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Energetics and Physiology of Microbial Iron Reduction

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2 Abstract / Summary

Dissimilatory iron reduction is a very common process in anoxic environments such as aquatic sediments, submerged soils and the terrestrial subsurface. Among those microorganisms reducing iron as terminal electron acceptor some species exist that can even use aromatic compounds as carbon source. From this point of view it becomes evident that microbial iron reduction may be a useful and manipulative tool to help on the remediation of contaminated environments.

The purpose of this thesis was to further elucidate the molecular processes of iron reduction on the one hand and to combine the process of iron reduction to the degradation of the polycyclic aromatic hydrocarbon naphthalene on the other hand. To investigate the molecular processes of the microbial reduction of iron, the question was assessed if and how microorganisms can adapt to the redox range offered by the electron acceptor iron. Growth experiments using the model iron reducers *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 on iron chelates with different redox potentials were performed. For *Geobacter sulfurreducens* a correlation of the biomass yield and enhanced reaction kinetics with the formal potential of the applied electron acceptor were observed. For *Shewanella oneidensis*, the same dependence of growth yield on the redox potential of the electron acceptor was observed. However, a higher growth rate was achieved with a lower redox potential, demonstrating the metabolic difference of the two microorganisms. These results show that iron-reducing bacteria can adapt their biomass yield to the obtainable energy.

These findings lead to the question how an iron-reducing organism can metabolically achieve this adaptation of biomass formation. To investigate this question in more detail the assumption was made that the respiratory system might be branched and that unknown compounds might be involved. This would mean that the organism had two respiratory pathways, one responsible for Fe(III)-electron acceptors with lower redox potentials, the other for Fe(III)-electron acceptors with higher redox

potentials. The latter possessing an additional compound that allows the conservation of more energy (e.g. an additional proton pumping step, or an alternative iron reductase). To find this unacquainted compound a random transposon mutagenesis on *Shewanella oneidensis* MR-1 was performed. The screen selected on mutants with the inability of either reducing an electron acceptor with a high redox or and electron acceptor with low redox potential. Mutants were sequenced and tested for initial reduction rates. Three types of mutants were found, 1) having a defect directly within a known compound of the respiratory chain, 2) having a defect in the biogenesis of a respiratory compound and 3) a defect within the secretion system of a compound located in the outer membrane. No mutants with mutations in unknown compounds were found. The reduction kinetics of mutants and wildtype strains showed a general difference of all types of strains between the compounds Fe-NTA (low redox potential) and Fe-Cit (high redox potential). The reduction rates were in average 50 % higher of Fe-NTA than of Fe-Cit. Evidence to verify the working hypothesis could not be found. However, the results confirm the resulting data from the growth experiments with *Shewanella* in the first chapter and underlined the differences between the organisms S*hewanella* and *Geobacter*. Aside from this, three groups were visible concerning the reduction rates. Wildtype strains or strains without a defect in the respiratory complex for iron were fastest with rates between 0.7 – 1.5 $mmol \cdot l^{-1} \cdot h^{-1}$. Strains with a defect in the secretory system of the terminal iron reductase showed mean reduction rates of about 0.1 – 0.3 $mmol \cdot l^{-1} \cdot h^{-1}$. Strains with mutations in essential respiratory compounds like cymA or menaquinone reduced iron only poorly (0.02 – 0.07 $mmol \cdot l^{-1} \cdot h^{-1}$). An evidence for the trueness of the working hypothesis could not be found.
In the last part of this thesis the microbial iron reduction and naphthalene degradation are assessed together. An iron reducing enrichment culture degrading naphthalene is presented. The culture showed growth on naphthalene accompanied by the depletion of the substrate and the reduction of ferric iron. Furthermore, the central metabolite of naphthalene degradation, 2-naphthoic acid, increased concomitantly with growth. The community within the culture was dominated by one bacterial organism closely related (99% 16S sequence similarity) to the major organism in the iron-reducing enrichment culture BF (Kunapuli*, et al.*, 2007,

Kunapuli*, et al.*, 2010). The classification of a new candidate species and genus of Gram-positive iron-reducers with the ability to degrade non-substitute aromatic hydrocarbons is suggested. As far as literature is concerned culture N49 is the first highly enriched, iron reducing microbial culture degrading naphthalene under anaerobic conditions.

In summary, the results of this thesis show that the role of Gram-positive iron-reducers in anaerobic PAH degradation might be more important than so far expected. Furthermore these results indicate that iron reducers are able to adapt the gain of their biomass to the energy available. A feature that might allow the survival and be a selective advantage even under conditions when only very few energy is available.

3 Zusammenfassung

Die dissimilatorische Eisenreduktion ist ein weit verbreiteter Prozess in anoxischen Lebensräumen wie zum Beispiel aquatischen Sedimenten, submersen Böden und der Erdoberfläche. Unter jenen Mikroorganismen, die Eisen(III) als Elektronenakzeptor nutzen gibt es sogar einige Arten, die selbst aromatische Substanzen als Kohlenstoffquelle heranziehen können. Aus dieser Perspektive betrachtet wird es offensichtlich, dass die mikrobielle Eisen(III)-reduktion zu einem nützlichen Hilfsmittel bei der Sanierung kontaminierter Standorte werden könnte.
Aufgabe und Ziel dieser Doktorarbeit war es einerseits, die molekularen Prozesse der mikrobiellen Eisenreduktion tiefer zu beleuchten, sowie andererseits Eisenreduktion und mikrobiellen Abbau des polyzyklischen aromatischen Kohlenwasserstoffs Naphthalin miteinander zu verbinden. Um die molekularen Prozesse hinter der mikrobiellen Eisenreduktion genauer zu untersuchen wurde die Frage gestellt, ob, und wenn ja, wie die Mikroorganismen sich dem Redoxbereich anpassen, der durch den Elektronenakzeptor Eisen(III) bereitgestellt wird. Es wurden daher Wachstumsexperimente mit den Modellorganismen *Geobacter sulfurreducens* und *Shewanella oneidensis* MR-1 auf Eisenchelatkomplexen mit unterschiedlichen Redoxpotentialen durchgeführt und dabei Biomassebildung und Eisenreduktion bestimmt. Für *Geobacter sulfurreducens* war eine Korrelation von Biomasseertrag und Reduktionskinetik mit dem formalen Redoxpotential des Elektronenakzeptors zu beobachten. Für *Shewanella oneidensis* war die gleiche Abhängigkeit von Wachstumsertrag und Redoxpotential des jeweiligen Akzeptors zu verzeichnen. Jedoch wurde mit einem geringeren Redoxpotential eine höhere Reduktionsrate festgestellt, was die Unterschiedlichkeit der beiden Organismen auf metabolischer Ebene veranschaulicht. Die Ergebnisse zeigen aber deutlich, dass Eisenreduzierer ihre Biomasseproduktion an die verfügbare Energie anpassen können, was aber weiter zu der Frage führt, wie dies metabolisch bewerkstelligt wird.
Um diese Fragestellung näher zu untersuchen, wurde die Arbeitshypothese aufgestellt, dass der respiratorische Weg gegabelt ist und bisher noch unbekannte

Komponenten existieren, die eine zusätzliche Energiekonservierung möglich machen. Also zwei Atmungskettenwege, der eine zuständig für Fe(III)-Elektronenakzeptoren mit niedrigerem Redoxpotential, der andere für jene mit einem höheren Redoxpotential. Wobei der letztere eine zusätzliche, unbekannte Komponente aufweist, so daß über diesen Weg eine höhere Energiekonservierung möglich ist. Dies könnte zum Beispiel ein zusätzlicher protonenpumpender Schritt oder eine alternative Eisenreduktase sein. Um diese unbekannte Komponente zu finden wurde eine randomisierte Transposonmutagenese mit dem Organismus *Shewanella oneidensis* MR-1 durchgeführt. Dabei wurde auf jene Mutanten selektiert, die die Fähigkeit auf einem Elektronenakzeptor mit hohem oder aber mit einem niedrigen Redoxpotential zu wachsen verloren hatten. Die Insertionsstellen der Transposons in den Mutanten wurden sequenziert. Die initalen Reduktionsraten der Mutanten und des Wildtyps wurden für Wachstum auf auf Fe(III)-Elektronenakzeptoren mit hohem bzw. mit niedrigem Redoxpotential ermittelt. Dabei wurden drei verschiedene Typen von Mutanten gefunden: 1) mit einem Defekt in einer bekannten Komponente des anaeroben Atmungswegs für Eisen(III), 2) mit einem Defekt in der Biogenese einer Fe(III)-Atmungskettenkomponente, 3) einem Defekt im Sekretionssystem für ein Außenmembranprotein der Eisen(III)-Atmungskette. Es wurden keine Mutationen in einer bisher unbekannten Komponente gefunden. Die Analyse der Reduktionskinetiken zeigte einen generellen Unterschied zwischen den Elektronenakzeptoren Fe-NTA (niedriges Redoxpotential) und Fe-Citrat (hohes Redoxpotential) für alle getesteten Mutantentypen als auch für den Wildtyp. Auf Fe-NTA waren die Reduktionsraten im Schnitt 50% höher als auf Fe-Cit. Ein Beweis für die Bestätigung der Arbeitshypothese konnte nicht gefunden werden. Die Ergebnisse bestätigen jedoch die Daten aus den Wachstumsexperimenten mit *Shewanella* im ersten Kapitel und verdeutlichen die Unterschiedlichkeit der beiden Bakterienarten.

