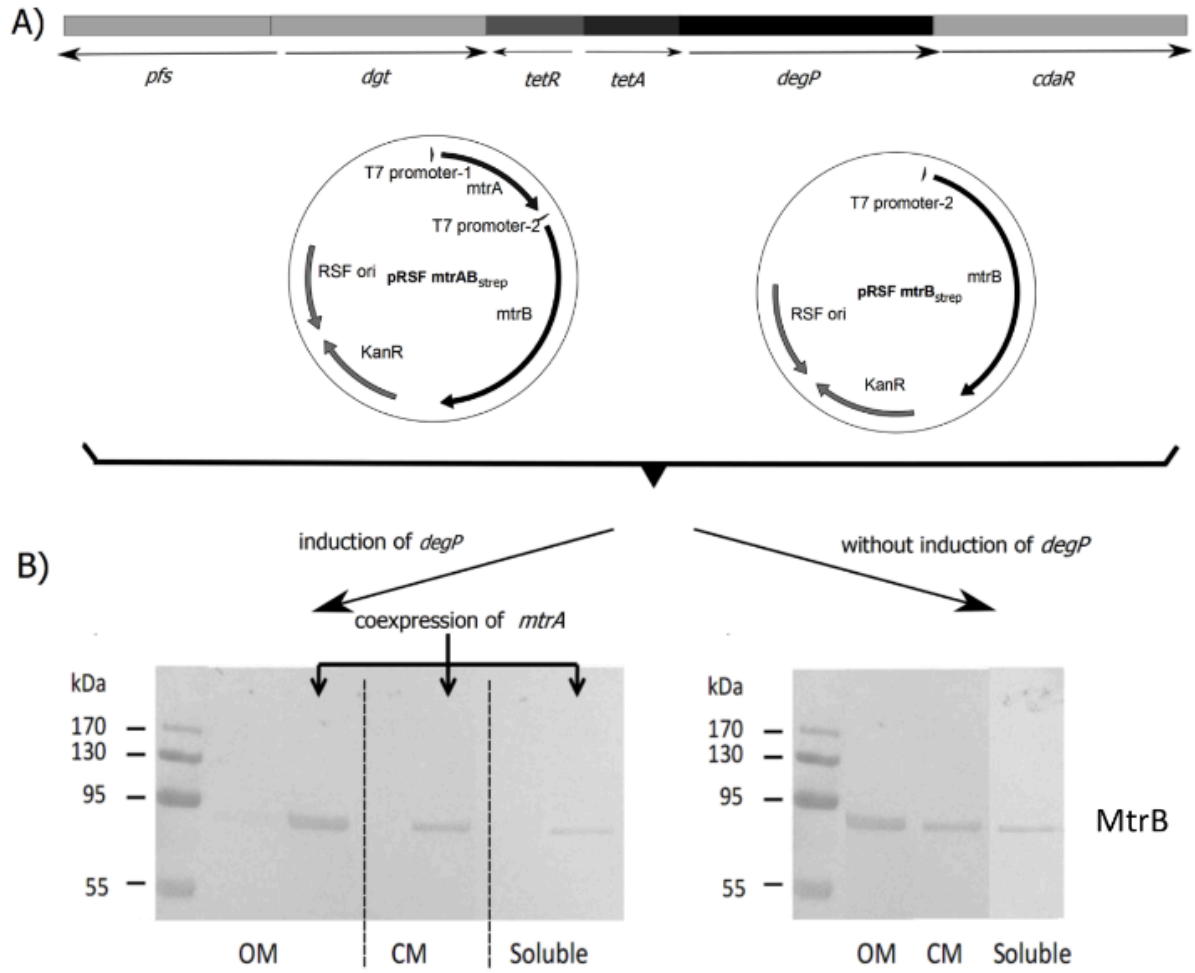


Fig. 7) A) Relevant genotype of the *E. coli* BL21 P_{tet} *degP* mutanat. The arrows indicate the orientation of the respesctive genes. Heterologous expression of $mtrB_{STREP}$ or $mtrAB_{STREP}$ was achieved using either plasmid pRSF $mtrB_{STREP}$ or pRSF $mtrAB_{STREP}$. The respective genes were under T7 polymerase dependant expression. T7 polymerase was expressied upon induction with 50 µM IPTG. B) Corresponding Immunoblots of MtrB in an induced *degP* background without or with co-expression of *mtrA* (left). Immunoblot of MtrB in an uninduced *degP* background without co-expression of *mtrA* (right). 20 µg of protein samples were loaded on a 10% SDS-PAGE.

1.6 Uncoupling of the MtrA/B dependence in *S. oneidensis* MR-1

To test whether the results obtained in the model organism *E. coli* are in agreement to those obtained in *S. oneidensis* MR-1 we constructed a markerless *degP* deletion mutant in strain ΔOMCA (Fig. 8A). The strain ΔOMCA is deficient in all outer membrane cytochromes and *mtrA* and carries an arabinose promoter-controlled version of *mtrB* with a 3` extension coding for the STREP-tag epitope. As expected,

MtrB was detectable in the outer membrane of ΔOMCA Δ*degP* in the presence of 1 mM arabinose as inducer for $mtrB_{STREP}$. In contrast, when the *degP* mutation was complemented *in trans* with a plasmid containing *degP*, MtrB was degraded and therefore not detectable in the outer membrane (Fig. 8B).

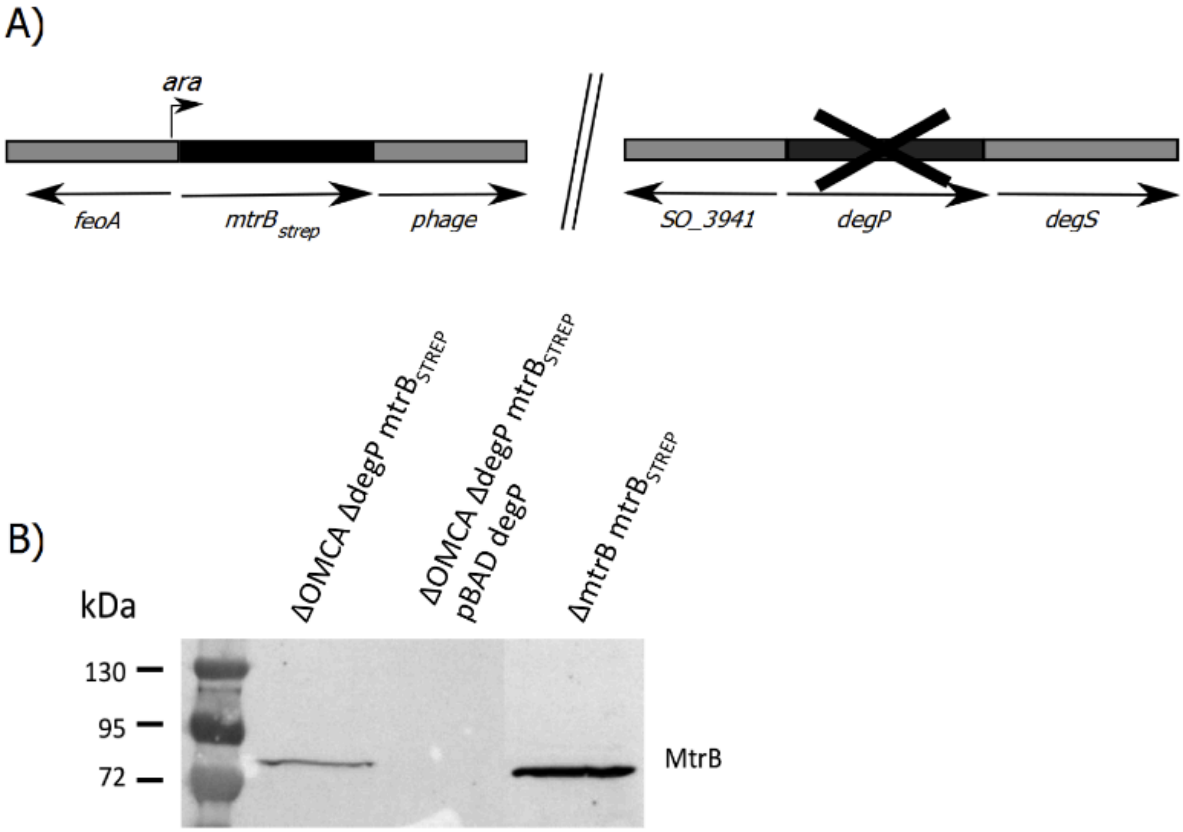


Fig. 8) A) Relevant genotype of *S. oneidensis* Δ*degP* Δ*mtrD-F* Δ*omcA* Δ*mtrCA::*P_{ara} $mtrB_{STREP}$ (referred to as ΔOMCA Δ*degP*). Arrows indicate the orientation of the respecting genes. The gene encoding for MtrB is under arabinose inducible promoter control. The deletion of the gene encoding for the homologous DegP protease is indicated by the cross. B) Corresponding Western-Blot of OM fractions of a *degP* deletion mutant and an *in trans* complemented mutant strain of *degP* expressing $mtrB_{STREP}$. A membrane fraction of a complemented Δ*mtrB* mutant strain was used as positive control. 50 µg of protein samples were loaded on a 10% SDS-PAGE. The positions of molecular mass markers (in kilodaltons) are indicated on the left of the gel.

However, although expressed and correctly incorporated in the outer membrane of *S. oneidensis* MR-1, the mutant lacking MtrA and DegP was not able to respire on ferric

citrate (data not shown), suggesting that the function of MtrA for MtrB stability might be curable by *degP* deletion while its function as electron transfer protein cannot be complemented by other periplasmic *c*-type cytochromes that are expressed under ferric iron reducing conditions.

2. Biochemical and genetic analysis of protein-protein interactions which might be responsible for MtrB stability

The ability of the Gram-negative bacterium *S. oneidensis* MR-1 to respire ferric iron relies on an integral membrane complex which has been shown to consist of the periplasmic oxidoreductase MtrA, the ß-barrel protein MtrB and the final reductase MtrC in a 1:1:1 ratio. Studies of the past two years have indicated a dual function of the *c*-type cytochrome MtrA. Next to the ability of transferring electrons through the periplasm to the final reductase MtrC, MtrA also seems to have a stabilizing function on MtrB and thus a high impact on the MtrCAB complex formation (see above). Deletion of the in Gram-negative bacteria ubiquitously distributed protease DegP in a *mtrA* null mutant strain uncoupled the dependence of MtrB on MtrA in terms of stability.

2.1 Investigation of possible chaperone like function of MtrA for MtrB during transport through the periplasm

One hypothesis which would explain the dependency of MtrB on MtrA might be a possible chaperone like function of MtrA which guides MtrB through the periplasm to the ß-barrel assembly complex, after secretion into the periplasm by the Sec system. This hypothesis also relies on the assumption of the initial complex formation in the periplasm before or while MtrB is transported to the outer membrane. To test this hypothesis we first mutagenized a Δ*mtrA* strain (Schuetz *et al.*, 2009) by introduction of a V5-epitope in the chromosome at the 3' end of the gene encoding for MtrB. The gene $mtrB_{V5}$ is under arabinose inducible promoter control. For immunoblot identification and affinity purification, we transformed a $mtrA_{\text{STREP/HIS}}$ construct under pBAD promoter control (Schuetz *et al.*, 2009) into the Δ*mtrA* $mtrB_{V5}$ strain. Prior to *in vivo* crosslinking, the ability for anaerobic iron respiration of this recombinant strain was tested and determined to be similar to wildtype (data not shown). For *in vivo*

crosslinking cells were grown anaerobically on 4 M minimal medium with 50 mM lactate as carbon and electron source and 50 mM fumarate as electron acceptor. The cells were harvested at exponential phase and aliquots were treated with different concentrations of Dithiobis-(succinimidylpropionate) (DSP or Lomant's Reagent). DSP is a membrane permeable crosslinking agent, which unspecifically links proteins by forming amide bonds which are ~15 Å apart from each other. The crosslink is reversible by the addition of ß-mercaptoethanol. The periplasmic fraction was isolated as described by Schuetz *et al.* and possible crosslinked protein complexes were affinity purified using a Ni-NTA superflow column (Qiagen) (Schuetz *et al.*, 2009). The elution profile showed one distinct peak. Flow through and purified fractions were separated by SDS-PAGE and subsequently blotted on a nitrocellulose membrane for immunodetection with either anti-V5-AB for detection of MtrB or anti-STREP-AB for tracking down MtrA (Fig. 9). Although a signal for MtrB in the periplasmic fraction could be observed, a co-elution of MtrA and MtrB was not detected. Thus, a transient complex of MtrA and MtrB in the periplasm most likely does not occur.

2.2 Identification of components interacting with periplasmic MtrA

An alternative hypothesis involves an interaction of MtrA with a yet unknown protein, which in turn is responsible for the stability of MtrB. Therefore, cells were grown anaerobically on 4 M minimal medium supplemented with lactate and fumarate and subsequently treated with DSP. The isolated periplasm was affinity purified as described above. The fraction resulting from a single distinct peak was subsequently loaded on an analytical gel filtration column (Fig. A1 (Appendix)). Fractions, which show overlapping signals at 420 nm – Soret peak of *c*-type cytochromes – indicating MtrA, and at 280nm were collected, pooled if possible, and subsequently subjected to SDS-PAGE. Two resulting bands of ~70 kDa and ~130 kDa were excised and submitted to LC-MS/MS. As shown in Table A1 (Tab. A1), again, no signal for MtrB was observed in either of the fractions, which confirms that there is no transient complex formation between MtrA and MtrB in the periplasm. A positive crosslink control was given by the co-elution of FccA with MtrA. Direct interaction of these

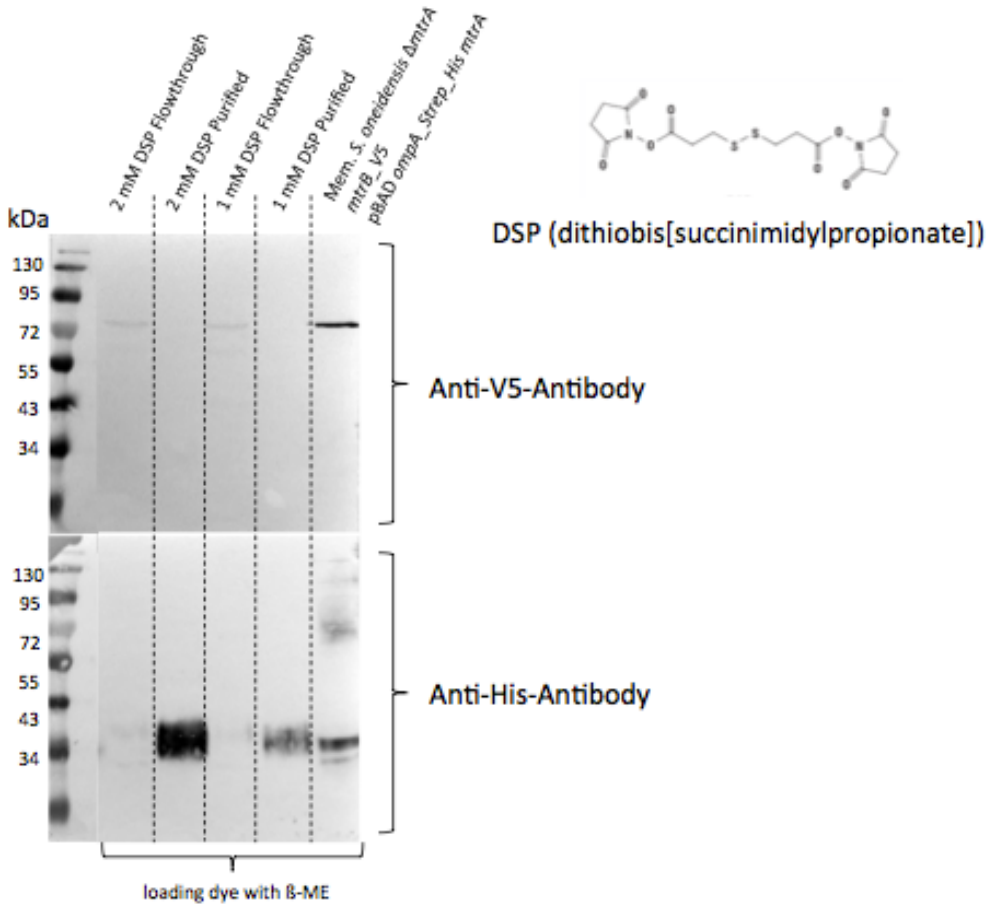


Fig. 9) Immunoblots of crosslinked and subsequently affinity purified periplasmic fractions. Whole cells were grown anaerobically under ferric citrate reducing conditions. Subsequently cells were treated with either 1 mM or 2 mM of DSP. The periplasm was isolated and affinity purified using a Ni-NTA column. Flowthrough and purified fraction were loaded under reducing (addition of ß-mercaptoethanol) conditions. Upper blot: detection of MtrB with anti-V5-AB; lower blot: detection of MtrA with anti-STREP-AB. As positive control a membrane fraction of untreated strain was used. The structure of DSP is shown in the upper right. The crosslink is reversible under reducing conditions due to the disulfide bond in the linker. 10 µg of protein samples were loaded on a 10% SDS-PAGE. The positions of molecular mass markers (in kilodaltons) are indicated on the left of the gel.

proteins with MtrA has been shown before (Schuetz *et al.*, 2009). Using *SignalP* analysis, 11 possible proteins that are secreted into the periplasm, which might interact with MtrA were detected. In the fraction which resulted in the ~70 kDa band, one single protein with a signal peptide, the 39.7 kDa putative outer membrane porin (encoded by SO3896), could be identified. The size of this protein also would match

to a complex with MtrA of ~70 kDa. In the fraction which resulted in the ~130 kDa band, the 95.8 kDa TonB-dependent receptor domain-containing protein (encoded by SO2907) was in the focus of our interest. In addition, it has been identified as a possible partner of a complex of ~130 kDa together with MtrA, since the gene product of SO2907 always co-eluted during purification of the MtrCAB complex (Stephan, 2011).