Im letzten Kapitel dieser Arbeit werden die mikrobielle Eisenreduktion und der anaerobe Abbau von Naphthalin verbunden und gemeinsam untersucht und es wird die eisenreduzierende, naphthalinabbauende Anreicherungskultur N49 präsentiert. Diese Kultur zeigte produktives Wachstum auf Naphthalin, begleitet von der Abnahme des Substrates und der Reduktion von Eisen(III). Des Weiteren fand sich

der Zentrale Metabolit des Naphthalinabbaus 2-Naphthoesäure, dessen Konzentration wachstumsbegleitend zunahm. Die bakterielle Gemeinschaft der Kultur wurde durch einen Hauptorganismus bestimmt. Sein nächster Verwandter erwies sich als der Hauptorganismus der BF Kultur, die ebenfalls Eisen reduziert, aber Benzol abbaut. Beide Organismen zählen zu den Gram-positiven Peptococcaceaen (Kunapuli*, et al.*, 2007, Kunapuli*, et al.*, 2010). Unserem Wissen nach handelt es sich bei der beschriebenen N49 Kultur um die erste, hoch angereicherte, naphthalinabbauende Kultur, welche Eisen reduziert. Aufgrund der phylogenetischen Nähe zur BF Kultur wird eine neue Kanditatenspezies und ein neuer Genus Gram-positvier Eisenreduzierer vorgeschlagen, welcher nicht substituierte, aromatische Kohlenwasserstoffe abbauen kann. Diese Ergebnisse machen die Variabilität unter den PAK abbauenden Mikroorganismen deutlich und geben einen Einblick in eine möglicherweise neue und bisher übersehene phylogenetische Gruppe.

Zusammenfassend zeigen die Ergebnisse dieser Doktorarbeit, dass Eisenreduzierer in der Lage sind, die Produktion der Biomasse der verfügbaren Energie anzupassen. Eine Eigenschaft die das Überleben unter Bedingungen ermöglichen könnte, unter denen nur sehr wenig Energie zur Verfügung steht. Sie könnte unter diesen Bedingungen sogar einen selektiven Vorteil bieten. Des Weiteren zeigt diese Arbeit, dass Eisenreduzierer auch beim anaeroben Abbau von Naphthalin eine bedeutende Rolle spielen die bisher nicht bekannt war. Möglicherweise ist die Rolle der eisenreduzierenden Mikroorganismen im Bereich der PAK-Abbauer wesentlich größer als angenommen; die Ergebnisse deuten in diese Richtung.

4 Outline and objectives of this thesis

The field of microbial dissimilatory iron reduction and the degradation of aromatic compounds are still quite young research areas. The capacity of microbes reducing iron has been know since the beginning of the 20th century but its environmental relevance has become discovered only about twenty years ago. Much less is known about the anaerobic degradation of PAHs. Only a decade ago the common understanding had been that PAHs, once entering the anoxic zone of an aquifer, remain as a persistent pollution. Yet this hypothesis has been disproven by several studies showing the mineralization of PAHs and is stoichiometric coupling to alternative electron acceptors like nitrate and sulfate (Langenhoff*, et al.*, 1989, Bregnard*, et al.*, 1996, Coates*, et al.*, 1996, Bedessem*, et al.*, 1997, Coates*, et al.*, 1997, Zhang & Young, 1997, Rockne & Strand, 1998). In this thesis these two fields will be combined and further elucidated by the following research questions:

1) How do iron reducers adapt to the ”redox range“ offered by iron oxides and how is that achieved by the organisms?

2) What kind of role do iron reducing bacteria play in anaerobic naphthalene degradation?

One part of this thesis will focus on question 1), addressing the thermodynamics and growth kinetics of the dissimilatory iron reducers *Shewanella oneidensis* MR-1 and *Geobater sulfurreducens* regarding the usage of the electron acceptor iron and its varying redox potentials.

A second part of this thesis follows the first one thematically but goes further into the details of how an organism could be able to conserve different energy yields according to the electron acceptor iron with its varying redoxpotentials. A hypothesis is made that a higher energy gain might be achieved with the modulation of the respiratory chain. The hypothesis is investigated through the construction and investigation of *Shewanella* oneidensis MR-1 mutants.

A third part combines the topic iron reduction and the topic anaerobic naphthalene degradation by focusing on question 2). An iron reducing enrichment culture degrading naphthalene is presented. Iron reducing naphthalene degraders have not been known so far. Growth, reduction behavior, the species involved and the putative mechanism of initial naphthalene degradation are investigated.

5 Introduction

5.1 Iron as electron acceptor

Ironoxides and ironhydroxides originate from weathering of igneous rocks, in which iron is siliciously bound in its two valent form meaning as pyroxene, biotite, olivine. It also occurs in the form of pyrite (FeS_2) (Schwertmann & Cornell, 2000). Sixteen different iron (hydr-)-oxides exist, e.g. goethite (α-FeOOH), ferrihydrite ($Fe_5HO_8x4H_2O$), hematite goethite (α-Fe_2O_3) or magnetite (Fe_3O_4), chemical compounds consisting of iron (Fe) with oxygen (O) or a hydroxyl group (OH). In most iron (hydr)-oxides iron is bound in its trivalent form. Magnetite in example is a mixture of trivalent (FeIII) and bivalent (FeII) iron (Cornell & Schwertmann, 1996).
In our environment, iron is widespread within soils and is responsible for the characteristic yellow, orange and red colorings therein. According to consistency of the respective soil the proportion of iron lies by approximately 0.2 – 5 % (Scheffer & Schachtschabel, 2002). Due to its high abundance in soils trivalent iron counts as a crucial electron acceptor for many bacterial species. The varying redoxpotential of iron is another reason why iron happens to be a suitable electron acceptor for bacteria (Pierre*, et al.*, 2002). The standard redox potential for the redox pair Fe^{2+}/Fe^{3+} at a pH of 2.5 is +770 mV. Depending on the bond with a variable ligand L the redoxpotential of L-Fe^{2+}/L-Fe^{3+} can assume values between -1 V and +1 V, a feature unique for iron as electron acceptor (Pierre & Fontecave, 1999, Pierre*, et al.*, 2002). However, the redox potential of iron is greatly influenced by the pH of the respective medium. In (Pierre*, et al.*, 2002) Ferrioxinamin is mentioned to gain an enhancement of the redox potential of about 300mV due to a change of pH from 7 to 3.5.
The reduction of iron (hydr)-oxides by the organism *Shewanella putrefaciens* takes course after the following equation (formate as substrate) (Lovley, 1991, Lovley, 1993).

Equation 1:

$HCOO^- + 2Fe(III) + H_2O \rightarrow HCO_3^- + 2Fe(II) + 2H^+$

Instead of formate, a variety of substances can be used as substrates by *Shewanella* (see figure 1) (lactate, pyruvate, hydrogen, formate etc) (Lovley, 1991, Lovley, 1993, Lovley*, et al.*, 1999, Nealson*, et al.*, 2002).

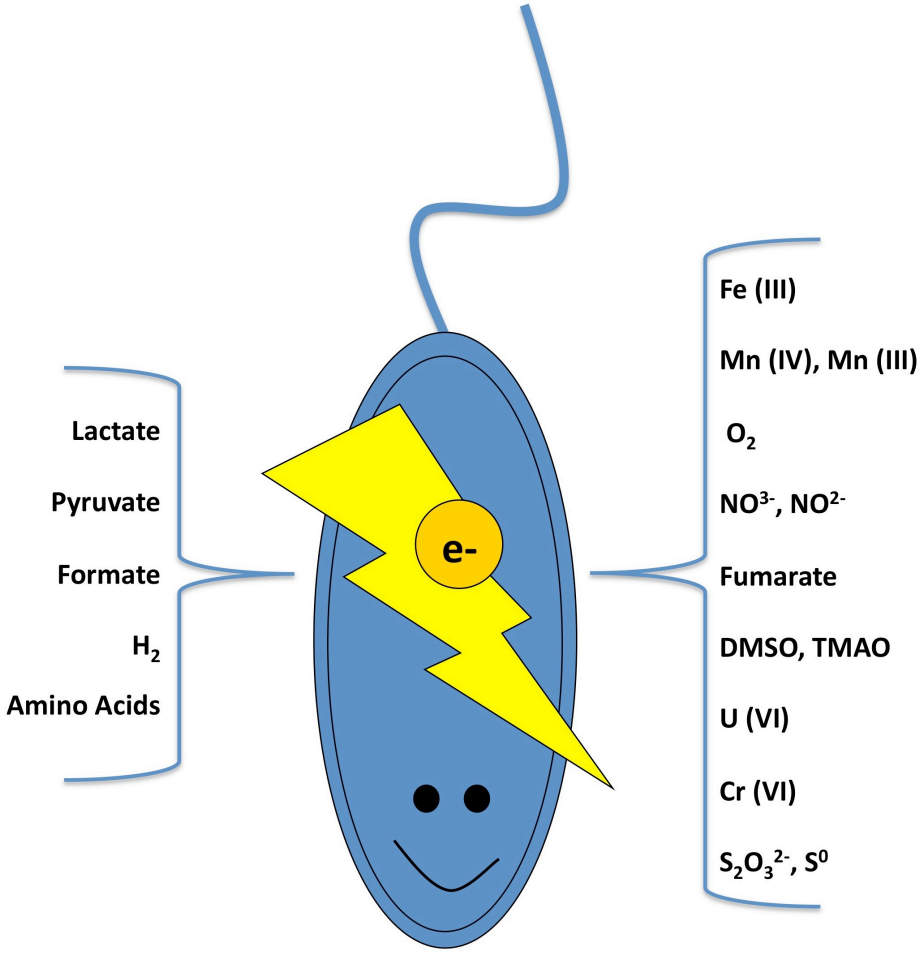

Figure 1: Versatility of *Shewanella* regarding electron acceptors and electron donors
Left panel shows the electron sources used by most of the *Shewanella* species. Most common electron acceptors of *Shewanella* species are shown on the right. TMAO= trimethylamine oxide, DMSO= dimethyl sulfoxide. Modified after (Nealson*, et al.*, 2002)

5.2 Dissimilatory iron reduction

Dissimilatory iron reduction is an anaerobic microbial respiration process during which inorganic or organic electron donors are oxidized and iron(III) as terminal electron acceptor gets reduced to iron(II) (Pierre*, et al.*, 2002). Even though it is known for almost a century that some microbes have the metabolic capability to reduce ferric iron, its importance and its environmental relevance has not been discovered until the last two decades (Harder, 1919, Runov, 1926, Alllison & Scarseth, 1942). Supposedly, microbial iron reduction is an evolutionary very old process of energy generation; geochemically it is one of the most relevant processes within aquatic sediments and wet soil (Lovley, 1991, Nealson & Saffarini, 1994, Lovley & Chapelle, 1995, Vargas*, et al.*, 1998). Phylogenetically, there is a high diversity amongst the group of microorganisms gaining energy through the reduction of ferric iron (Lovley*, et al.*, 1997, Rooney-Varga*, et al.*, 1999).

Within the domain *Bacteria* the capability of dissimilatory iron reduction is found in many different phyla and e.g. in four subgroups of the proteobacteria (alpha, delta, gamma and epsilon) (Lovley*, et al.*, 1997). The family of the *Geobacteraceae* with the *Geobacter* and *Desulfuromonas* clusters are located among the delta-proteobacteria (Lovley*, et al.*, 1997, Rooney-Varga*, et al.*, 1999). The *Geobacteraceae* are known to also reduce elemental sulfur, next to ferric iron. Furthermore, the genus *Shewanella, Ferrimonas, Aeromonas, and Thiobacillus* are situated within the gamma-proteobacteria

(Lovley*, et al.*, 1997). Likewise, members of the epsilon-proteobacteria like *Campylobacter, Geospirillum, Acrobacter, and Helicobacter* species could be identified as iron reducers (Lovley*, et al.*, 1997). Also, the phototrophic *Rhodobacter* (belonging to the alpa-proteobacteria), as well as the lines *Geothrix* and *Geovibrio* are iron reducers (Lovley*, et al.*, 1997). *Bacillus infernus* (in contrast to all other bacilli a strict anaerobe) is the only known gram positive known to be capable of iron reduction so far (Lovley*, et al.*, 1997).

Within the domain of the *Archaea* the members shown to be iron reducers belong to the kingdoms of the *Crenarchaeota* and *Euryarchaeota*. Among the *Crenarchaeota*

they belong within the order *Thermoproteales*, and among the *Euryarchaeota* within the orders *Archaeoglobales* and *Thermococcales*.
Also bacterial consortia consisting of nitrate-, fumarate- or sulfate reducers that live syntrophically with iron reducers are known to exist (Lovley & Coates, 2000).

5.3 Respiratory chain in *Shewanella oneidensis* MR-1

While the organism *Shewanella oneidensis* MR-1 is more limited about the energy sources, it is very versatile towards the electron acceptors used. As substrates lactate, pyruvate, amino acids, formate and H_2 can be used by the organism to gain energy (Ringo*, et al.*, 1984, Lovley*, et al.*, 1989).
As electron acceptors next to Fe(III) O_2, NO_3^-, NO_2^-, Mn(IV), Mn(III), fumarate, trimethylamine n-oxide (TMAO), dimethyl sulfoxide (DMSO), S^0, $S_2O_3^{2-}$, U(VI), Cr(VI), V(V) can be reduced (Myers & Nealson, 1988, Myers & Nealson, 1988, Lovley*, et al.*, 1989, Lovley*, et al.*, 1991, Moser & Nealson, 1996, Carpentier*, et al.*, 2005, Gralnick*, et al.*, 2006, Daulton*, et al.*, 2007). A detailed list about used electron acceptors and its reaction products can be found in (Nealson & Scott, 2006).
As known so far, if Fe(III) as an electron acceptor is used, protons and electrons get separated by a dehydrogenase and are transferred to a menaquinone pool (Myers & Myers, 1993). From there they make their way over a membrane bound tetraheme cytochrome CymA (Myers & Myers, 1997) which delivers them to a small soluble cytochrome (MtrA) within the periplasm which shuttles the electrons to the outer membrane (Pitts*, et al.*, 2003, Schuetz*, et al.*, 2009). At the outer membrane, electrons are transferred to the terminal iron reductase, which is an oligomeric protein complex between two decaheme c-type cytochromes OmcA and MtrC (formerly OmcB) (Shi*, et al.*, 2006, Shi*, et al.*, 2009).
For other metals like Mg(V), As(V), Vanadium(V), as well as fumarate, nitrate, and DMSO electrons take the same way through the redox chain to the CymA (Myers & Myers, 1997, Myers & Myers, 2000, Myers & Myers, 2003, Schwalb*, et al.*, 2003, Myers*, et al.*, 2004). For TMAO it is known that no menaquinone and no CymA seem to be required for reduction (Myers & Myers, 1997).