2.3 Determination of possible protein-protein interactions of MtrB in the outer membrane due to disulfide bond formation

In order to identify factors that mediate an indirect interaction between MtrA and MtrB, a complementary approach to the DSP crosslinking strategy mentioned above was performed to elucidate possible interaction partner of MtrB.
In this experiment, isolated membrane fractions of Δ*mtrA* $mtrB_{V5}$ pBAD $mtrA_{STREP/HIS}$ cells treated with different concentrations of DSP were separated under non-reducing conditions using SDS-PAGE and subsequently transferred onto a nitrocellulose membrane. As negative control, membrane fractions of untreated cells of the same genotype and of a Δ*mtrB* strain were used. Developing of the blot with antibodies specific for the V5 epitope – representing MtrB – revealed a shift for the MtrB signal (Fig. 10A). Attempts to purify the complex in order to reverse the crosslink and identify individual components were not successful (data not shown). However, remarkably, a shift of MtrB could be observed even in the membrane fraction of the untreated cells, which disappeared when samples were reduced with ß-Mercaptoethanol (Fig. 10B).

MtrB contains two cysteine residues, which are localized at the N-terminus of its amino acid sequence forming a CXXC motif. Next to MtrB, the homologous proteins MtrE, DmsF and the gene product of SO4359 from *S. oneidensis* MR-1 also contain only two cysteine residues in their amino acid sequence after signal sequence processing. A multiple sequence protein alignment of MtrB with MtrB homologs of *S. oneidensis* MR-1 revealed that the N-terminal CXXC motif is highly conserved in all four ß-barrel proteins (Fig. 11). Further ClustalW alignment of all MtrB proteins and MtrB homologs from all sequenced *Shewanella* species (except of *S. denitrificans*)

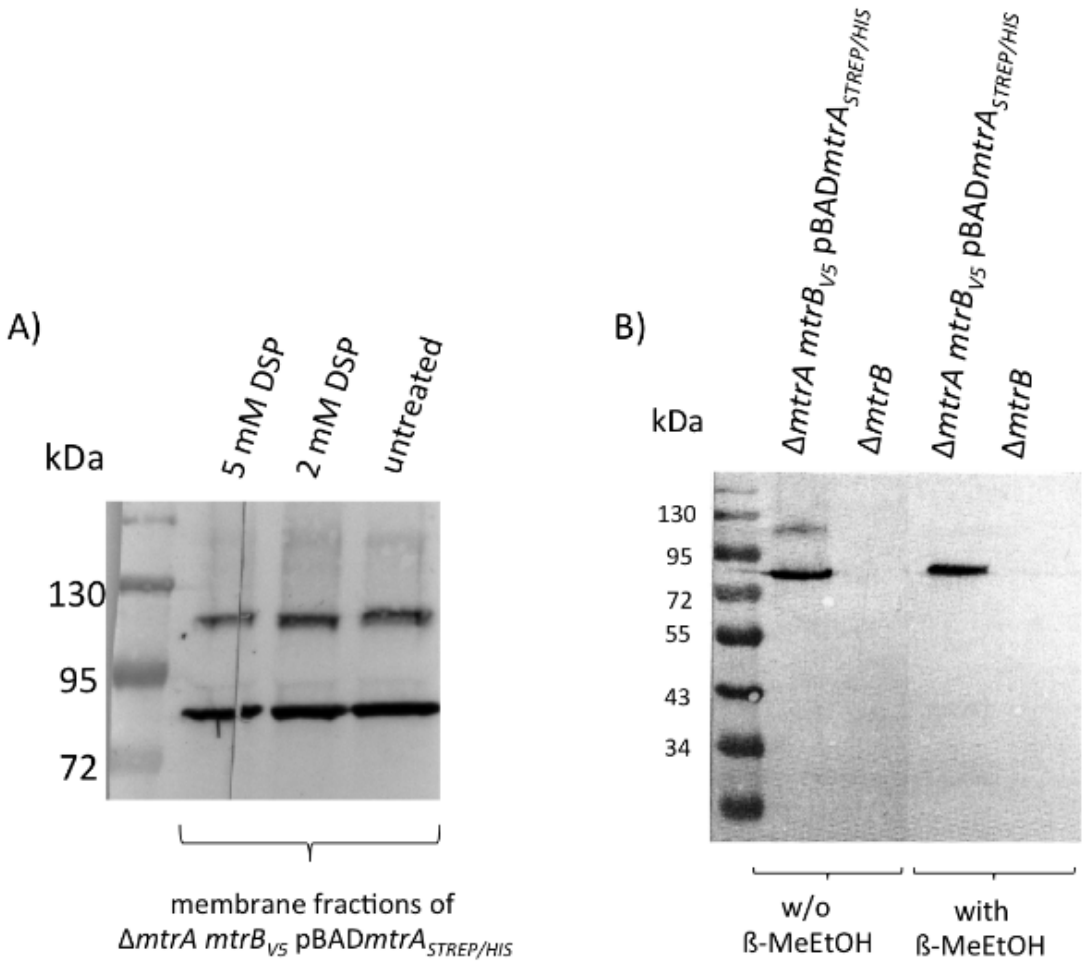


Fig. 10) A) Membrane fractions of Δ*mtrA* *mtrB*$_{V5}$ pBAD *mtrA*$_{STREP/HIS}$ cells treated with different concentrations of DSP (5 mM or 2 mM) or untreated, respectively. Membrane fractions were separated under non-reducing conditions using SDS-PAGE. MtrB was detected using antibodies specific for the V5 epitope. B) Membrane fractions of untreated Δ*mtrA* *mtrB*$_{V5}$ pBAD *mtrA*$_{STREP/HIS}$ and Δ*mtrB* respectively (line 1 through 4). Reduction of the untreated sample with ß-Mercaptoethaol (ß-MeEtOH, line 3 and 4) resulted in a band of the expected MW of MtrB. In addition, the absence of a signal in the membrane fraction of Δ*mtrB* illustrates that the signal at ~120 kDa is due to MtrB. 50 µg of protein samples were loaded on a 10% SDS-PAGE. The positions of molecular mass markers (in kilodaltons) are indicated on the left of the gel.

emphasize this highly conserved motif suggesting a structurally important formation of a disulfide bond or an involvement of this motif in protein-protein interactions (Fig. 12). Since we could show that cysteine residues are responsible for the shift of the MtrB signal and therefore these residues might be involved in protein-protein interactions of MtrB, we submitted the band of ~120 kDa deriving from untreated cells under non-reducing SDS-PAGE conditions to LC-MS/MS and synchronized the data to the *S. oneidensis* MR-1 proteome.

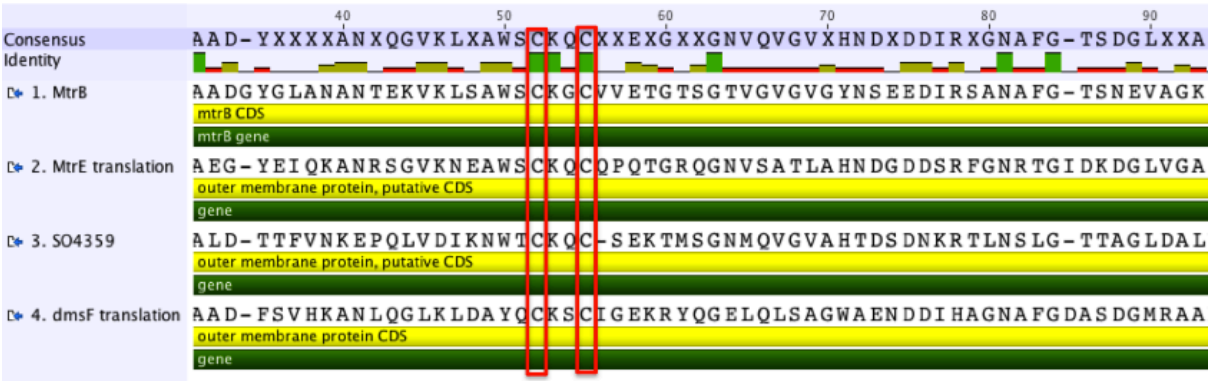


Fig. 11) ClustalW protein alignment of *S. oneidensis* MR-1 MtrB homologs. Red boxes highlight conserved cysteine residues.

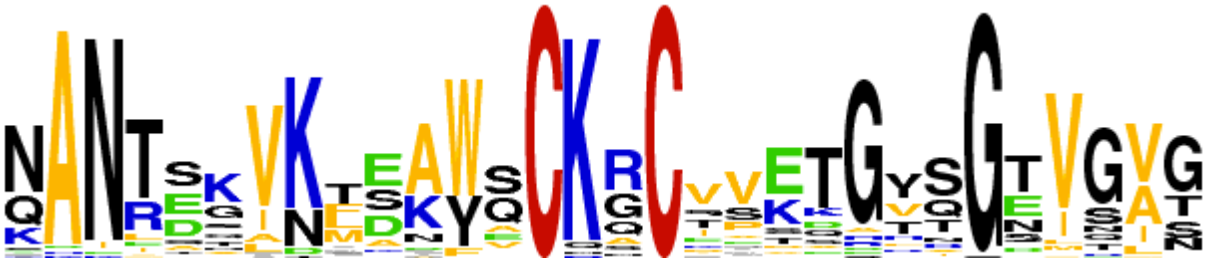


Fig. 12) LOGO Diagram of CXXC region of MtrB. Diagram generated from ClustalW alignment of all MtrB homologs from all 18 sequenced *Shewanella* strains. Letter height corresponds to percentage of sequences with specific residue at the indicated position. Numbering is according to *S. oneidensis* MR-1. The two cysteine residues are conserved in **all** 53 MtrB-like proteins.

2.4 Comparison of LC-MS/MS data of untreated membrane fractions and MtrA pulldown and genetic analysis of identified targets

Among all the detected proteins, revealed by LC-MS/MS, several TonB-dependent receptors were identified. These proteins are encoded by the genes SO2907, SO2469, and SO2427 (Tab. A2). An additional protein encoded by SO3896, a 40 kDa OM ß-barrel protein, also was one of the observed hits. The proteins corresponding to genes SO2907 and SO3896 were also found to possibly interact with MtrA in the periplasmic fraction.

Subsequently single or double deletion mutant strains were constructed that were devoid of either SO2907 or SO3896 or both genes together. None of the mutants displayed a detectable phenotype (neither was the ability of ferric iron reduction nor the stability of MtrB affected) suggesting either a complementation by other redundant proteins or no involvement in the MtrB stability/assembly in *S. oneidensis* MR-1. Towards a redundancy driven complementation would speak the high similarity of two other proteins to the gene product of SO2907 (e-value of SO2469: $2e^{-131}$ and SO2427: $6e^{-63}$).

3. Genomic plasticity responsible for dissimilatory iron reduction in *S. oneidensis* MR-1

3.1 Isolation of a *mtrB* suppressor mutant strain

The goal of the following study was to identify alternative or less dominant pathways for iron reduction, which are encoded within the *S. oneidensis* MR-1 chromosome.
It is known that the integral outer membrane protein MtrB is necessary for the dissimilatory iron reduction in *S. oneidensis* MR-1 (Myers & Myers, 2002, Beliaev & Saffarini, 1998, Hartshorne *et al.*, 2009). However, the chromosome of *S. oneidensis* MR-1 contains three homologs of the gene encoding for MtrB: MtrE, DmsF and the gene product of SO4359. Partial functional replacement experiments for MtrA by MtrA homologs, MtrB by MtrE as well as partial redundancy of CymA by the quinol

dehydrogenase complex (SirCD) have been shown recently (Coursolle & Gralnick, 2012, Coursolle & Gralnick, 2010, Cordova *et al.*, 2011).

To investigate how *S. oneidensis* MR-1 adapts to the challenge of iron respiration without the ß-barrel protein MtrB, we screened for a *mtrB* suppressor strain which regained the ability to respire on iron. Therefore, a *mtrB* null mutant strain was inoculated in serum vials containing anoxic 4 M minimal medium supplemented with 50 mM lactate as electron donor and 50 mM ferric citrate as electron acceptor. After incubation of 30 days, the medium turned to a greenish color, indicating ferrous iron formation. The cells were then harvested and an aliquot was used for inoculation of additional ferric citrate vials. This step was repeated twice until the incubation time for complete ferric iron reduction was decreased to 48 hours (data not shown). Aliquots were plated on ferric citrate plates supplemented with 50 mM lactate and incubated under anoxic conditions for isolation of possible suppressor mutant stains. An isolated single colony, referred to as Δ*mtrB*^{S}, was chosen for molecular and biochemical characterization. We determined the ability of Δ*mtrB*^{S} to respire on ferric citrate by monitoring the ferrous iron formation and the optical density over the time (Fig. 13). The *mtrB* null mutant strain and the wildtype strain were monitored simultaneously to Δ*mtrB*^{S} as a negative and positive control, respectively.