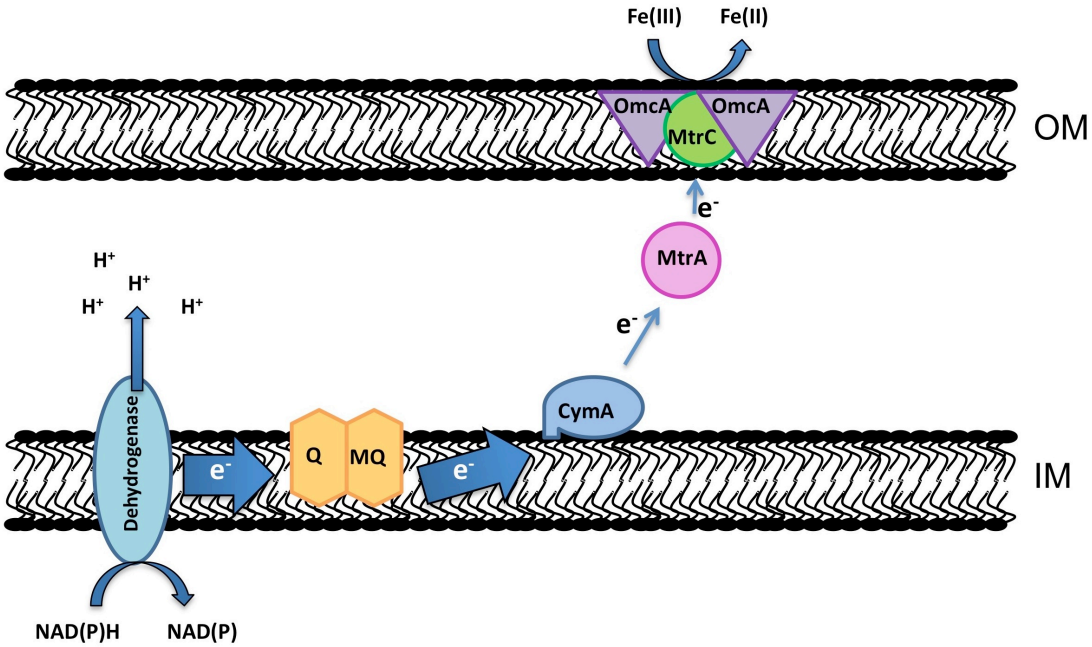

Figure 2: Image of the respiratory chain in *Shewanella oneidensis* MR-1.

IM = inner membrane, OM = outer membrane, Q = quinone pool, MQ = menaquinone pool. See text for detailed description. Modified after (Schwalb*, et al.*, 2003, Weber*, et al.*, 2006)

5.4 Energetics of microbial iron reduction

In the case of iron used as electron acceptor, the microorganisms need special characteristics. As already mentioned before, iron exhibits various redox potentials according to ligands and pH. Iron reducers therefore might face a redox range when using iron as terminal electron acceptor. Out of this, the question arises if these microorganisms can adapt on that feature and gain more biomass out of a reaction that offers more free energy. If the redox chain was fixed for the usage of iron, a greater amount of energy will be lost by heat dissipation. A modulation of the respiratory system could be possible as well. Achieved in example with an additional proton-pumping step. However, all this remains speculative. Little is known about the adaptation of the respiratory complex so far, but experiments with *Shewanella* showed a branching and overlapping of the respiratory chain according to the electron acceptor used (Myers & Myers, 2003, Lies*, et al.*, 2005) and even alternative routes have been described for the Mtr-pathway (Coursolle & Gralnick, 2010). These findings might make an adaptation possible, but yet, the molecular mechanism of how bacteria might modulate and adapt their respiratory system and metabolism still remains unclear.

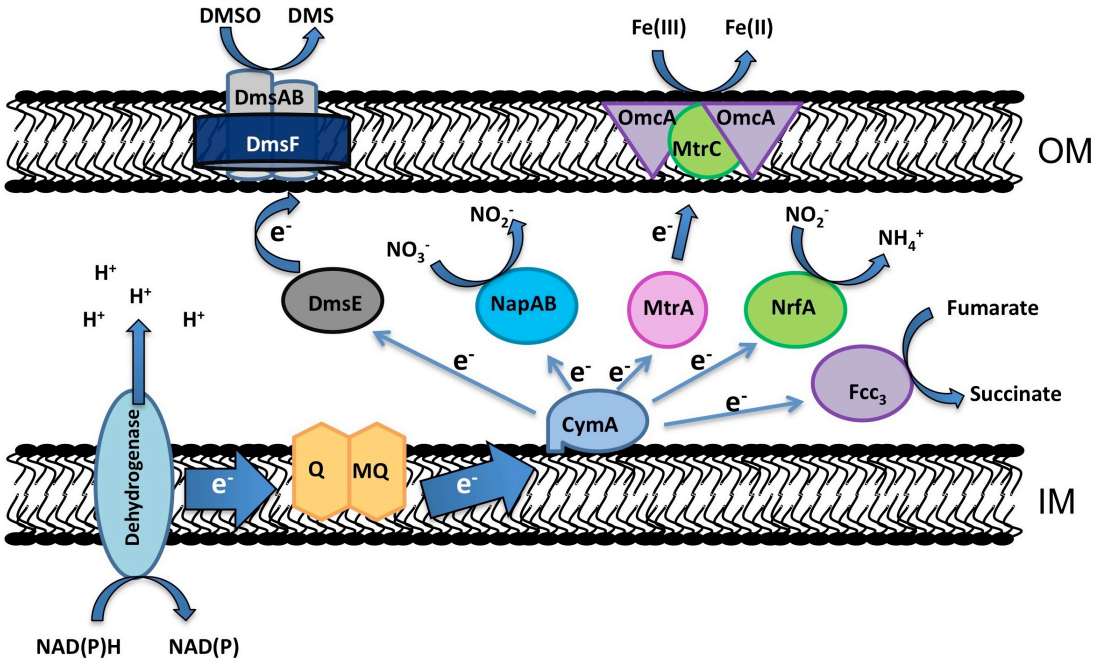

Figure 3: CymA, a central branching point in the respiratory system of *Shewanella oneidensis* MR-1

IM = inner membrane, OM = outer membrane, Q = quinone pool, MQ = menaquinone pool, Fcc_3 = fumarate reductase, NrfA = nitrite reductase, NapAB = nitrate reductase, DmsAB = DMSO reductase. See text for detailed description. Modified after (Schwalb*, et al.*, 2003, Weber*, et al.*, 2006)

5.5 Electron transfer mechanisms by iron reducing bacteria

In natural environments neutral pH values are predominant. In contrast to other electron acceptors like nitrate, sulfate or oxygen ferric iron is almost insoluble at neutral pH values and therefore exists only in its crystalline form (e.g. goethite, hematite, ferrihydrite), so this unsolubility of iron at neutral pH values forms an obstacle for the bacteria to use iron as electron acceptor. Transport of iron into the cell can be excluded because of its inefficiency. The exact mechanism, how the cells transfer their electrons onto iron minerals is still not fully understood. Several hypotheses of electron transfer have been in the center of discussion (Hernandez & Newman, 2001, Nevin & Lovley, 2002).

Direct Contact might be necessary for electron transfer onto iron minerals. For bacteria of the genus *Geobacter* it could be shown that a lack of electron acceptors yields to iron oxide chemotaxis as well as to the production of pili like structures, so

called nanowires (Childers*, et al.*, 2002, Reguera*, et al.*, 2005). The existence of nanowires could be shown for *Shewanella* as well and just recently the electron transport along these structures could be demonstrated (Gorby*, et al.*, 2006, El-Naggar*, et al.*, 2010). For *Shewanella,* protein mediated adhesion was also shown to happen between bacteria and iron crystals (Caccavo Jr, 1999, Das & Caccavo, 2000, Das & Caccavo, 2001, Caccavo & Das, 2002). Furthermore, it was observed that *Shewanella* reduces distinct areas on the iron mineral in the distance of the cell (Rosso*, et al.*, 2003).This leads to the second hypothesis that direct transport may not be needed. Another possibility to transfer electrons onto iron minerals would be the presence of **chelating agents** in the medium, which solubilize the mineral through complexation and make it more bioavailable (Dobbin*, et al.*, 1995, Dobbin*, et al.*, 1996). *In vitro* experiments with synthetic complexing agents like ethylendiamintetraacetic acid (EDTA) and nitrilotriacetic acid (NTA) could demonstrate an accelerative impact on microbial iron reduction(Lovley & Woodward, 1996, Lovley*, et al.*, 1996). Another suggested way to transfer electrons onto iron minerals would be the use of **electron shuttles** that are either produced by the organism itself or already exist in the environment. Electrons might leave the cell surface to be directly transferred onto the shuttle molecule, which thereby is reduced. The shuttle then diffuses to the surface of the iron oxide. Here the received electrons are abiotically transferred to the iron oxide. Through diffusion processes the electron shuttles are transported back to the surface of the bacterial cell and the cycle can start anew

(Lovley*, et al.*, 1996). Organic material, melanin, sulfur compounds or cytochromes are supposed to take on the action of such shuttles. Humic substances (or natural organic matter = NOM) are ubiquitious in the environment. The percentage of NOM within sediments and soils averages out at approximately 10% (Gaffney*, et al.*, 1996). As NOM is already available within the environment this might be an energetic and selective advantage. However, there are indications about bacteria producing

shuttle molecules themselves (Nevin & Lovley, 2002, Straub & Schink, 2003). The abiotic reduction of crystalline iron oxides mediated by humics or humic acid analogs could be shown both, in the lab and in the environment (Lovley*, et al.*, 1996, Lovley*, et al.*, 1998, Nevin & Lovley, 2000, Chen*, et al.*, 2003). Hence, it could be shown that hydroquinone groups within humic substances were responsible for the electron transfer whereas iron within humic substances is not (Dunnivant*, et al.*, 1992, Curtis & Reinhard, 1994, Lovley & Blunt-Harris, 1999). **Quinones,** due to the their function as reactive groups within humic acids, are on the one hand considered as assistants for the electron transfer reaction, on the other hand experiments with *Shewanella putrefaciens* suggested that quinones are produced and excreted by bacteria.
In addition to that, it could be shown that *Shewanella* mutants with mutations within the men (menaquinone) genes, unable to reduce ferric iron, could be functionally complemented by the addition of menaquinone to medium (Saffarini*, et al.*, 2002). For some *Shewanella* species it has been shown that the organisms are able to use extracellular melanin as electron shuttling agent. In this case, melanin can be provided with the medium or produced and excreted by the cells themselves (Turick*, et al.*, 2002, Turick*, et al.*, 2003). Concerning this, the uptake and release of electrons might be mediated through functional groups similar to quinones. Aside from this, **sulfur compounds**, like the redox couples cysteine/cystine and S-II+x/HS-, are discussed as electron shuttles as well. In (Doong & Schink, 2002) it could be demonstrated that *Geobacter sulfurreducens* is able to reduce cysteine to cystine by dint of electron transfer. Whereby cystine transfers the electrons abiotically to Fe(III) under reoxidation to cysteine which then is available to accept electrons again (Doong & Schink, 2002). The sulfurreducer *Sulfurospirillum delyianum* is not able to reduce Fe(III) directly. However, it is able to transfer electrons to sulfides thereby producing HS^-. Now HS^- being able to transfer the electrons abiotically to Fe(III), closing the cycle and starting anew (Straub & Schink, 2004). Moreover **cytochromes** have been discussed as possible electron carrier. In (Seeliger*, et al.*, 1998) the function of a small c-type cytochrome in *Geobacter sulfurreducens* has been described. But these results have been disproven in a following publication (Lloyd*, et al.*, 1999).

The function of **colloidal iron particles** as electron shuttles has been discovered just recently and is investigated at present (Jahn, 2006, Bosch*, et al.*, 2009, Bosch*, et al.*, 2010, Bosch*, et al.*, 2010). Experiments with the colloidal pigment Prussian Blue (iron(III)-hexacyanoferrate) in artificial groundwater gave evidence that the pigment can serve as electron shuttle (Jahn, 2006). Recent studies demonstrated iron oxide nanoparticles being able to enhance the process of microbial iron reduction to a great extent. It is hypothesized that the special properties of the nanoparticles like particle size, surface energy, peculiarities in the surface topography and the state of suspension are sustaining the process (Bosch*, et al.*, 2009, Bosch*, et al.*, 2010, Bosch*, et al.*, 2010).

Colloidal iron can be found in many marine and freshwater habitats, but its role as electron shuttle still has to be elucidated (Tipping*, et al.*, 1981, Degueldre*, et al.*, 1996, Degueldre*, et al.*, 1996, Wu*, et al.*, 2001).

5.6 Working hypothesis: HIGH- and LOW- energetic pathways

As already mentioned before, iron reducing microorganisms are versatile towards their electron acceptors which can have various redox potentials. Logically, with an adaptaple respiratory chain, the organisms should be able to adapt their biomass yields to the available amounts of energy.

Out of this , the question arose, what might be the physiological basis for such a metabolism. Therefore, the hypothesis of the existence of different energetic pathways was assumed: A high energy pathway which is active while iron chelates with high redox potentials are used. And a low energy pathway, active while iron chelates with lower redox potentials are available. The decision, which pathway is to be used, would be made by an energetic regulation.

The hypothesized respiratory scenario is depicted in figure 4.

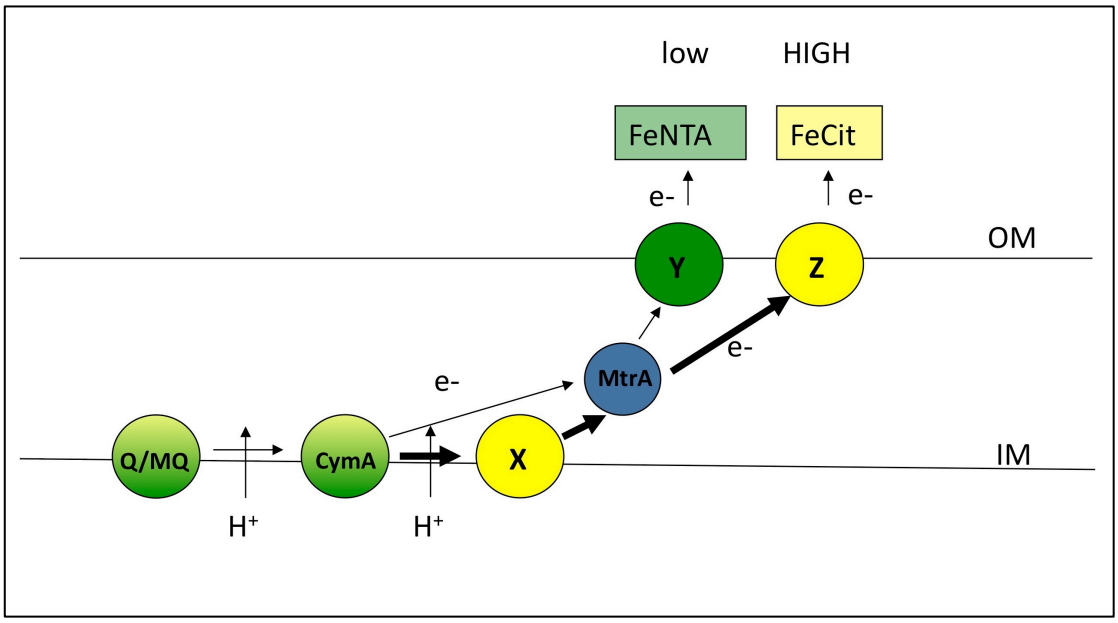

Figure 4: Hypothetical high and low energetic pathways in the respiratory chain of *Shewanella oneidensis* MR-1.

The organisms could achieve higher biomass yields on electron acceptors with higher redox potentials (e.g Fe-Cit) through additional proton pumping steps within the respiratory pathyway or by using different terminal reductases. The quinone/menaquinone pool would be used by both pathways with a branching point at CymA and the high energy pathway having an additional compound X that allowes an additional proton pumpig step. Both pathways would then share MtrA and evetually the same iron reductase. But the scenario with different reductases (Y and Z) as depicted here could be happening as well. Q = quinone pool, MQ = menaquinone pool, X = an unknown compound of the high engergy pathway, Y = Fe-NTA reductase, Z = Fe-cit reductase, OM = outer membrane, IM = inner membrane.

To assess this hypothesis experimentally, a random transposon mutagenesis was performed with the organism *Shewanella oneidensis* MR-1. The goal of the mutagenesis was to destroy an integral part of either one of the hypothetical pathways (figure 5) and to select on mutants with phenotypes characterized by the absence of the ability to grow on iron chelates with high- or low redox potentials. A detailed description about the methodical background of the mutagenesis is given in the section „Materials and Methods“ of this thesis.