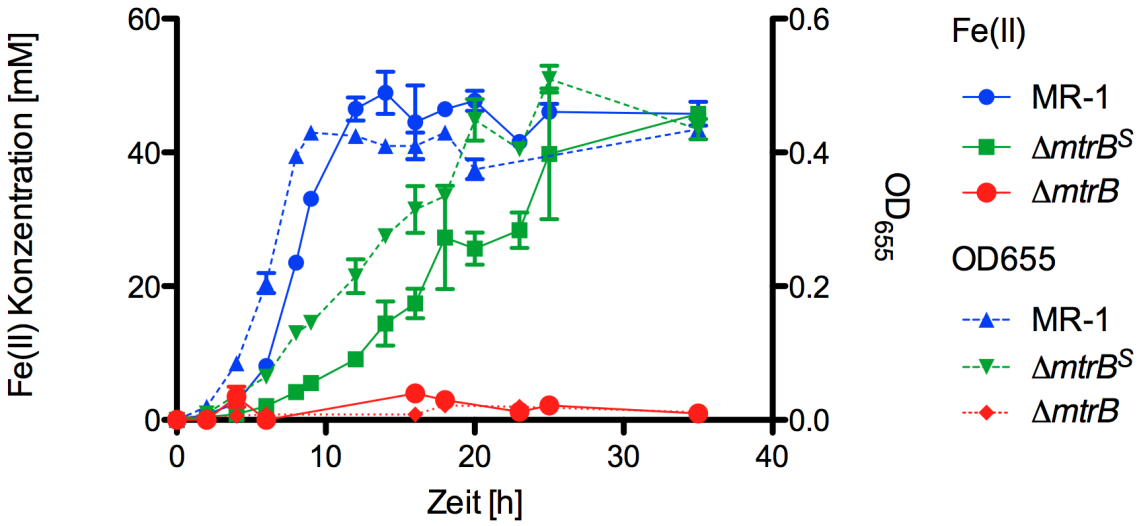


Fig. 13) Correlation between ferric citrate reduction (left y-axis, solid lines) and increase of the optical density (right y-axis, dashed lines) of similar grown *S. oneidensis* MR-1 and an isolated Δ*mtrB* suppressor strain on anaerobic 4 M minimal medium supplemented with 50 mM lactate and 50 mM ferric citrate. The assay was done over 35 hours.

The Δ*mtrB*^{S} strain had a growth rate of ca. 0.02 h^{-1} compared to 0.06 h^{-1} of the wild type strain when grown anaerobically with ferric iron as electron acceptor. Aerobic growth on LB medium and anaerobic growth on 4 M minimal medium with fumarate or DMSO as terminal electron acceptor grew at wildtype levels (data not shown).

3.2 Involvement of SO4359 in ferric iron reduction

To identify possible key players responsible for the suppression of the *mtrB* mutation, Δ*mtrB*^{S} and wild type cells growing independently on ferric citrate were harvested at exponential phase and fractionated. Equal amounts (50 µg) of membrane and periplasmic fractions of both strains were separated using SDS-PAGE. Due to previously described homologs encoded in the *S. oneidensis* MR-1 chromosome, we first focused on proteins having about the same size as MtrA and MtrB. Protein bands running at approximately 75 kDa (roughly the size of MtrB) and 35 kDa (roughly the size of MtrA) were excised, submitted to LC-MS/MS analysis and synchronized to the *S. oneidensis* MR-1 proteome. The comparison of the mass spectrometry data obtained from the wildtype and the Δ*mtrB*^{S} strain revealed upcoming signals in the suppressor sample from the MtrB homolog encoded by SO4359 in the 75 kDa membrane fraction (Tab. A3). The MtrA homolog encoded by SO4360 also could be detected in the 35 kDa bands of both the membrane and the soluble fractions of the suppressor strain. Supplemental Solexa sequencing data affirmed a possible involvement of SO4359 in recovery of the Δ*mtrB* mutant by showing a gap upstream of the gene cluster encoding for SO4362-SO4357 when aligned to the genome sequence of the ancestral strain (Fig. A2). To resolve whether the suppression is mediated due to the activity of the gene product of SO4359, we created a markerless deletion mutant of the MtrB homolog in Δ*mtrB*^{S}. Subsequently, we tested the resulting strain for the ability to respire on ferric citrate and could show that indeed SO4359 is involved in the suppression of the *mtrB* null mutant strain (Fig. 14). To exclude any downstream effects due to the *mtrB* homolog gene deletion, we expressed SO4359 *in trans* in the Δ*mtrB*^{S} ΔSO4359 strain using a pBAD expression system. To enable immunodetection of the ß-barrel protein encoded by SO4359, we introduced a C-terminal STREP-tag as a 3' extension of SO4359 (herein after referred to as $SO4359_{STREP}$). Cell viability only was achieved when expression of $SO4359_{STREP}$ was induced with 0.1-0.2 mM arabinose. Higher concentrations of

arabinose in 4 M minimal medium supplemented with lactate as electron donor and ferric citrate, fumarate or DMSO as electron acceptor had a lethal impact on the cells indicating cell lysis due to instability of the outer membrane (data not shown). However, the ability to respire on ferric citrate of the *in trans* complemented Δ*mtrB*^{S} ΔSO4359 with SO4359$_{STREP}$ supports the observation of a direct involvement of the gene product of SO4359 in the DIR of the suppressor strain (Fig. 14).

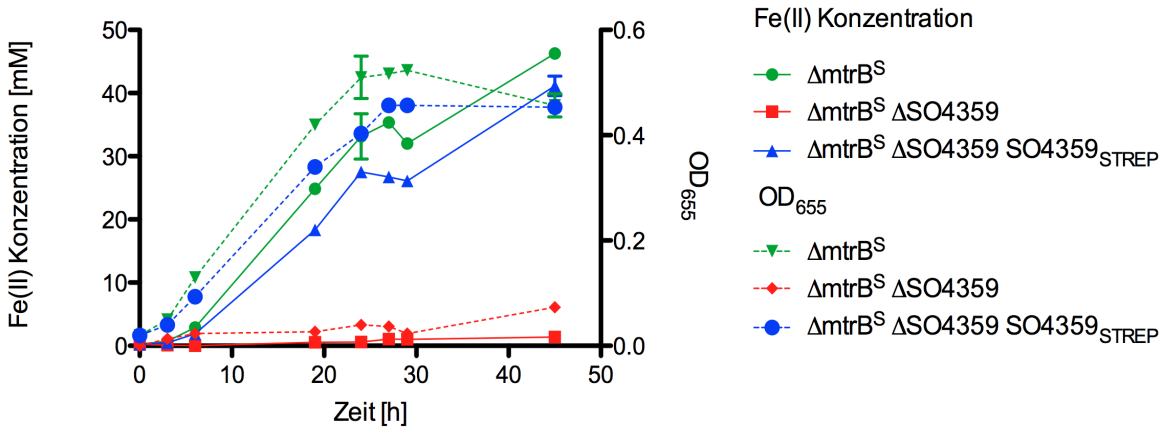


Fig. 14) Correlation between ferric citrate reduction (left y-axis, solid lines) and increase of the optical density (right y-axis, dashed lines) of similar grown Δ*mtrB*^{S}, Δ*mtrB*^{S} ΔSO4359 and Δ*mtrB*^{S} ΔSO4359 pBAD SO4359$_{STREP}$ strains on anaerobic 4 M minimal medium supplemented with 50 mM lactate and 50 mM ferric citrate. The assay was done over 45 hours.

3.3 Investigation of the underlying mechanism for gene upregulation

In *S. oneidensis* MR-1 only the activity associated with the gene cluster SO1774-SO1777 as well as the gene cluster SO1427-SO1429 for extracellular electron transfer during anaerobic respiration has been described so far (Beliaev *et al.*, 2001, Gralnick *et al.*, 2006). We reasoned that an upregulation of SO4359 in the suppressor stain might be due to a mutation event. Supporting evidence for this hypothesis was given by Solexa genome sequencing data. Sequence assembly visualization to the Δ*mtrB* reference genome using Tablet software (Milne *et al.*,

2010), revealed a gap upstream of the gene cluster SO4362-SO4357. Recently, a *cymA* suppressor mutant strain was isolated and the suppression was found to be due to transcriptional activation of SirC and SirD by an insertion of an IS-element forming a hybrid constitutive promoter (Cordova *et al.*, 2011). Sequencing analysis of the region containing the identified gap upstream of SO4362 revealed an insertion sequence (ISSod1) introduced in the SO4362 gene. Upregulation of downstream genes through formation of a hybrid promoter by a ISSod1 mobile genetic element has been previously described by Cordova *et al.*. Analysis of the hybrid region using bioinformatics tools (BPROM, Softberry, Mt. Kisco, NY) revealed a putative σ^{70} dependent promoter, whereby the predicted -35 region is derived from the IS-element and the predicted -10 region is part of the SO4362 gene. (Fig. 15)

Fig. 15) Incorporation of the ISSod1 insertion sequence into the gene encoding for SO4362. Yellow arrows: coding sequence; blue arrows: Newly fromed sigma70 depending promoter with the respecive -35 and -10 promoter regions; N: indicate the intrinsic part of the ISSod1 sequence.

To test whether the newly formed constitutive promoter was affecting the expression of the *mtrAB* homologous gene cluster we fused the region including the putative promoter to a *mCherry* reporter gene. The entire region upstream of SO4360 including the native promoter upstream of SO4362 (position 411,554-413,284 of the *S. oneidensis* MR-1) from the suppressor strain (containing the ISSod1) or the wildtype was cloned upstream to the reporter gene (Fig. 16).

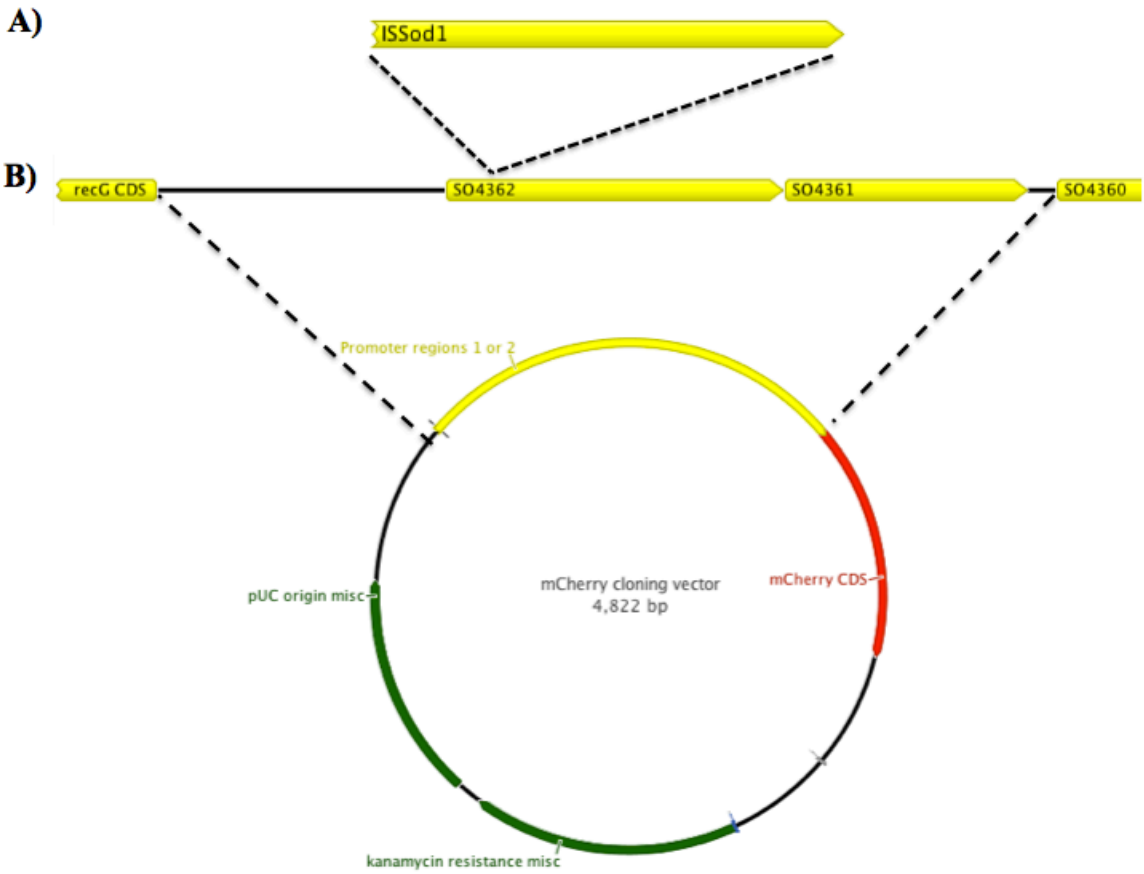


Fig. 16) Fragment upstream of SO4360 was cloned upstream of the reporter gene *mCherry* either with the ISSod1 sequence representing the region and putative promoter in the suppressor strain (A, referred to as ISSod1::mCherry), or without the ISSod1 sequence representing the region and putative promoter in the wildtype (B, referred to as wt::mCherry).

Both designed vectors were transformed into *E. coli* and expression of the *mCherry* reporter was monitored using fluorescent microscopy. Samples were detected using a Cy3 filter and an HCX PL Fluotar 100.0x1.30 Oil objective with an exposure time of 957 ms. As Figure 17 shows, only the construct including the genomic region from the suppressor strain, and the ISSod1 sequence, leads to an expression of *mCherry*. To exclude that the missing signal in the negative control is due to a lethal side effect, viability of the cells was monitored using a N Plan 100.0x1.25 oil objective for phase contrast microscopy (Fig. 17).

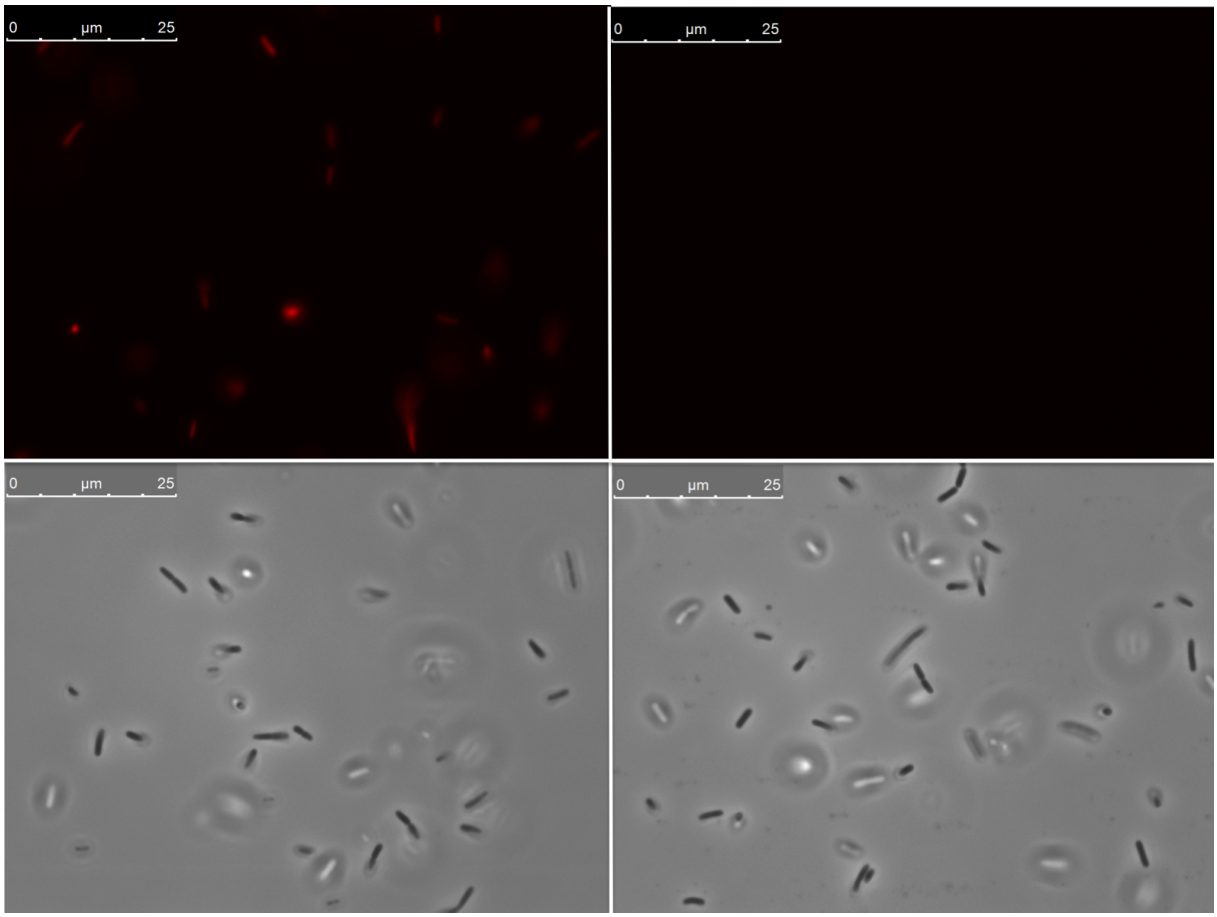


Fig. 17) Probe for functionality of the newly formed constitutive hybrid promoter of the suppressor strain. Genomic region upstream of SO4359/60 of the suppressor or wildtype strain fused to the *mCherry* reporter gene. Cells were grown aerobically on LB medium and moitored with an HCX PL Fluotar 100.0x1.30 Oil objective with an exposure time of 957 ms (upper panel). Viability of the cells was illustrated by phase contrast using a N Plan 100.0x1.25 oil objective (lower panel). Only the construct including the genomic region from the suppressor strain, and the ISSod1 sequence, leads to an expression of *mCherry* (upper left).