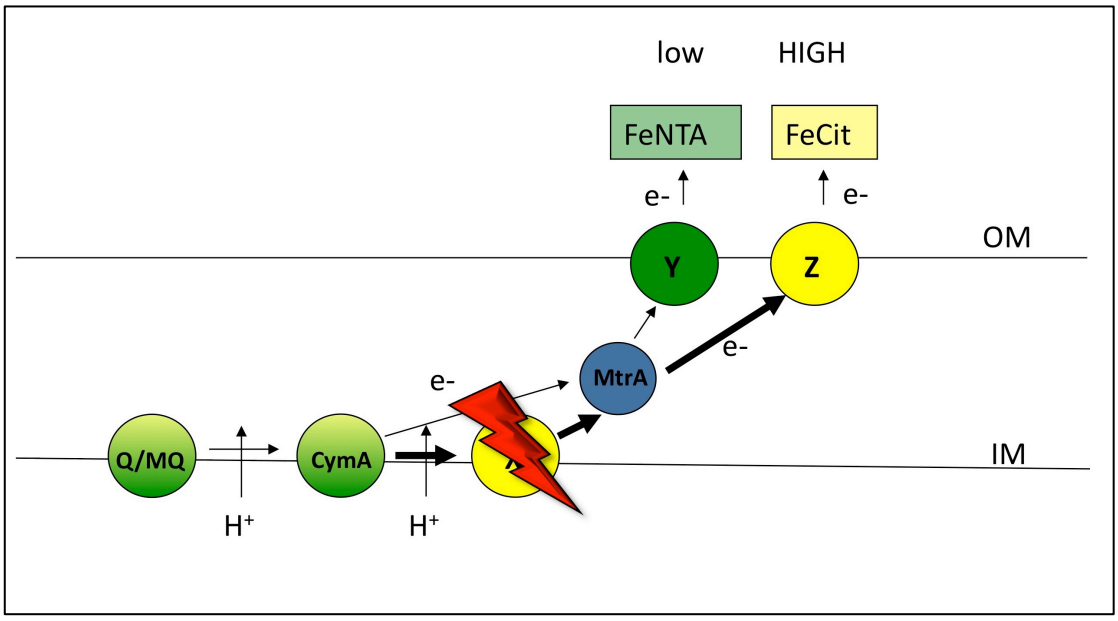

Figure 5: Possible scenario of a mutation in the unknown compound X of the high energy pathway. Through random mutagenesis and selection on either FeNTA or Fe-cit mutants defective in an unknown compound specific for one or the other pathway were constructed. In this specific scenario shown the compound X is destroyed. The high energy pathway therefore is affected. Fe-cit is still reduced following the low energy pathway with the cost of a smaller biomass yield. Q = quinone pool, MQ = menaquinone pool, X = an unknown compound of the high engergy pathway, Y = Fe-NTA reductase, Z = Fe-cit reductase, OM = outer membrane, IM = inner membrane.

5.7 Naphthalene and anaerobic naphthalene degradation

The chemical compound naphthalene ($C_{10}H_8$) is a polycyclic aromatic hydrocarbon (PAH) consisting of two fused benzene rings. The aromatic ring structure with its five conjugated double bonds makes it chemically inert and due to its apolarity it is poorly soluble in water (250 µmol·l^{-1} at 20 °C). The chemical characteristics leading to a high persistence in the environment are followed by a low bioavailability and degradability. According to the U.S Environmental Protection Agency (EPA) naphthalene has been classified as carcinogen commonly known to cause severe health defects e.g. haemolytic anemia, vomiting and diarrhea (EPA, 1998). Naphthalene is mainly produced in incomplete combustion processes. In example naphthalene is a byproduct in coal gas production (round about 10% of coal tar consist of naphthalene) but it can also derive from the combustion of crude oil,

wood or tobacco. Smaller amounts of naphthalene were even found in male white deer, termite nests, magnolia species and a fungus
(Azuma*, et al.*, 1996, Gassett*, et al.*, 1997, Daisy*, et al.*, 2002).
These days, naphthalene contaminated sites form a public health risk to our water resources. The remediation and decontamination of many former gas works sites, coking plants and refineries have become an important topic. As oxygen in contaminated groundwater is generally rather low than abundant, anaerobic microbial degradation processes make up an integral part of the natural attenuation of aromatic hydrocarbons (Meckenstock*, et al.*, 2004, Winderl*, et al.*, 2008, Bauer*, et al.*, 2009, Meckenstock*, et al.*, 2010). The function of microorganisms as PAH degraders in natural attenuation processes has been an object of study for some time.
However, only a few microorganisms that are able to degrade PAHs anaerobically have been identified until today (Zhang & Young, 1997, Meckenstock*, et al.*, 2000, Rockne*, et al.*, 2000, Eriksson*, et al.*, 2003) and only two sulfate-reducing pure cultures have been described so far (Galushko*, et al.*, 1999, Rockne*, et al.*, 2000, Musat*, et al.*, 2009). Organisms involved were shown to be members of the *Desulfobacteriaceae* within the delta-Proteobacteria (Galushko*, et al.*, 1999, Rockne*, et al.*, 2000, Musat*, et al.*, 2009). Although, ferric iron is an important and highly abundant electron acceptor in anoxic aquifers, PAH-degradation with ferric iron as terminal electron acceptor has only been described with sediment incubations (Anderson & Lovley, 1999). Yet, microorganisms belonging to the genera *Geobacter*, *Desulfitobacterium* and the family *Peptococcaceae* have been reported to be involved in the degradation of other aromatic compounds, like toluene or xylene, with iron as electron acceptor (Lovley*, et al.*, 1993, Christiansen & Ahring, 1996, Coates*, et al.*, 1996, Wiegel*, et al.*, 1999, Coates*, et al.*, 2001, Kunapuli*, et al.*, 2007, Abu Laban*, et al.*, 2009, Kunapuli*, et al.*, 2010). The initial biochemical activation reaction for naphthalene is still not conclusively elucidated, but two different pathways have been proposed (figure 6). One possible mechanism is a carboxylation in position 2 yielding 2-naphthoic acid.

The latter can be further activated by co-enzyme-A ligation and subsequent ring reduction by napthoyl-CoA reductase (Raulf, 2009) similar to the benzoyl-CoA reductase pathway (Boll*, et al.*, 2002). Experimental evidence for a carboxylation was given by (Zhang & Young, 1997, Meckenstock*, et al.*, 2000, Bergmann*, et al.*, 2011). A second possible pathway, a methylation of naphthalene to 2-methyl-naphthalene was proposed (Safinowski & Meckenstock, 2006). The produced 2-methyl-naphthalene was suggested to be degraded by the known methylnaphthalene degradation pathway, which comprises an addition of fumarate to the methyl group producing napthyl-2-methyl-succinic acid and a subsequent *beta*-oxidation to 2-naphthoic acid (Annweiler*, et al.*, 2002, Safinowski & Meckenstock, 2006). For the sulfate-reducing strains NaphS2, NaphS3, and NaphS6 a methylation was excluded (Musat*, et al.*, 2009).

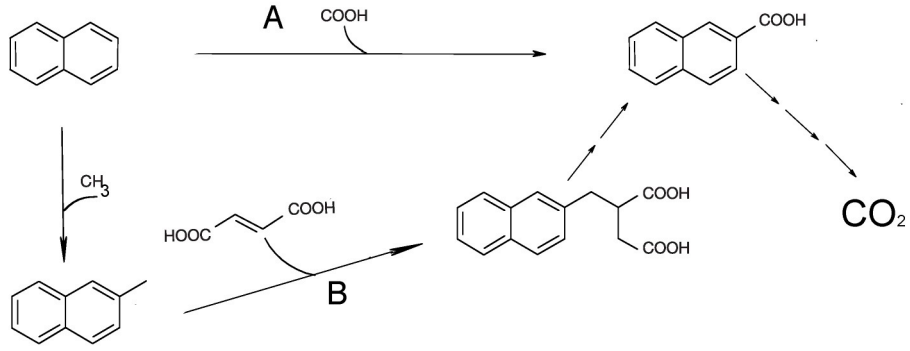

Figure 6: Two initial reaction mechanisms proposed for the anaerobic degradation of naphthalene
A) Carboxylation of naphthalene to 2-naphthoic acid. B) Methylation to 2-methylnaphthalene. An addition of fumarate would follow here and produce the compound 2-naphthylmethylsuccinic acid which is then further transformed into 2-naphthoic acid. In both cases 2-naphthoic acid is oxidized to CO_2.