Taken together, it seems likely that the ISSod1 sequence forms a constitutive hybrid promoter together with SO4362, as shown recently, and consequently results in the upregulation of the gene cluster SO4362-SO4357. This supports the hypothesis of an involvement of SO4359 in the DIR of the suppressor stain.

3.4 MtrA or gene product of SO4360 as periplasmic electron carrier?

As previously observed in *S. oneidensis* MR-1, MtrA seems to have beyond the function as periplasmic electron carrier a fundamental function in the establishment of an outer membrane spanning complex (Schicklberger *et al.*, 2011, Hartshorne *et al.*, 2009). Due to the involvement of MtrA in complex formation, several possibilities for the reacquired ability to transfer electrons to ferric citrate arise. (1) MtrA interacts with the protein encoded by SO4359. Therefore, MtrA is involved in both the stability of the ß-barrel protein and function as the periplasmic electron carrier. Thus, the gene product of SO4359 alone would then functional replace MtrB. (2) MtrA serves as electron carrier such as in the wildtype, but the protein encoded by SO4360 is crucial for complex formation. (3) Alternatively, the gene product of SO4360 replaces MtrA as the periplasmic electron carrier, but MtrA is necessary for complex formation. (4) The protein encoded by SO4360 serves as electron carrier and assists in establishment of the outer membrane complex.

To investigate whether MtrA or the MtrA homolog encoded by SO4360 is essential for the suppression of MtrB, we constructed mutants in Δ*mtrB*^{S} lacking either *mtrA* or SO4360 and SO4359. SO4359$_{STREP}$ was expressed in the double mutant strain *in trans* by the addition of 0.15 mM arabinose to exclude a downstream effect of SO4360 on SO4359. Subsequently we tested the strains on their ability to respire ferric citrate and monitored the Fe(II) formation as well as the optical density over the time. The resulting growth curve revealed that a deletion of the *mtrA* allele in the Δ*mtrB*^{S} chromosome did not affect the strain in its ability to reduce ferric citrate at all, whereas a deletion of SO4360 in Δ*mtrB*^{S} severely impaired the ability to grow on ferric citrate (Fig.18).

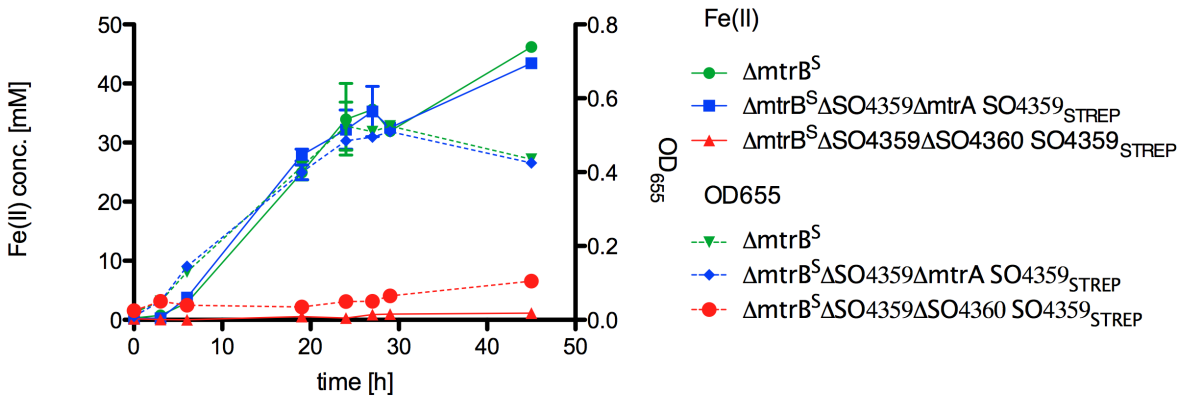


Fig. 18) Correlation between ferric citrate reduction (left y-axis, solid lines) and increase of the optical density (right y-axis, dashed lines) of similar grown Δ*mtrB*^{S}, Δ*mtrB*^{S} ΔSO4359 ΔmtrA pBADSO4359$_{STREP}$ and Δ*mtrB*^{S} ΔSO4359 ΔSO4360 pBADSO4359$_{STREP}$ strains on anaerobic 4 M minimal medium supplemented with 50 mM lactate and 50 mM ferric citrate.

In addition, sole expression of SO4359$_{STREP}$ *in trans* in a *mtrB* mutant strain also was not able to complement for iron respiration (data not shown). As a result, we reasoned that MtrA is not involved in either process of the iron respiration pathway in a Δ*mtrB*^{S} strain, neither as periplasmic electron carrier nor as an essential part for complex formation.

To answer the question concerning an involvement of the gene product of SO4360 in the stability of the SO4359 encoded protein, we expressed SO4359$_{STREP}$ *in trans* in a Δ*mtrB*^{S} ΔSO4359 ΔSO4360 mutant strain. As positive control for immunodetection of the protein encoded by SO4359$_{STREP}$ was expressed *in trans* in a Δ*mtrB*^{S} ΔSO4359 Δ*mtrA* mutant strain. Cells were anaerobically grown on 4 M minimal medium supplemented with 50 mM lactate and 50 mM fumarate. Subsequently, the cells were harvested at exponential phase and fractionated. Membrane factions were separated in outer membrane and cytoplasmic membrane using *N*-lauroylsarcosine (Leisman *et al.*, 1995). Equal amounts of protein (50 µg) were separated using SDS-PAGE. Subsequent immunodetection revealed a sharp band in the Δ*mtrB*^{S} ΔSO4359 Δ*mtrA* mutant stain, whereas the signal for SO4359$_{STREP}$ was scattered over the entire blot in the Δ*mtrB*^{S} ΔSO4359 ΔSO4360 sample (Fig. 19 A). However, a complete loss of the signal as shown recently for MtrB in the absence of MtrA was not observed (Schicklberger *et al.*, 2011). For additional heterologous expression of SO4359$_{STREP}$

and SO4360, we constructed a vector bearing the genes encoding for SO4360 and $SO4359_{STREP}$ (herein after referred to as $SO4360/59_{STREP}$). In contrast to the previously observed MtrA dependent stability of MtrB, expression of $SO4359_{STREP}$ was not or even negatively affected by a coexpression of SO4360 in *E. coli* (Fig. 19B).

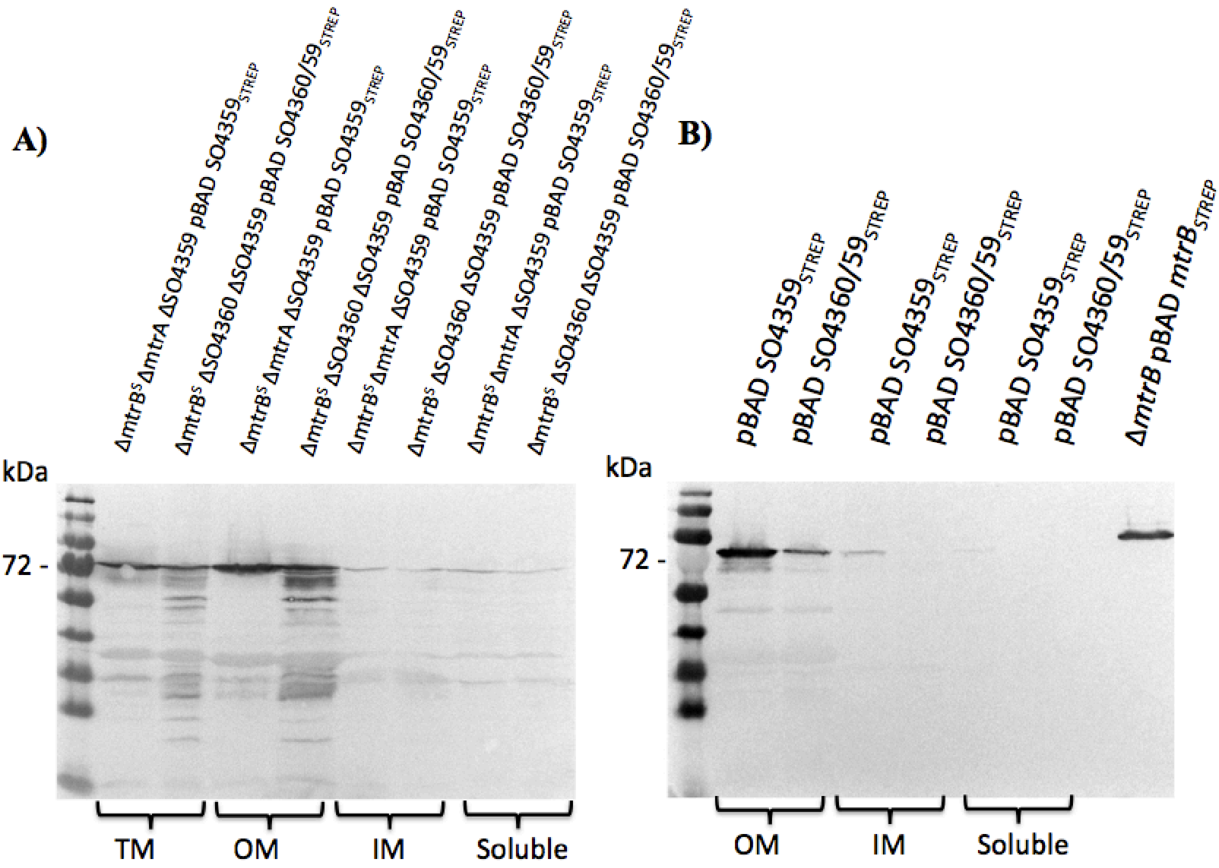


Fig. 19) A) Immunodetection of $SO4359_{STREP}$ in total membrane (TM) outer membrane (OM) inner membrane (IM) and soluble fractions of anaerobically grown cells of either Δ*mtrB* Δ*mtrA* ΔSO4359 $pBADSO4359_{STREP}$ or Δ*mtrB* ΔSO4360 ΔSO4359 $pBADSO4359_{STREP}$. B) OM, IM and soluble fractions of heterologous expression of either $SO4359_{STREP}$ or SO4360-$SO4359_{STREP}$ in *E. coli*. 50 µg of protein samples were loaded on a 10% SDS-PAGE, respectively.

Thus, the results suggest an involvement of SO4360 in the electron transfer, but the periplasmic electron carrier is not involved in the stability of SO4359 in the suppressor stain.

3.5 The module SO4359 SO4360

Having shown that MtrA is not involved in the electron transfer and that a expression of $SO4359_{STREP}$ alone does not complement either a Δ*mtrB*^{S} ΔSO4359 ΔSO4360 strain or a Δ*mtrB* strain the next question was if the SO4359-SO4360 module could complement a Δ*mtrB* strain for DIR. Therefore, we used the vector containing the genes encoding for SO4360 and $SO4359_{STREP}$, and transformed it into a *S. oneidensis* MR-1 Δ*mtrB* strain. Subsequently we inoculated the strain in 50 mM ferric citrate 4 M minimal medium with 50 mM lactate as electron donor. Gene expression was achieved by supplementing the media with of 0.15 mM arabinose. Induction of $SO4360/59_{STREP}$ resulted in the ability to respire on ferric citrate to the same activity than the Δ*mtrB*^{S} strain does as shown in Fig. 20. Without the addition of arabinose, the Δ*mtrB* strain is severely deficient in respiring on iron. We therefore concluded, that the lack of MtrB is compensated in the suppressor strain by SO4359 and SO4360 and it is sufficient and necessary to express $SO4360/59_{STREP}$ to recover the ability of a Δ*mtrB* strain for iron respiration.

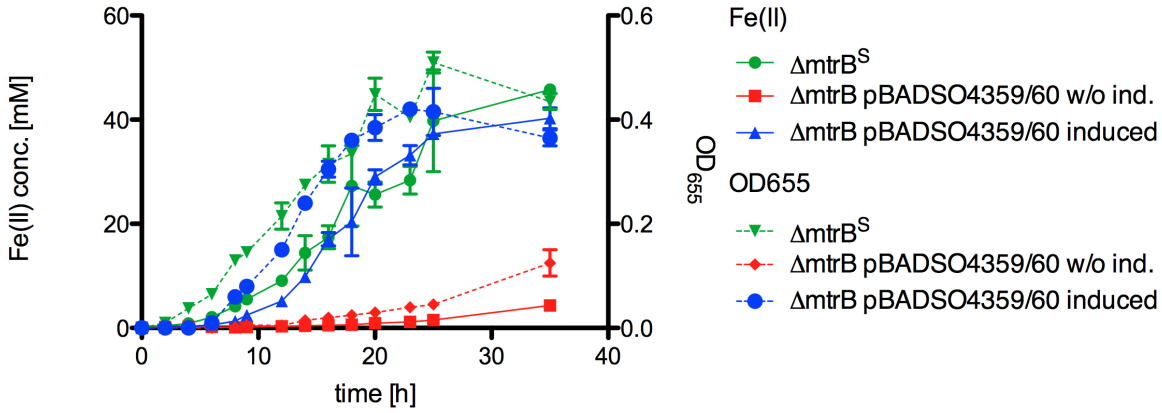


Fig. 20) Correlation between ferric citrate reduction (left y-axis, solid lines) and increase of the optical density (right y-axis, dashed lines) of similar grown Δ*mtrB*^{S}, and induced or uninduced Δ*mtrB* pBAD $SO4360/59_{STREP}$ strains on anaerobic 4 M minimal medium supplemented with 50 mM lactate and 50 mM ferric citrate. The assay was done over 35 hours.

3.6 Deciphering the final reductase in Δ*mtrB*^{S}

In *S. oneidensis* MR-1 electron transfer over the outer membrane under anaerobic iron reducing conditions only occurs when MtrCAB form an integral membrane spanning complex. Having shown that SO4359 and SO4360 are essential for the electron transfer to extracellular electron acceptors, the remaining question we addressed was which final reductase catalyzes the reduction of ferric citrate in the suppressor strain. Our hypotheses considered two possible enzymes, which could be responsible for the electron transfer on iron under anoxic conditions. The first one would be the *c*-type cytochrome MtrC, which today is well established to have the biggest impact in ferric iron reduction (Beliaev *et al.*, 2001, Bretschger *et al.*, 2007, Coursolle & Gralnick, 2012). Specifically, SO4359 could substitute MtrB and due to the high similarity of the ß-barrel proteins, MtrC could also interact with SO4359. Here, SO4359 would serve as a sheath in which SO4360 and MtrC exchange electrons. The second hypothesis centers on the DmsAB homologs SO4358 and SO4357 facilitating electron transfer directly to ferric citrate since they are part of the upregulated operon. An influence of DmsAB in extracellular electron transfer has been shown earlier (Gralnick *et al.*, 2006) and it is reasonable to think, that the homolog enzymes SO4358 and SO4357 in turn could be responsible for ferric citrate reduction in the Δ*mtrB*^{S} strain. In order to test the two hypotheses we created in frame gene deletion strains lacking either MtrC or both SO4357 and SO4358 in a Δ*mtrB*^{S} background and tested their ability to grow on ferric citrate. The strain deficient in the genes encoding for SO4358 and SO4357 showed an increase in the Fe(II) concentration and also in the optical density which indicates the ability to respire ferric iron. However, the suppressor strain bearing a deletion in *mtrC* also did not show any deficiencies in ferric iron respiration. Moreover, it was able to respire iron to about the same level than the ancestral strain Δ*mtrB*^{S} does (Fig. 21).

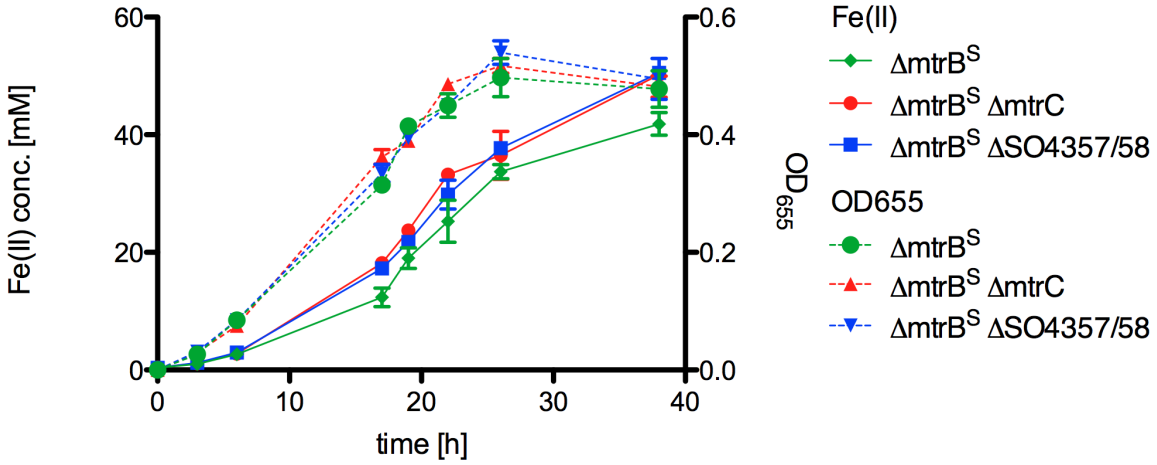


Fig. 21) Correlation between ferric citrate reduction (left y-axis, solid lines) and increase of the optical density (right y-axis, dashed lines) of similar grown Δ*mtrB*^{S}, Δ*mtrB*^{S} Δ*mtrC* and Δ*mtrB*^{S} ΔSO4357/58 strains on anaerobic 4 M minimal medium supplemented with 50 mM lactate and 50 mM ferric citrate. The assay was done over 38 hours.

Previous publications have suggested the establishment of hybrid complexes between components of MtrCAB and MtrDEF, which transfer electrons to extracellular electron acceptors via interaction with the termini of the decaheme *c*-type cytochromes MtrC or MtrF (Bücking *et al.*, 2010, Clarke *et al.*, 2011, Coursolle & Gralnick, 2010, Coursolle & Gralnick, 2012). MtrC and MtrF, are both predicted to be outer membrane *c*-type cytochromes and also show high similarity to each other (Coursolle & Gralnick, 2012, Clarke *et al.*, 2011). Under these circumstances, it might also be possible, that SO4359 and SO4360 are forming a complex together with the *c*-type cytochrome MtrF.

To exclude any interactions of SO4360 and SO4359 with MtrF or any other remaining outer membrane *c*-type cytochrome, we transformed SO4360/59 $_{STREP}$ in a *S. oneidensis* MR-1 strain deficient in all outer membrane *c*-type cytochromes and *mtrA* (ΔOMCA) (Schicklberger *et al.*, 2011). Therefore, we tested a possible ability to respire on ferric citrate by monitoring the increase of Fe(II) and the optical density over the time. Expression of SO4360/59$_{STREP}$ was achieved by adding 0.15 mM arabinose. The growth curve clearly revealed that the strain lacking any outer membrane *c*-type cytochrome is able to respire on ferric citrate, when SO4360/59$_{STREP}$ is expressed. When expression of the periplasmic electron carrier

and the ß-barrel protein is omitted, the ΔOMCA strain lacks the ability to respire on ferric citrate (Fig. 22). This would support a hypothesis in which SO4360 has taken over the function as the final reductase and SO4359 being necessary to access the poorly soluble ferric iron minerals.

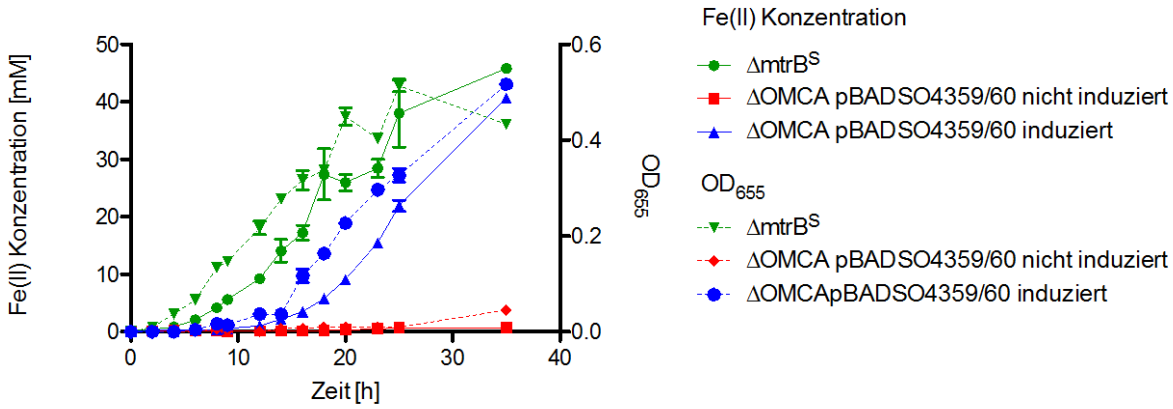


Fig. 22) Correlation between ferric citrate reduction (left y-axis, solid lines) and increase of the optical density (right y-axis, dashed lines) of similar grown Δ*mtrB*^{S}, and induced or uninduced ΔOMCA pBAD SO4360/59$_{STREP}$ strains on anaerobic 4 M minimal medium supplemented with 50 mM lactate and 50 mM ferric citrate. The assay was done over 35 hours.

DISCUSSION

Dissimilatory iron reduction is a respiratory process in which proton gradient dependent energy generation at the cytoplasmic membrane is coupled to the reduction of ferric iron (Myers & Nealson, 1990). Microbial catabolic iron reduction has been studied intensively since its discovery in the 1980s as a respiratory process (Myers & Nealson, 1988, Myers & Nealson, 1990, Nealson & Myers, 1992, Lovley *et al.*, 2004). For dissimilatory iron reducing bacteria, the physiological challenge of this form of respiration is the existence of ferric iron at neutral pH as primarily crystalline iron (hydr)oxides (Cornell & Schwertmann, 2003). As an answer to the physiological challenge, microbes have evolved an extended respiratory chain to the insoluble electron acceptor at the cell surface (Shi *et al.*, 2006).

The aim of this study was to characterize the ß-barrel protein MtrB regarding its involvement in the establishment of an outer membrane complex responsible for DIR. Integral membrane proteins play a fundamental role in electron exchange over the outer membrane in *Shewanella* species as well as in other Gram-negative bacteria. MtrB could be shown as a necessary part of an integral membrane complex consisting of MtrABC and a lack of MtrB impaired *S. oneidensis* MR-1 in its ability to respire on ferric iron under anoxic conditions (Hartshorne *et al.*, 2009, Beliaev & Saffarini, 1998, Myers & Myers, 2002, Bretschger *et al.*, 2007).

1. Involvement of the *S. oneidensis* MR-1 decaheme cytochrome MtrA in the periplasmic stability of the beta-barrel protein MtrB

1.1 Dual function of MtrA under ferric iron reducing conditions

Next to the importance of MtrB in the establishment of an outer membrane spanning complex, the periplasmic decaheme *c*-type cytochrome MtrA was furthermore known to be necessary. It was believed that this necessity was caused by the function of MtrA as electron transferring protein. We could show a novel role of MtrA. It is crucial for the stability of MtrB. Consequently MtrA is – beyond its role as electron transfer

component – necessary for the assembly of the MtrCAB complex (Hartshorne *et al.*, 2009, Schicklberger *et al.*, 2011). In past publications MtrA was shown to be the only essential soluble periplasmic *c*-type cytochrome of *S. oneidensis* MR-1 under dissimilatory metal reducing conditions (Beliaev *et al.*, 2001, Bretschger *et al.*, 2007). The extremely high amount of cofactor bound to the 36.06 kDa protein via 10 heme attachment sites and its localization in the periplasm as well as at the outer membrane clearly points towards a role in electron transfer from the periplasm towards outer membrane cytochromes (Pitts *et al.*, 2003, Schuetz *et al.*, 2009). At the same time our experiments show that periplasmic presence of MtrA seems to be necessary for resistance against DegP protease degradation of MtrB. Thus, MtrA seems to have a dual function under ferric iron reducing conditions, which could also explain the essential role of MtrA in iron reduction (Bretschger *et al.*, 2007).

DegP catalyzes the hydrolysis of unfolded or at least partially unfolded proteins in the periplasm. Typical cleavage sites seem to be usually buried in the core of the protein and consequently become only acceptable in the unfolded state (Kolmar *et al.*, 1996). It was shown that ß-barrel proteins are usually guided through the periplasm via the interaction with chaperons like SurA or Skp (Sklar *et al.*, 2007). Binding of these chaperones seems to prevent protease hydrolysis. Several hypotheses can be considered based on the observed requirement of MtrA. One would include a direct role for MtrA, which accelerates MtrB-chaperone interaction and another one could be a direct binding of MtrA to unfolded MtrB. In this case MtrA itself would act as a protective chaperone.

Previous studies have indicated that MtrA forms a stable complex with MtrB at the outer membrane, which favors the second hypothesis (Hartshorne *et al.*, 2009, Ross *et al.*, 2007). Formation of this complex could likely be initiated in the periplasm when MtrB is in an unfolded state. This periplasmic complex of MtrA with precursor MtrB could mask hydrophobic amino acid residues that when exposed to the hydrophilic periplasmic environment would likely lead to misfolding and hence a target for DegP hydrolysis. While further work is required to delineate the exact mechanism of MtrA-dependent MtrB stability, a direct role of MtrA in MtrB stability is supported by the findings in the non-dissimilatory iron reducing bacteria, *E. coli*.

1.2 Hybrid complex formation and complementation assays by MtrAB homologs

Since the loss of MtrB in a *mtrA* null mutant strain due to MtrA-dependent stability of MtrB has been discovered, it is reasonable to think that the only reason as to why MtrA homologs are incapable to complement for MtrB production under ferric iron reducing conditions is that they are not expressed. However, a recent study by Coursolle *et al.* demonstrated partial complementation by hybrid outer membrane complexes (Coursolle & Gralnick, 2012). The hybrid complexes composed of different combinations of homologous components of the two *mtr*-clusters are able to complement a strain depleted in all identified outer membrane cytochromes (ΔOMCs) and periplasmatic electron carrier (PEC), which show homology to MtrA. In addition, a *mtrB* null mutant could be functionally replaced only by *mtrE* and only when expressed *in trans* under constitutive promoter control (Coursolle & Gralnick, 2012). These findings are consistent with the higher similarity of MtrDEF to MtrABC, respectively (Coursolle & Gralnick, 2012). In contrast, additional complementation experiments of a strain deficient in all periplasmic MtrA homologs by overexpression of *mtrD* and *dmsE* revealed only a partial compensation, whereas a substitution with SO4360 does not show any reduction activity at all (Coursolle & Gralnick, 2010). Moreover, overexpression of SO4360 was unable to compensate for the loss of DmsE in growth with DMSO (Coursolle & Gralnick, 2010). It was shown that MtrA paralogs able to functionally replace MtrA in ferric citrate reduction are in the hierarchical order MtrD>DmsE>>SO4360 gene product (Coursolle & Gralnick, 2010). The inability for partial complementation by the expression of SO4360 in a mutant deficient in all periplasmic MtrA homologs was suggested to be due to the evolutionary distance of the protein encoded by SO4360 to other MtrA paralogs (Coursolle & Gralnick, 2010). Moreover, in a strain that expresses *mtrB* and contains no other encoded ß-barrel paralogs, complementation was observed only by *in trans* expression of *mtrD* and not by *dmsE* and SO4360 (Coursolle & Gralnick, 2012). Therefore, we hypothesize that MtrD must be able to substitute for MtrA in terms of stability of MtrB. Still, until now, the production of MtrB in the presence of MtrD has never been shown.

2. Biochemical and genetic analysis of protein-protein interactions which might be responsible for MtrB stability

Since we have shown that MtrA is essential for the stability of MtrB it could be that MtrA and MtrB interact already prior to the formation of a holo-protein complex including MtrC. To investigate a possible chaperone like function of MtrA on MtrB we conducted *in vivo* crosslink experiments on cells expressing tagged versions of *mtrA* and *mtrB*, respectively, to probe for possible periplasmic interactions of both. Isolation of the periplasm followed by affinity purification of the $MtrA_{STREP/HIS}$ and subsequent LC-MS/MS analysis did not indicate a possible transient complex of MtrA and MtrB.

In order to find targets involved in MtrB stability, we used two complementary strategies of DSP crosslinking, regarding MtrA and MtrB, which revealed similar results. For proteins interacting with MtrA, a DSP crosslink-affinity purification approach with $MtrA_{STREP/HIS}$ was used. For MtrB, the shift, which indicated possible complex formation through DSP crosslinking also appeared in membrane fractions under non-reducing conditions of cultures in which the crosslinking reagent was omitted prior fractionation. Here a protein-protein formation could be due to a disulfide bond formation. Also, DiChristina could show, that only one of the two cysteine residues is crucial for proper function (“A Single Cysteine Residue of a Thioredoxin-Like Motif in the Outer Membrane Protein MtrB Is Required for Metal Respiration by *S. oneidensis* MR-1” Abstract DiChristina). Since an intramolecular disulfide bond formation seems not to be fundamental in the stability of MtrB, these findings indeed would suggest a protein-protein interaction due to a native disulfide bond formation resulting in the stability of the protein.

Further validating our results based on the complementary biochemical strategies was that similar proteins were identified including two key proteins of further interest – the gene product of SO2907, a ~96 kDa TonB receptor protein and the gene product of SO3896, a ~40 kDa outer membrane protein. Both proteins belong to the ß-barrel superfamily. A previous study could show an upregulation of the proteins encoded by the genes SO2907, SO2469, SO3896 and two more TonB-depentent receptor proteins encoded by SO2427 and SO0815 under ferric iron conditions

(Ruebush *et al.*, 2006). Additionally, a recent study from Qian *et al.* has indicated an involvement of SO2907 in the respiration of ferric citrate and ferric-NTA in *S. oneidensis* MR-1 (Qian *et al.*, 2011). They discovered an iron binding site in truncated versions of the protein encoded by SO2907, which is capable of binding soluble Fe(III) forms. In addition, a SO2907 mutant was diminished in the respiration of ferric citrate and ferric-NTA, suggesting SO2907 encodes for a low affinity iron transporter inside the periplasm for DIR (Qian *et al.*, 2011). According to our crosslink data both, MtrA and MtrB, possibly can interact with the gene products of SO2907 and SO3896, which seem to be upregulated under ferric iron reducing conditions. However, single or double deletion mutant strains of SO2907 and/or SO3896 were able to produce MtrB. A reason for this could also be the high similarity of the TonB receptor proteins in *S. oneidensis* MR-1 and thus redundancy during iron respiration. More investigation in this field would be necessary to understand the exact role of those proteins in ferric iron respiration and how they could be involved in the stability of MtrB, if at all. In addition, it appears that the elucidation of the interaction of MtrA and MtrB may require different methods such as fluorescence resonance energy transfer (FRET) microscopy. An influence of the C42 residue in protein-protein interaction could be visualized by site directed photo-crosslinking.