Another proposed initial reaction mechanism was proposed in (Bedessem*, et al.*, 1997). Here the authors reported naphthalene degradation under sulfate reducing conditions with naphthalenol as potential metabolic intermediate. Therefore hydroxylation was suggested as first degradation step. The authors in (Zhang*, et al.*, 2000) suggested a hydroxylation to 2-napththoate and then stepwise reduced to 2-naphthoic acid.

6 Materials and methods

6.1 Strains and cultures:

6.1.1 Geobacter sulfurreducens

DSMZ 12127 (Caccavo Jr*, et al.*, 1994) was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

6.1.2 JG 247

Shewanella oneidensis-MR1 (wt), *S.oneidensis* MR-1 which was originally isolated from Oneida Lake in New York (Myers, Nealson, & Wisconsin Univ., 1988) was pprovided by Jeffrey A. Gralnick (University of Minnesota, USA).

6.1.3 JG 31:

Shewanella oneidensis-MR1, Amp sensitive strain of MR-1 constructed by Jeff Gralnick by deleting SO0837 using the plasmid pSMV3.

6.1.4 JG 187:

Escherichia coli WM3064, ΔdapA, kanamycine resistant, diaminopimelic acid auxotroph Plasmid pSMV3 (Saltikov & Newman, 2003)

6.1.5 JG 107:

Shewanella oneidensis-MR1, cymA$^-$, constructed by Jeff Gralnick using the transposon Tn*phoA* (Manoil & Beckwith, 1985)

6.1.6 JG 299:

Shewanella oneidensis-MR1, mtrB$^-$, Km, constructed by Jeff Gralnick using the transposon Tn*phoA* (Manoil & Beckwith, 1985)

6.1.7 JG 300:

Shewanella oneidensis-MR1, MQ^-, Km, constructed by Jeff Gralnick using the transposon Tn*phoA* (Manoil & Beckwith, 1985)

6.1.8 RK 45, RK 52, RK 100, RK 236, RK 457:

Shewanella oneidensis-MR1 mutants, this study, constructed with the assistance of Jeffrey Gralnick and his staff.

6.1.9 Culture N49

was enriched from a former manufactured gas plant site (Testfeld Süd) in Stuttgart, Germany, an aquifer shown to be contaminated with BTEX compounds. Sediment from the bottom of a monitoring well was taken as inoculum. For more information about the site see (Herfort*, et al.*, 1998, Bockelmann*, et al.*, 2001, Zamfirescu & Grathwohl, 2001).

6.2 Culture conditions & media

6.2.1 Stockcultures

Frozen stocks (*Shewanella* and *E.coli* strains) were made by mixing Glycerol (50%) and a grown LB (Bertani, 1951) (Caccavo Jr*, et al.*, 1994) overnight culture in a 3:1 ratio. Stocks were stored at -80°C in the dark.

6.2.2 Overnight Cultures

For overnight cultures (all *Shewanella* and *E.coli* strains) single colonies were picked and transferred into glass tubes containing 2 ml of liquid LB medium (Bertani, 1951) and shaken for 16 hours at 30°C (for *E.coli* 37 °C) at 225 rpm.

6.2.3 Solid Media (Agar):

All *Shewanella* and *E.coli* strains were streaked out from glycerol stocks onto LB (lysogeny broth agar plates (Bertani, 1951) and incubated at 30°C (37°C for *E. coli* strains) for until small colonies were visible. After incubation, agar plates were stored at 4°C in the fridge and discarded after a week and a new plate was made.
In the case of strains containing kanamycine (km) resistence, 50 $\mu g \cdot ml^{-1}$ of the antibiotic were added to the growth medium.
Ampicilline was added at a final concentration of 10 $\mu g \cdot ml^{-1}$.
Iron containing plates and media were complemented with the iron chelates Fe-citrate or Fe-NTA (Fe-nitrilotriaceticacid) at a final concentration of 50 mM.
As substrate for anaerobic growth of *Shewanella* strains lactate was added at a final concentration of 10 mM.
In case of DAP (diaminopimelic acid) usage the concentration was 60 mM, the substance was plated onto the respective plate.

6.2.3.1 SBM (Standard Minimal Medium):

Shewanella was cultivated anaerobically in standard basal media (SBM) which consisted of 0.225 $g \cdot l^{-1}$ K_2HPO_4, 0.225 $g \cdot l^{-1}$ KH_2PO_4, 0.46 $g \cdot l^{-1}$ NaCl, 0.225 $g \cdot l^{-1}$ $(NH_4)_2SO_4$, 0.117 g $MgSO_4x7H_2O$. HEPES buffer was added to a final concentration of 10 mM. The media were supplemented with trace elements solution SL10, selenite-tungsten and vitamins solutions (Widdel & Pfennig, 1982, Widdel*, et al.*, 1983) and buffered at pH 6.8 – 7.0..

In case of DAP (diaminopimelic acid) usage the concentration was 60 mM.

6.2.4 Widdel standard anaerobic media:

6.2.4.1 Geobacter, Shewanella:

For pre-cultivation and experiments, *Geobacter* was grown at 30°C in anaerobic medium consisting of (per liter) 1.0 g NaCl, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 0.5 g KCl, 0.15 g $CaCl_2 \cdot 2H_2O$, 10 µM Na_2SO_4, 30 mM $NaHCO_3$ buffer, and 10 mM acetate as electron donor. The media were supplemented with trace elements solution SL10, selenite-tungsten and vitamins solutions (Widdel & Pfennig, 1982, Widdel*, et al.*, 1983), and buffered at pH 6.8 – 7.0. For pre-cultivation, 50 mM ferric citrate was used as electron acceptor. Grown cultures served as inoculum, which was transferred into fresh media in a 1:10 ratio using a sterile syringe and standard anaerobic techniques.

6.2.4.2 Culture N49

was grown under standard anoxic conditions in the dark at 30 °C. The growth medium consisted of 1.0 $g \cdot liter^{-1}$ NaCl, 0.4 $g \cdot liter^{-1}$ $MgCl_2 \cdot 6H_2O$, 0.2 $g \cdot liter^{-1}$ KH_2PO_4, 0.25 $g \cdot liter^{-1}$ NH_4Cl , 0.5 $g \cdot liter^{-1}$ KCl, 0.15 $g \cdot liter^{-1}$ $CaCl_2 \cdot 2H_2O$, trace elements (SL10), vitamins as well as a selenite and tungsten solution (Widdel & Pfennig, 1982, Widdel*, et al.*, 1983). $FeCl_2$ was added to a final concentration of 3 mM as a reducing agent and to scavenge produced oxygen. The medium was buffered with 30 mM $NaHCO_3$ (pH 7.0). Bottles were flushed with a mixture of N_2/CO_2 (80/20), and closed with butyl rubber stoppers. The adsorber resin Amberlite® XAD7HP (Sigma Aldrich, Germany) was added at a final concentration of 6 $g \cdot liter^{-1}$ to ensure a constant and

low concentration of naphthalene within the medium. Readily prepared medium bottles were shaken for two weeks in the dark to let the naphthalene adsorb to the resin before inoculation. If not otherwise mentioned, ferrihydrite was used as an electron acceptor and added to a final concentration of 50 mM (Lovley & Phillips, 1986). For a better accessibility of the electron acceptor 243 µmol/L 2.6-anthraquinone-disulfonate were added. All substrates except methanol (10 mM) were added in 2 mM concentrations. Alternative electron acceptors were added in the according stoichiometric ratios. Elemental sulfur was added as powder.
Grown cultures served as inoculum, which was transferred into fresh media in a 1:10 ratio using a sterile syringe and standard anaerobic techniques.
For pasteurization, 10 ml of a grown culture was heated to 80 °C for 10 min in a closed reagent vial and used as inoculum.