2.1 A hypothesis for the underlying mechanism of MtrA assisted MtrB stability

The mechanism by which ß-barrel proteins are incorporated is not well understood and is at present in focus of intense research. A recent study by the group of Prof. Thomas J. Silhavy revealed the dependency of the ß-barrel protein LptD, on LptE, a lipoprotein anchored to the outer membrane and facing the periplasm (Chimalakonda *et al.*, 2011). LptE and LptD are responsible for LPS incorporation in the outer membrane and form a tight OM complex like MtrA and MtrB. LptD and LptE are targeted to the outer membrane by two different pathways (Bam and Lol respectively). In their work, they could isolate suppressor mutations of a mutated *lptE* (which impairs LptD biogenesis) in either *lptD* or in one of the POTRA (**po**lypeptide **tr**ansport **a**ssociated) domains of *bamA*. Due to the identification of compensatory mutations both in *lptD* and *bamA*, they concluded that LptE interacts with the precursor LptD while it is being held in the Bam complex. LptE was suggested to

participate in LptD folding by either acting as a scaffold or stabilizing the newly formed protein (Chimalakonda *et al.*, 2011).

Based on our findings that (1) MtrB relies on MtrA for proper incorporation into the outer membrane, (2) that a deletion of *degP* in a *mtrA* null mutant background uncouples this dependency and (3) the LptD/E/Bam complex findings from other groups, I postulate the following mechanism for MtrA(D) mediated incorporation of MtrB. When MtrA(D) is present, it interacts with the Bam-complex, which leads to an fast and efficient incorporation of MtrB into the outer membrane (Fig. 23). Here, even when expressed, degradation by DegP would be inhibited by the fast kinetics of incorporation.

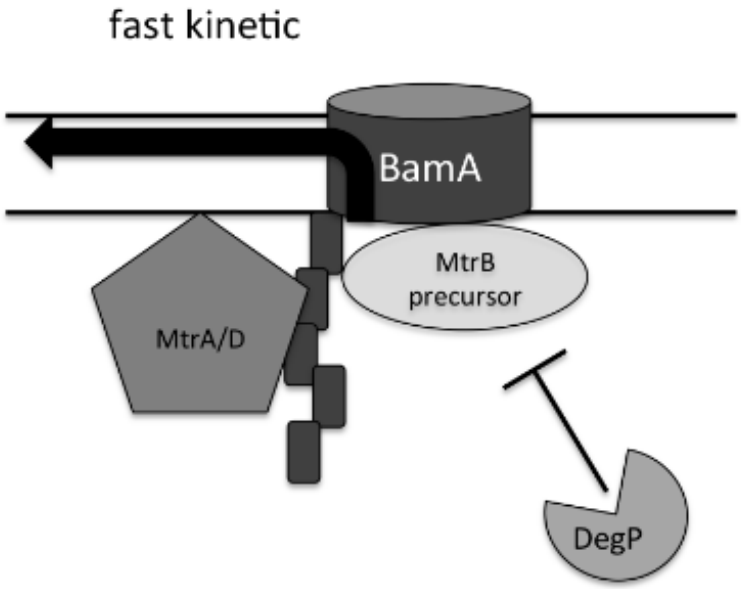


Fig. 23) Fast kinetic incorporation of MtrB due to MtrA.

Deletion of MtrA would result in slow incorporation of MtrB, resulting in an aggregation of the outer membrane protein in the periplasm and thus cause a sigma E dependent upregulation of the periplasmic protease *degP* (Fig. 24). This would also explain the lack of the MtrB signal observed in our experiments in a degP complemented Δ*mtrA* $mtrB_{STREP}$ strain.

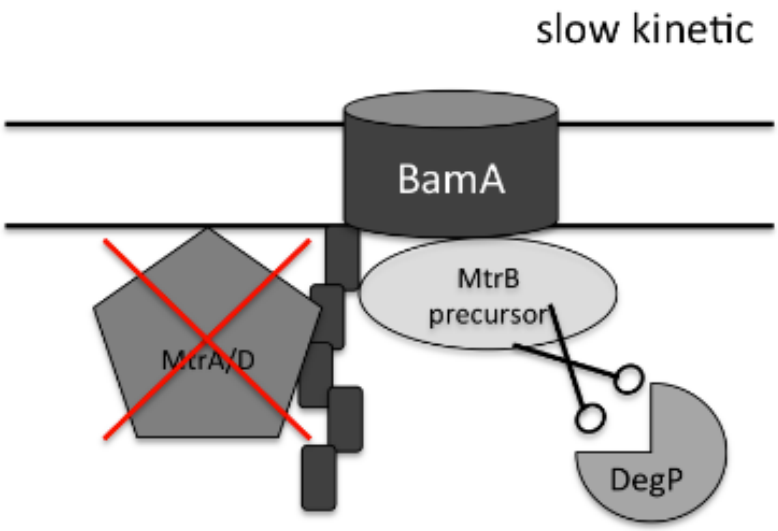


Fig. 24) Degradation of MtrB by DegP due to slow kinetic of the incorporation.

The subsequent deletion of *degP* in a *mtrA* null mutant strain would then allow for proper incorporation into the outer membrane. The incorporation would also be kinetically slower. A lethal effect due to aggregation of the precursor MtrB in the periplasm would be omitted by the sigma E depended downregulation of the ß-barrel protein expression (Fig. 25). Supporting the idea of varying kinetics for beta barrel protein incorporation is a study on the *E. coli* porin PhoE which revealed that a SNP in PhoE resulted in cell death and protein aggregation during high-level expression, whereas low-level expression was tolerated and allowed for assembly into the OM. Here the mutagenized PhoE required slower assembly into the outer membrane (Bos *et al.*, 2007).

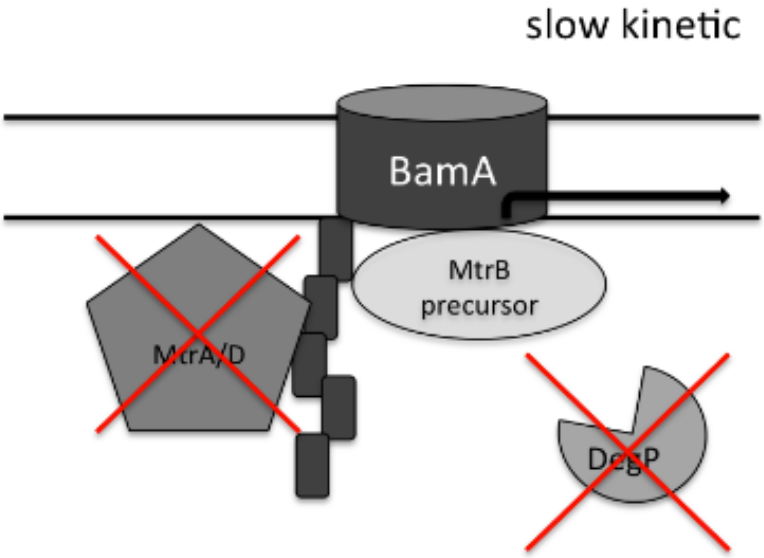


Fig. 25) Slow kinetic incorporation of MtrB in absence of MtrA and DegP.

This hypothetical mechanism also would explain the fainter signal of MtrB in immunoblot analysis of membrane fractions from anaerobic grown cultures of a ΔOMCA Δ*degP* $mtrB_{STREP}$ mutant strain compared to the wildtype.

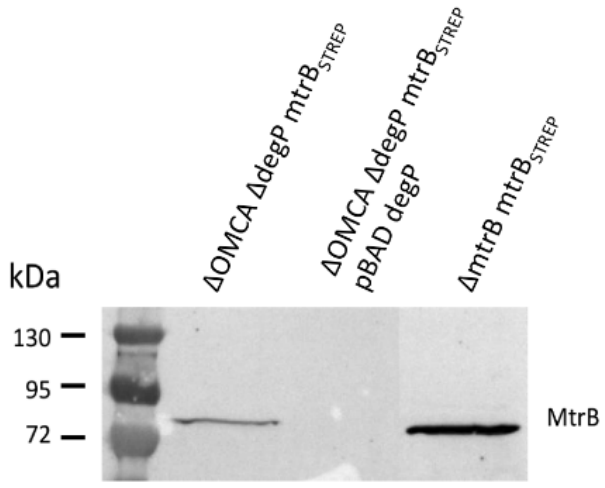


Fig. 26) Expression of $mtrB_{STREP}$ in a ΔOMCA Δ*degP* strain compared to complemented Δ*mtrB* strain with pBAD$mtrB_{STREP}$.

The fact that the production of the SO4359 encoded protein does not rely on the gene product of SO4360 in *S. oneidensis* MR-1 and also not when heterologously expressed in *E. coli* could be due to later evolution of a post-translational control mechanism in the highly expressed *mtr*-clusters. Here, *S. oneidensis* MR-1 would ensure the electron transfer from CymA through the periplasm by MtrA prior to generate an integral membrane protein which eventually is needed for a membrane spanning complex. The fact that protein encoded by SO4359 is not subjected to this kind of mechanism could be due to the rare expression of this gene cluster. This would mean on the other hand that the gene product of *dmsF*, which encodes for a ß-barrel protein and is expressed under DMSO respiration, should also rely on DmsE, the periplasmic decaheme c-type cytochrome, since this polycistronic operon was shown to be active under DMSO reducing conditions.

3. Genomic plasticity responsible for dissimilatory iron reduction in *S. oneidensis* MR-1

An additional goal of this work was to identify alternate or secondary pathways for iron reduction, which are encoded by the *S. oneidensis* MR-1 chromosome. Four individual pathways predicted to facilitate electron flow for respiration of extracellular electron acceptors are encoded by the genome of *S. oneidensis* MR-1 (Gralnick *et al.*, 2006). So far, only two of the pathways (OmcA/MtrCAB and DmsEFABGH) have been described to be active.

3.1 Functional redundancy of MtrAB homolog modules

Previous work has demonstrated that ferric citrate reduction rates close to wildtype levels are achieved only when MtrA and MtrB are co-expressed. Coursoulle *et al.*, showed, via *in trans* hybrid complex expression, a hierarchical order of MtrABC>MtrABF>MtrAEC>MtrAEF for ferric citrate reduction (Coursolle & Gralnick, 2012). While ferric reductase activity is greatly affected by interchanging components within the MtrAB module, the choice of the final reductase in form of MtrC or MtrF seems to have only minor effects, since expression of either one in combination with MtrAB resulted in reduction rates close to the wildtype (Coursolle & Gralnick, 2012, Bücking *et al.*, 2010). This highlights the strict requirement of the native MtrAB

module for efficient ferric iron reduction and not MtrC despite the fact that the *mtrCAB* cluster is an operon.

It could be that single component exchanges of the MtrAB module within the MtrCAB complex might not be functional with homologs from the SO4357-SO4362 or *dms*-clusters due to the evolutionary distance of these two clusters relative to the two *mtr*-clusters. Based on the observed strict requirement of the MtrAB module for maximal ferric iron reduction efficiency, we hypothesize that complementation by the other non *mtr*-clusters can take place only when the MtrAB homolog module from these clusters would be expressed. Accordingly, we have revealed that expression of the respective MtrAB homolog module from the gene cluster SO4357-SO4362 is necessary for a functional replacement of MtrAB(C/F). This also might explain our result whereby the protein encoded by SO4359 interacts solely with the gene product of SO4360, and MtrA does not show any interaction with SO4359 encoded protein – neither in terms of stability and complex formation nor in electron transfer reactions. In line with this hypothesis is also the fact, that expression of SO4359 alone is unable to complement neither a *mtrB* null mutant strain nor a Δ*mtrB*^{S}ΔSO4360 strain for the ability to respire on ferric iron. Specifically, we determined that an exchange of soluble iron species or electron shuttle components over the outer membrane due to pores formed by the SO4359 encoded protein seems to be unlikely since an expression of SO4359 alone, although incorporated into the outer membrane, was insufficient to complement for ferric iron reduction. Consequently, an increase of ferrous iron due to previously described periplasmic or cytoplasmic membrane bound iron reductases (Gescher *et al.*, 2008, Schuetz *et al.*, 2009, Pitts *et al.*, 2003) could not be observed when SO4359 is expressed alone. Further supporting our hypothesis is the observed compensatory evolution within the MtrAB module in the recent data by Bücking *et al.* (submitted), which showed the necessity of a single nucleotide polymorphism (SNP) in *mtrA* **and** *mtrB*, respectively, to suppress a ΔOMC strain (Bücking *et al.* submitted). The suppression did not occur by an expression of a combination of wildtype and mutant alleles of MtrA and MtrB or *vice versa*. In our study, Solexa sequencing data also revealed, that genes encoding for functional components involved in the suppression of a Δ*mtrB* mutant as well as genes encoding for components involved in ferric iron reduction in the wildtype strain, were not affected by SNPs or other mutations. Therefore, these results indicate that,

contrary to previous work, the MtrAB homolog module encoded within the SO4357-SO4362 cluster does represent an alternative route for electron transfer over the outer membrane.

3.2 Expression of SO4360/59 is necessary and sufficient for ferric iron reduction

As our results suggest, the swapping of the MtrAB module is sufficient and necessary for the suppression of a deletion in the gene encoding for the ß-barrel protein MtrB. Since expression of $SO4360/59_{STREP}$ *in trans* in either the Δ*mtrB* null mutant or the ΔOMCA strain increases the ferrous iron concentration, a direct interaction of the MtrA homolog with ferric iron could be possible. Recently, small-angle X-ray scattering and analytical ultracentrifugation of the decaheme *c*-type cytochrome MtrA revealed a 104 Å "extended 'wire' like shape" (Firer-Sherwood *et al.*, 2011). Given the dimensions of MtrA and the estimated pore size of MtrB on the basis of FepA, the authors suggest that MtrA at least insert partially into the ß-barrel protein, if not span the entire outer membrane. Assuming that the MtrA homolog encoded by SO4360 possesses the same dimensions as MtrA and the pore size of the SO4359 encoded protein is equal to or larger than pore size of MtrB, it could be possible that the protein encoded by SO4360 protrudes from the gene product of SO4359 and can directly interact with ferric citrate. Supporting this hypothesis are data by Pitts *et al.*, which revealed a ferric iron reductase activity for periplasmic electron carrier MtrA in *E. coli* (Pitts *et al.*, 2003). It has been shown before that CymA also has ferric iron reductase activity in *E. coli* spheroblasts and that heterologous expression of *cymA* is necessary and sufficient to convert *E. coli* into a dissimilatory iron respiring organism (Gescher *et al.*, 2008). Co-expression of *cymA* and *mtrA* heterologously in *E. coli* increases iron reduction activity, when soluble ferric-NTA is used as electron acceptor, indicating a direct electron transfer from CymA to MtrA and an enlargement of the iron reducing surface due to ferric iron reductase MtrA (Schuetz *et al.*, 2009).
We presume that the additional reductase in form of the periplasmic decaheme *c*-type cytochrome evolved to interact with CymA. In our case it might be that the protein module encoded by SO4360 and SO4359 reflects the third stage of dissimilatory iron reduction by transferring the electrons from the periplasm to the extracellular side of the outer cell membrane.

3.3 Phylogenetic distribution of MtrAB modules and implications for electron transfer from/to the outer membrane

Today, 18 *Shewanella* strains have been sequenced. With the exception of *S. denitrificans*, the chromosome of *Shewanella* spp. contain up to 9 copies of the genes encoding for a MtrAB homolog module. *S. denitrificans* lacks the ability to use ferric iron as terminal electron acceptor, which might be explained by the missing MtrAB homologs. *Gallionella ferruginea* and *Sideroxydans lithotrophicus* also contain a module similar to MtrAB. Both microorganisms are well known lithotrophic ferrous iron oxidizers. The cluster of the iron oxidizer *R. palustris* TIE1 in which the *mtrAB* module is embedded contains a gene *pioC* encoding for a periplasmic high potential iron sulfur protein with a twin arginine translocation signal sequence. A deletion of *pioC* alone results only in partial loss of Fe(II) oxidation (Jiao & Newman, 2007). Therefore, it could be possible that the MtrAB homologs are sufficient for ferrous iron oxidation as we have suggested it is the case for the ferric iron reduction in the strains complemented with SO4360/59$_{STREP}$.

4. Research outlook

The possibility of a different mechanism in ß-barrel assembly of MtrB and gene product of SO4359 opens the door for further research. A construction of a chimeric *mtrB*-SO4359 encoded protein for example could reveal possible MtrA interaction sites of MtrB. Additionally, a chimeric ß-barrel protein and thus a hybrid complex together with the gene product of SO4360 and MtrC maybe would allow for a more efficient iron reduction activity due to an increase of the surface of an already efficient ferric iron reductase (encoded by SO4360) by MtrC. Another way to elucidate which part of MtrA is required for MtrB stability/assembly would be the generation of different MtrA truncated versions from heme 7 through 10, since a protein encoding for a heme 6 truncated version, although stable, does not cause MtrB stability (Probst, 2010). Recent studies indicated that truncated versions of MtrA form stable proteins, which contained covalently bound hemes but were not able to stabilize MtrB (Clarke *et al.*, 2008b). Thus the crucial part of MtrA, which is responsible for stabilization/assembly of MtrB most likely is localized in the C-terminal part of the periplasmic oxidoreductase. To prove the hypothesis of the interaction of MtrA with

BamA, the α-helical anchor from CymA could be used to tether the MtrA protein to the cytoplasmic membrane, which then should result in the loss of the MtrB signal and in a deficient ferric iron reduction activity. Furthermore, importance of a disulfide bond formation for the stability or incorporation of MtrB in the outer membrane could be investigated by a deletion of disulfide bond forming protein *dsbA*. Deletion of *dsbA* at least does not show a lethal phenotype in *E. coli,* which would allow for DsbA to be a potential target for further research regarding the incorporation of MtrB into the outer membrane (Ruiz *et al.*, 2010).

5. Broader impact of studies on DMRB similar to *S. oneidensis* MR-1

5.1 Environmental implications

This research focused on the mechanism of how an outer membrane spanning complex in *S. oneidensis* MR-1 is established. The importance of an elucidation of the underlying mechanism for an electron transfer over the outer membrane of *S. oneidensis* MR-1 or other DMRB becomes clear by considering their environmental implications. In many environments, the microbial iron cycle is linked to the cycling of carbon and the degradation and transformation of organic and inorganic pollutants. Some ferric iron reducing strains have the ability to oxidize aromatic organic pollutants such as benzene, toluene, phenol, *p*-cresol and *o*-xylene. (Lovley *et al.*, 1989, Lovley & Lonergan, 1990, Lovley & Anderson, 2000, Jahn *et al.*, 2005) If at those sites ferric iron oxides and other nutrients (e.g. nitrogen, phosphor, sulfur) are present, DMRB can significantly contribute to the degradation of those toxic compounds. DMRB were also shown to convert toxic metal ions like chromium and uranium from the more soluble form Cr(VI) and U(VI) into the less soluble forms Cr(III) and U(IV) (Lovley *et al.*, 2004, Lovley *et al.*, 1993). Those immobilized toxic metal ions remain in the subsurface and do not lead to groundwater contamination.
Additional to the coupling of the oxidation of pollutant to the reduction of iron, ferric iron minerals can serve as adsorbents. Many ferric iron mineral surfaces are

positively charged at neutral pH due to their high points of net zero charge[1]. Those iron oxides are therefore good adsorbents for negatively charged compounds like bicarbonate (HCO_3^-) and oxyanions such as arsenate (AsO_4^{3-}), arsenite (AsO_3^{3-}) or chromate (CrO_4^{2-}). A transformation of the ferric iron minerals and the change in pH due to DMRB activity affect also the capacity of those minerals to adsorb negatively charged compounds. A dramatic example is given in Bangladesh and India. Due to the reductive dissolution of arsenic loaded iron oxides arsenic (As) become released and accessible to microorganisms that transform As between arsenate As(V) and arsenite As(III). Arsenite is more mobile than arsenate (Croal *et al.*, 2004) and therefore arsenate reducing bacteria contribute to the release of As(III) into groundwater, which results in highly contaminated potable water (Cummings *et al.*, 1999, Smedley & Kinniburgh, 2002, Harvey *et al.*, 2005). The World Health Organization (WHO) recommends as guideline values of 10 µg/l. In Bangladesh the concentration of 1000 µg/l exceeds the recommended guideline by two orders of magnitude. In contrast to the reductive dissolution which leads to the release of negatively charged compounds into the environment, Fe(II) oxidation can lead to the immobilization of toxic compounds like arsenic.

5.2 Generation of electricity using DMRB

Another example of the importance to elucidate the mechanism and function of the extended respiratory chain over the outer membrane is given by an application producing electricity. Since the Industrial Revolution in the 1700s, the use of fossil carbon sources and deforestation have increased the carbon dioxide (CO_2) concentration in the atmosphere dramatically. The CO_2 concentration in the atmosphere rose from approximately 280 parts per million (ppm) in pre-industrial times to 382 ppm in 2006 according to the National Oceanic and Atmospheric Administration's (NOAA). Shocking data have been presented by the

[1] For each iron oxide there is a pH at which the charge on the surface arising from all sources is zero. This pH is also often referred to as the point of zero charge (pzc) or isoelectric point (iep), but these terms can only be used interchangeably, if there is no specific adsorption. The point of zero charge is the pH at which the net adsorption of potential determining ions on the oxide is zero. It is obtained by the potentiometic titration oft he oxide in an indifferent electrolyte and is taken as the pH at which the titration curves obtained at several different electrolyte concentrations intersect Cornell, R. M. & U. Schwertmann, (2003) *The iron oxides : structure, properties, reactions, occurrences, and uses,* p. xxxix, 664 p. Wiley-VCH, Weinheim.

Intergovernmental Panel on Climate Change (IPCC), which revealed that the CO_2 concentration is higher than any time in **at least** the last 650,000 years (http://www.ipcc.ch/publications_and_data/publications_and_data.shtml). Knowledge on the mechanisms relevant for DIR can assist in the development of microbial fuel cells (MFCs), which would allow for exploitation of renewable energy sources. Bacteria in MFC convert chemical energy produced by the oxidation of organic or inorganic compounds (potentially obtained from waste water) to electrical current (Watson & Logan, 2010). MFCs represent a completely new method of renewable energy recovery. They provide a direct method of obtaining bioelectricity from cellulose and other biodegradable organic matter by a process called electrogenesis (Watson & Logan, 2010, Gorby *et al.*, 2006). Exoelectrogenic bacteria transfer electrons obtained from the oxidation of organic matter to the MFC anode while releasing protons into solution (Bretschger *et al.*, 2007). Electrons, protons, and oxygen react at the cathode, producing water. In addition, hydrogen gas can be produced with the same exoelectrogenic bacteria by modifying the MFC by adding a small voltage to that produced by the bacteria and omitting oxygen from the cathode. This process is referred to as electrohydrogenesis (Cheng & Logan, 2007). A higher understanding of how the mechanism of the extracellular electron transfer gets accomplished is important to increase the efficiency of such applications and thus broaden the interest for MFC as alternative energy source.

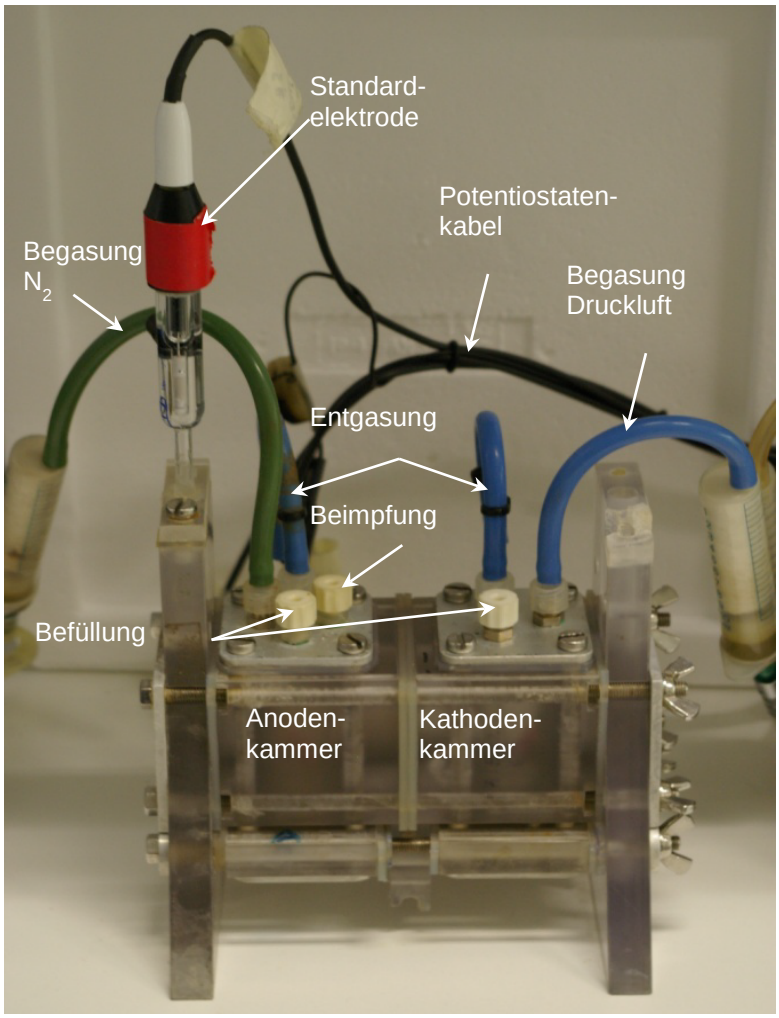


Fig. 27) Microbial fuel cell (MFC) as it is used in the laboratory. Photo and Construction: Frederik Golitsch.

APPENDIX

Tables and figures

Tab. A1) LC-MS/MS data of ~120 kDa band after crosslink, affinity purification and analytical gelfiltration of MtrA.

Fraction 7-9						
Protein name	**EMBL**	**Mass**	**Queries matched**	**Score**	**Gene locus**	**SignalP**
hypothetical protein	Q8E8E6	29348	12	398	SO4719	aa 28/29
HemB-1	Q8EE03	36779	9	381	SO2587	no
FccA	P83223	62409	10	295	SO0970	aa 25/26
CysK	Q8ED64	34399	10	248	SO2903	no
Spb	Q8E8L1	37797	10	235	SO4652	aa25/26
CysP	Q8EBC6	37086	6	193	SO3599	aa 23/24
BccP	Q8EF24	36177	6	192	SO2178	aa22/23
hypothetical protein	Q8EHM9	29955	8	186	SO1190	aa20/21
TufA	Q8EK70	43257	2	140	SO0229	no
MoeA	Q8E8E2	64686	3	109	SO4723	no
MtrA	Q8EG35	36035	5	103	SO1777	aa 34/35
GapA-2	Q8EEN3	36458	3	73	SO2345	no
Crp	Q8EJ49	23691	2	72	SO0624	no
hypothetical protein	Q8EB67	20589	1	50	SO3667	no
MoeA	Q8EKF5	44728	2	47	SO0138	no
PurU	Q8EGI0	30324	2	46	SO1624	no
hypothetical protein	Q8EJ53	29521	1	46	SO0620	no
MoaC	Q8E942	17283	2	46	SO4451	no
GlmS	Q8CX33	66687	1	43	SO4741	no
FdnG	Q8EKJ1	111987	1	42	SO0101	aa 31/32
SodB	Q8ED83	21482	2	41	SO2881	no
hypothetical protein	Q8E8R4	52245	2	40	SO4597	no
hypothetical protein	Q8EAJ5	17744	1	37	SO3907	aa 28/29
Alcohol dehydr.	Q8EEB0	39410	1	36	SO2477	no
TrxA	Q8EJQ6	11881	1	36	SO0406	no
HypB	Q8EF92	29374	1	33	SO2093	no
TonB receptor	Q8ED60	95702	2	32	SO2907	aa 31/32
hypothetical protein	Q8E903	19118	1	28	SO4492	no
GntR family	Q8EDB0	24983	2	27	SO2852	no

Fraction 13						
Protein name	**EMBL**	**Mass**	**Queries matched**	**Score**	**Gene locus**	**SignalP**
outer membrane porin	Q8EAK6	39874	3	124	SO3896	aa 23/24
hypothetical protein	Q8EJ53	29521	1	33	SO0620	no
SucA	Q8EFP0	104798	1	28	SO1930	no

Tab. A2) LC-MS/MS data of shifted MtrB signal in *S. oneidensis* MR-1 membrane.

Protein name	EMBL	Mass	Queries matched	Score	Gene locus
TonB-dependent receptor	Q8EEB8	97700	21	834	SO2469
TonB-dependent receptor	Q8EEF5	91901	14	653	SO2427
TonB-dependent receptor	Q8EIN9	79683	13	634	SO0798
Fe-S cluster binding protein	Q8EGS5	51399	17	633	SO1519
AcrB/AcrD/AcrF family protein	Q8E8H2	112473	11	585	SO4692
secA	Q8E9Q5	102559	12	502	SO4211
outer membrane porine	Q8EAK6	39874	9	443	SO3896
TonB-dependent receptor	Q8ED60	95702	12	341	SO2907
Fumarate reductase flavoprotein	P83223	62409	7	275	SO0970
GroL	Q8CX48	57044	7	174	SO0704
OmpA familiy protein	Q8EBH3	40157	4	157	SO3545
OmcA	Q8EG33	78567	3	130	SO1779
Peptid-prolyl cis-trans isom D	Q8EG15	67456	3	94	SO1798
DmsA	Q8EH03	91061	1	83	SO1429
MtrC	Q8EG34	71191	2	75	SO1778
MtrA	Q8EG35	36035	2	25	SO1777

Tab. A3) LC-MS/MS data of 75 kDA or 35 kDa soluble or membrane fractions from wildtype and suppressor strain grown under ferric citrate reducing conditions.

~75 kDa membrane wildtype					
Protein name	**EMBL**	**Mass**	**Queries matched**	**Score**	**Gene locus**
MtrB	Q8CVD4	77629	39	2282	SO1776
OmcA	Q8EG33	78567	30	1608	SO1779
fumarate reductase flavoprotein subunit	P83223	62409	26	1468	SO0970
MtrC	Q8EG34	71191	28	1367	SO1778
Fe-S cluster binding protein	Q8EGS3	99787	25	1239	SO1521
DmsF	Q8EHO4	74451	9	496	SO1428

~35 kDa membrane wildtype					
Protein name	**EMBL**	**Mass**	**Queries matched**	**Score**	**Gene locus**
cyt c oxidase cbb3-type subunit III (Ccop)	Q8EEL8	34995	11	573	SO2361
OmpA family protein	Q8EBH3	40157	12	439	SO3545
MtrA	Q8EG35	36035	7	259	SO1777
CcpA	Q8EF24	36177	7	240	SO2178
DmsE	Q8EH05	34634	2	123	SO1427

~75 kDa supernatant wildtype					
Protein name	**EMBL**	**Mass**	**Queries matched**	**Score**	**Gene locus**
OmcB	Q8EG34	71191	6	235	SO1778
Fe-S cluster binding protein	Q8EGS3	99787	6	184	SO1521

~35 kDa supernatant wildtype					
Protein name	**EMBL**	**Mass**	**Queries matched**	**Score**	**Gene locus**
Ccyt family protein	Q8EA62	38340	7	264	SO4047
MtrB	Q8CVD4	77629	2	155	SO1776
OmcA	Q8EG33	78567	1	90	SO1779

~75 kDa membrane suppressor					
Protein name	**EMBL**	**Mass**	**Queries matched**	**Score**	**Gene locus**
MtrB-like protein	Q8E9C5	74509	35	1848	SO4359
DmsA	Q8EHO3	91061	35	1572	SO1429
hypothetical protein	Q8E9C8	74024	21	1051	SO4356
DmsA-2	Q8E9C6	95638	26	1007	SO4358
Fe-S binding protein	Q8EGS3	99787	21	876	SO1521
OmcA	Q8EG33	78567	16	698	SO1779
DmsF	Q8EHo4	74451	9	679	SO1428
MtrC	Q8EG34	71191	12	645	SO1778

~35 kDa membrane suppressor					
Protein name	**EMBL**	**Mass**	**Queries matched**	**Score**	**Gene locus**
CcpA	Q8EF24	36177	20	868	SO2178
MtrA-like	Q8E9C4	33150	3	172	SO4360
Fe-S cluster binding protein	Q8EGS5	51399	4	156	SO1519
OmpA	Q8EBH3	40157	6	138	SO3545
DmsE	Q8EHO5	34634	2	116	SO1427

~75 kDa supernatant suppressor					
Protein name	**EMBL**	**Mass**	**Queries matched**	**Score**	**Gene locus**
DmsA	Q8EH03	91061	21	269	SO1429
DmsA-2	Q8E9C6	95638	19	257	SO4358
OmcA	Q8EG33	78567	5	217	SO1779
MtrC	Q8EG34	71191	3	194	SO1778

~35 kDa supernatant suppressor					
Protein name	**EMBL**	**Mass**	**Queries matched**	**Score**	**Gene locus**
CcpA	Q8EF24	36177	47	2569	SO2178
DmsB-2	Q8E9C7	22491	4	257	SO4357
MtrA-like	Q8E9C4	33150	3	202	SO4360
Cyt c family protein	Q8EA62	38364	2	193	SO4047
MtrB-like	Q8E9C5	74509	2	178	SO4359
MtrA	Q8EG35	36035	2	156	SO1777
DmsB	Q8EH02	24402	1	78	SO1430
CymA	Q8E8S0	20822	1	68	SO4591

Fig. A1) A) Elution profile of affinity purified crosslinked $MtrA_{STREP/HIS}$ using a Ni-NTA superflow column. B) Elution profile of the affinity purified crosslinked $MtrA_{STREP/HIS}$ using a Superdex^{200} 10/300 cloumn with a flowrate of 1 ml min^{-1}. Green line indicates absorption at a wave length of 280 nm to track proteins in general. Orange line indicates absorption at 420 nm to follow elution of *c*-type cytochromes. AU: absorption units; numbers on top reflect the respective fractions.

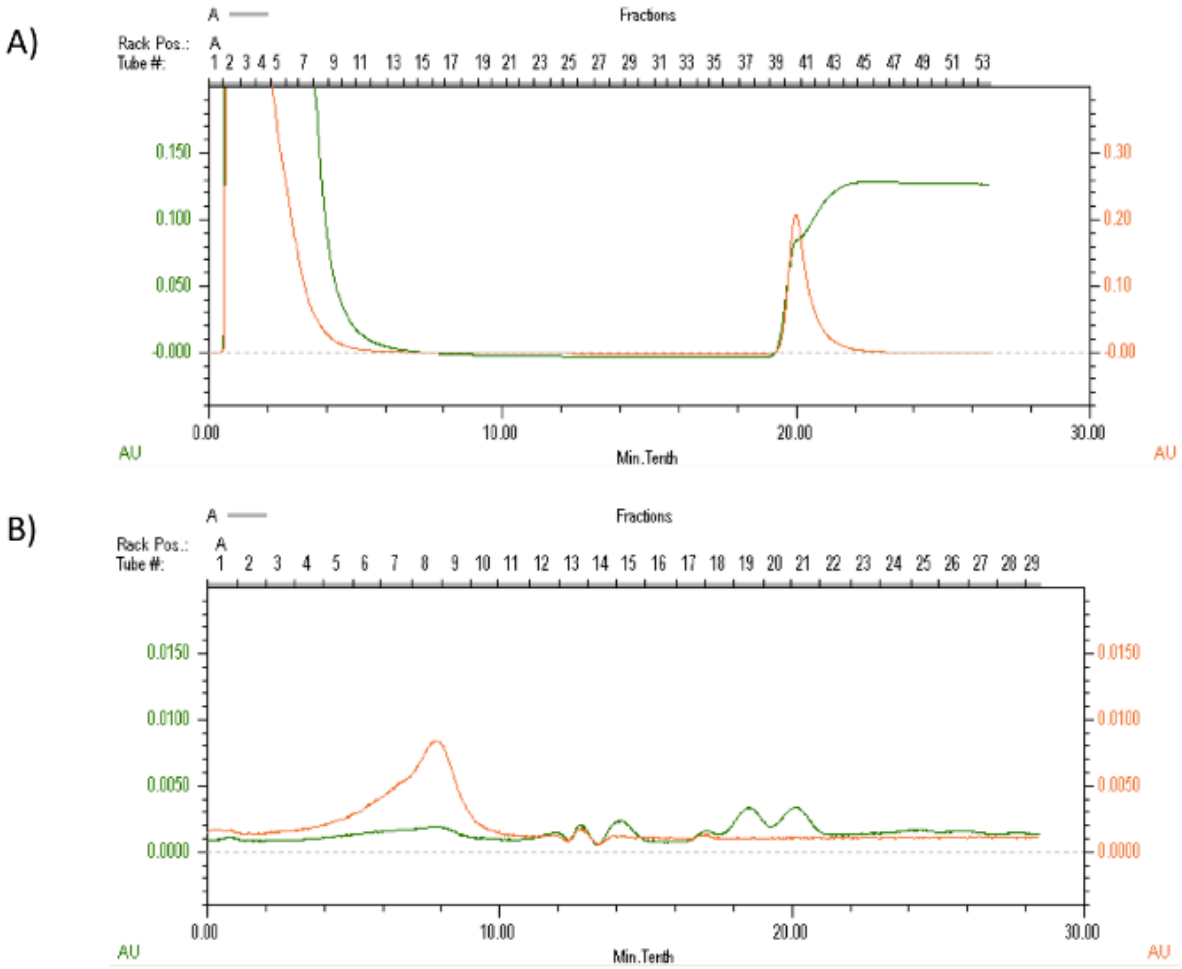
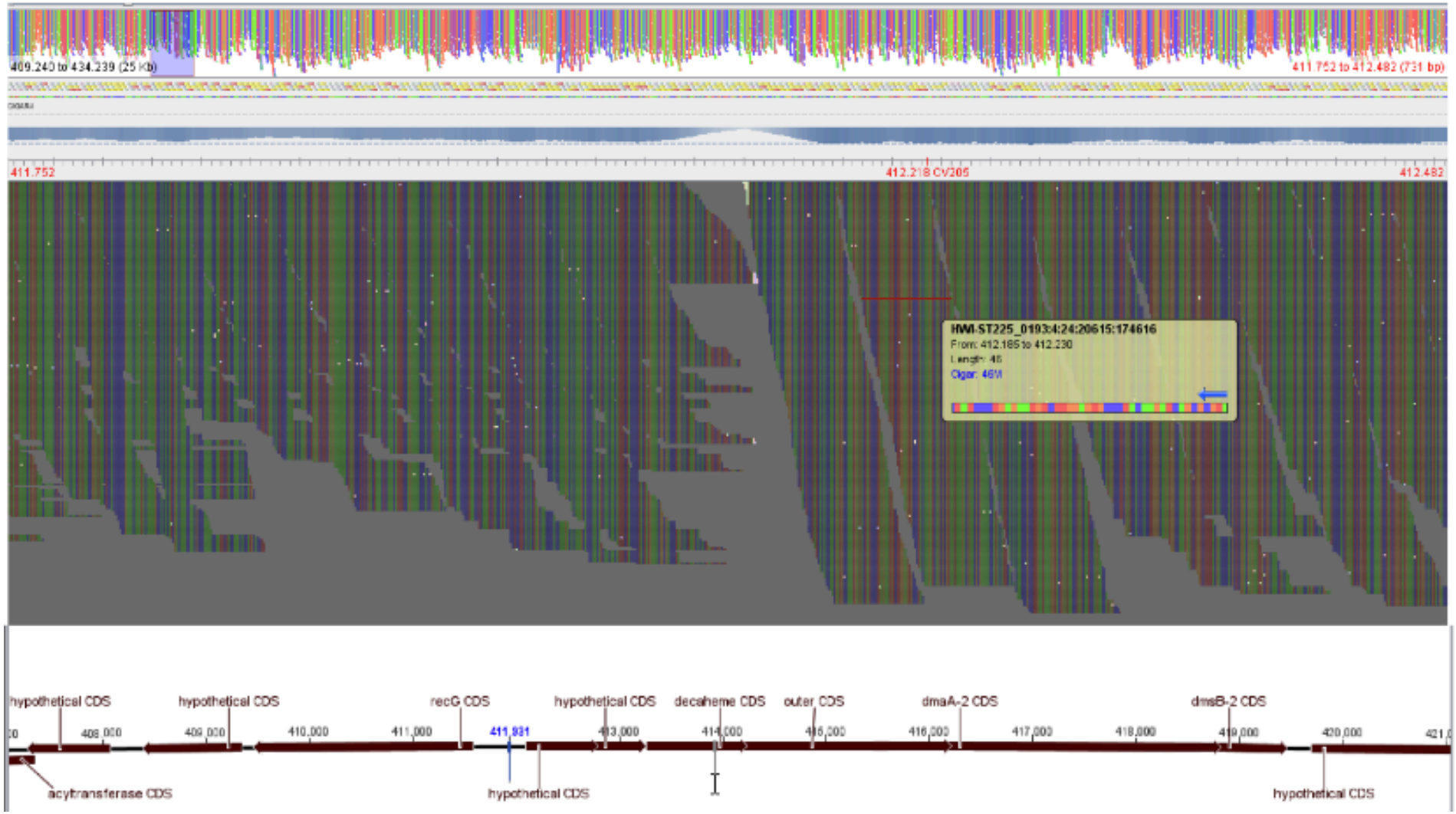


Fig. A2) Assembly of reads obtained from Solexa sequencing to the *S. oneidensis* MR-1 Δ*mtrB* genome as reference. A gap in the assembly is indicates genomic rearrangements. An overview of the affected gene cluster is shown at the bottom.



Curriculum vitae

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Postgraduate study

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7-2008 to 7-2011:	PhD thesis: "The Involvement of ß-barrel proteins in the dissimilatory iron reduction in *Shewanella oneidensis* MR-1." at the University of Freiburg i.Br., Institute of Biology II, Microbiology, supervised by Dr. Johannes Gescher.
6-2010 bis 7-2010:	Internship at the Stanford University, Hopkins Marine Station, Pacific Grove, CA, Prof. Alfred Spormann, Prof. Chirs Francis and Prof. Paul Rainey.
7-2008 to 9-2008:	Internship at the University of Stanford, Department of Civil & Environmental Engineering, Prof. Alfred Spormann.

Graduate study

7-2007 to 7-2008:	Diploma Thesis: "The involvement of the ß-barrel protein MtrB on the Localisation of the outer membrane cytochromes in *Shewanella oneidensis*" at the University of Freiburg i.Br., Institute of Biology II, Microbiology, supervised by Dr. Johannes Gescher (Grade 1.1)
7-2007:	Examination in Biology (Diploma) with focus on Biochemistry (Grade 1.3, Prof. Wolfgang Haehnel), Genetics and Molecular Biology (2.0, Prof. Karl-Friedrich Fischbach), Developmental Biology (1.7, Prof. Wolfgang Driever) and Clinical Chemistry (2.3, Prof. Heinrich Wieland).
10-2004 to 7-2008:	Biology (Diploma) at the Albert-Ludwigs-University Freiburg i.Br. (Germany)
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Publications and Manuscripts

1. Schütz B., Schicklberger M., Kürmann J., Spormann A.M., Gescher J.: “Periplasmic electron transfer via the *c*-type cytochromes MtrA and FccA of *Shewanella oneidensis* MR-1”. *Applied and Environmental Microbiology*, 2009.

2. Schicklberger M., Bücking C., Schuetz B., Heide H., Gescher J. “Involvement of the Shewanella oneidensis decaheme cytochrome MtrA in the periplasmic stability of the beta-barrel protein MtrB.“ *Applied and Environmental Microbiology*, 2010.

3. Richter K., Bücking C., Schicklberger M., Gescher J.: “A simple and fast method to analyze the orientation of *c*-type cytochromes in the outer membrane of Gram negative bacteria”. *Journal of Microbiological Methods*, 2010.

4. Cordova CD., Schicklberger MF., Yu Y., Spormann AM. “Partial functional replacement of CymA by SirCD in Shewanella oneidensis MR-1.” *Journal of Bacteriology*, 2011.

5. Richter K.*, Schicklberger M.*, Gescher J. „Dissimilatory reduction of extracellular electron acceptors in anaerobic respiration.“ *Applied and Environmental Microbiology*, 2011. (*Shared first author)

6. Bücking C., Schicklberger M., Gescher J. “The biochemistry of dissimilatory ferric iron and manganese reduction in *Shewanella oneidensis*” *Book chapter* (in progress)

7. Schicklberger M., Gescher J. “Genomic Plasticity Responsible for Dissimilatory Iron Reduction in *Shewanella oneidensis* MR-1”, (in progress)

Poster

1. Marcus Schicklberger, Carmen D. Cordova, Alfred Spormann, Johannes Gescher: “A synthetic biology approach to resolve the minimal protein set for dissimilatory iron reduction.” *Biospektrum*, 2008; Poster presentation at the annual meeting of the “Vereinigung für Allgemeine und Angewandte Mikrobiologie“ (VAAM) in Frankfurt, (2008).

2. Marcus Schicklberger, Johannes Gescher: “The Role of the ß-Barrel Protein MtrB in the Dissimilatory Metal Reduction of *Shewanella oneidensis* MR-1” *Biospektrum*, 2010; Poster presentation at the annual meeting of the “Vereinigung für Allgemeine und Angewandte Mikrobiologie“ (VAAM) in Hannover, (2010).

3. Marcus Schicklberger, Johannes Gescher: “The Role of the ß-Barrel Protein MtrB in the Dissimilatory Metal Reduction of *Shewanella oneidensis* MR-1” *Biospektrum*, 2011; Poster presentation at the annual meeting of the “Vereinigung für Allgemeine und Angewandte Mikrobiologie“ (VAAM) in Karlsruhe, (2011).

4. Marcus Schicklberger, Johannes Gescher: “The Role of the ß-Barrel Protein MtrB in the Dissimilatory Metal Reduction of *Shewanella oneidensis* MR-1“ Poster presentation at the congress of the european microbiologists from the “Federation of European Microbiological Societies“ (FEMS) in Genf, (2011)

5. Simon Stefan, Marcus Schicklberger, Johannes Gescher: “Genomic Plasticity Responsible for Dissimilatory Iron Reduction in *Shewanella oneidensis* MR-1, *Biospektrum*, 2012; Poster presentation at the annual meeting of the “Vereinigung für Allgemeine und Angewandte Mikrobiologie“ (VAAM) in Tübingen, (2012).

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Marcus Schicklberger, 7.3.2012

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