6.2.5 Synthesis of Fe^{3+} complexes

A 0.5 M Fe-citrate solution was prepared by dissolving Fe-citrate over night and adjusting it to pH 7 with 10 M NaOH. The solution was filtered with paper mesh to remove residual iron oxide precipitates. For Fe-NTA, a 1 M nitrilotriacetic acid solution was added to a 1 M $FeCl_3 \cdot 6H_2O$ solution in a 1:2 ratio. Then, the pH was adjusted to 7 with 2 M Na_2CO_3. The mixture was centrifuged at 6000 g for 1 h and then filtered with paper mesh filter. Fe-ethylenediaminetetraacetic acid (EDTA), Fe-methyliminodiacetic acid (MIDA), and Fe-Diethylenetriaminepentaacetic acid (DTPA) syntheses were performed similarly but in lower concentrations of 5-10 mM. Iron was determined using a 1 M HCl extraction followed by the ferrozine assay (Stookey, 1970) adapted to the individual chelating agent.
Cell suspension experiments with *Shewanella* were performed as described in (Coursolle*, et al.*, 2010). Final concentration of lactate was 20 mM and for iron chelates 5 mM. Microplates were incubated in a glove bag with an atmosphere of 1 % hydrogen and 99 % nitrogen.

6.3 Experimental setups and analytical methods

6.3.1 Growth- and cell suspension experiments

In conventional batch long-term growth experiments, a small aliquot of a grown *G. sulfurreducens* culture was taken to inoculate fresh medium bottles in a 1:10 dilution.

For growth experiments with *S. oneidensis*, LB grown cells were transferred into SBM containing lactate (50mM) and fumarate (100mM) to adapt the cells to anaerobic conditions. After 16 hours of incubation at 30°C, cells were harvested and washed three times in an anoxic glove box.

These cultures were monitored for biomass increase, and ferrous iron increase as a measure for iron reduction. By this method, biomass formation could be related to the moles of respired electrons. Additionally, the electron transfer over time could be monitored as Fe^{2+} increase and was calculated as the growth rate μ_{max}.

For cell suspension experiments with *Geobacter*, 3L of grown culture were harvested by centrifugation and resuspended in fresh medium. This concentrated biomass was then introduced to the reaction bottles containing 5mM of the respective ferric compound.

Cell suspension experiments with *Shewanella oneidensis* MR-1 *and Shewanella* mutants were performed on plates in a glove bag with an atmosphere of 5 % hydrogen, 20 % carbon dioxide and 75 % nitrogen as described in (Coursolle & Gralnick, 2010).

Using these methods, a maximum reaction rate (v_{max}) was observed over the first 4 hours.

The final concentration of added Fe-chelate was 5mM in cell suspension experiments and 50mM in long-term growth experiments for both organisms.

6.3.2 Detection of bacterial biomass

Cell numbers were determined with an A-LSRII flow cytometer (Becton Dickinson Bioscience, Franklin Lakes). Nucleic acid stain 1x SYBR® Green I (Molecular Probes, Eugene) was diluted 1:30,000 in sterile filtered phosphate buffered saline (PBS) (140 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4) and was used to stain bacterial cells. Reference beads were used as internal standard (TrueCount™, Becton Dickinson Bioscience, Franklin Lakes). Cells were fixed in 2.4 % paraformaldehyde, stained with SYBR® Green for 10 min in the dark and counted at a wavelength of 510 nm.

6.3.3 Determination of dry mass:

Cultures of Fe-citrate and Fe-NTA grown cells were collected on glass microfiber filters (Whatman, UK) by vacuum filtration. The filters were dried at 60°C for a minimum of 24 hours and weighed before and after filtration to determine the dry weight of the filtered cells. The resulting mass increase was transferred to carbon content according to (Liu*, et al.*, 2007) and divided by the Fe^{2+} increase to determine the growth yield in terms of gram biomass carbon formed per mol of transferred electrons.

6.3.4 Fe(II)-determination

Iron concentrations were determined with the Ferrozine-assay after (Stookey, 1970). At each sample point 0.1 ml were taken from the culture bottle, mixed with 0.9 ml 1M HCl. Samples were shaken for 24 hours at 25,000 x g. Then, 0.1 ml aliquots were diluted in 1:10 ratio with ferrozine and incubated for five minutes. The absorbance at 560 nm was determined in a Wallac 1420 Victor3 micro-plate reader (Perkin Elmer, Massachusatts).

6.3.5 Redox potentials

Standard redox potentials were taken from (Pierre*, et al.*, 2002). For Fe-citrate the redox potential was measured by a procedure adapted from (Dhungana*, et al.*, 2003). All potentials reported here are given versus NHE (normal hydrogen electrode). The redox-potentials of the net reactions were calculated according to the following equation: $\triangle G^{\theta'} = -n\ F\ (E^{\theta'}_{acc} - E^{\theta'}_{don})$.

6.4 Molecular methods:

6.4.1 Mutagenesis

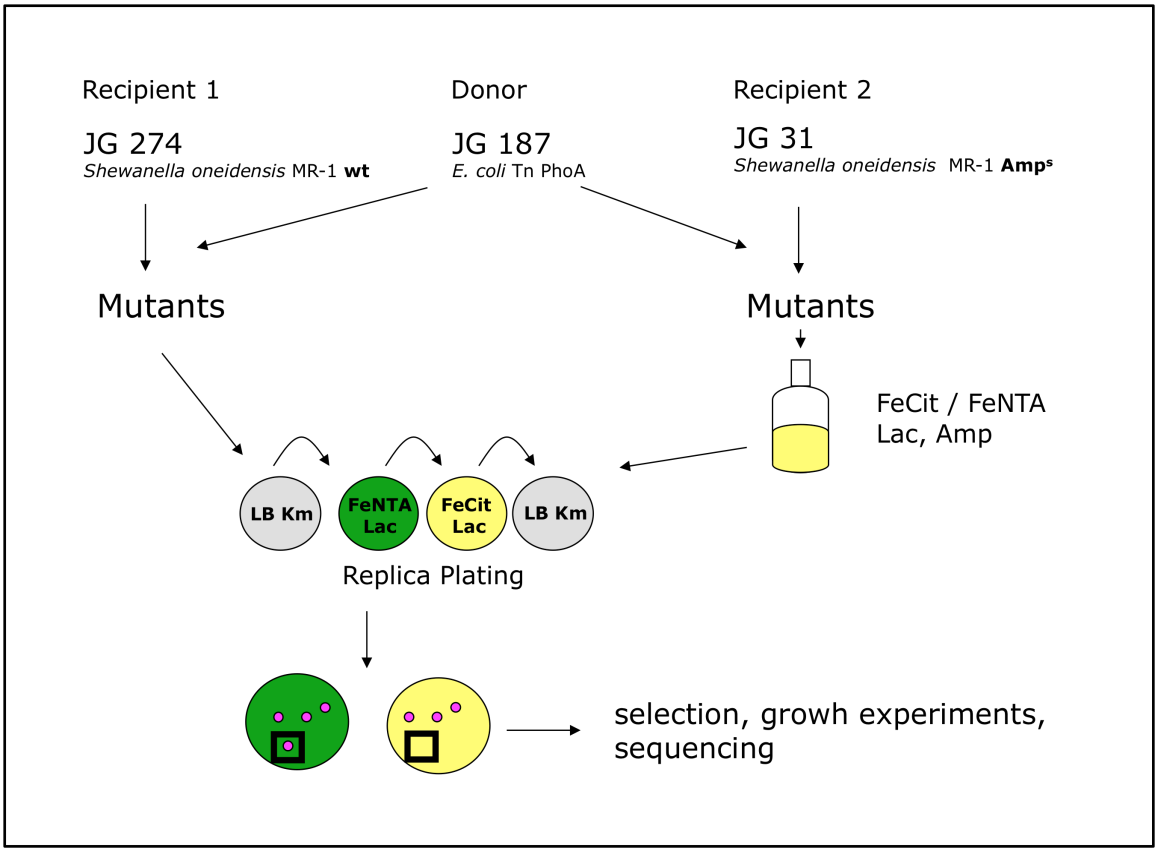

Figure 7: Random transposon mutagenesis and additional screen

Two attempts were made to gain mutants. The first is depicted on the left and included the mating of the wildtype Shewanella oneidensis MR-1 strain with the donor strain that inherited the transposon (*E.coli* strain JG187). The second attempt was the mating of the donor strain with an ampicilline sensitive *Shewanella oneidensis* MR-1 strain (JG 31). Mutants underwent a specific ampicilline treatment after the mating in which those defective in reducing Fe-cit or FeNTA would not grow on the respective compound and therefore survive the antibiotic treatment. The following screen was the same for both mating attempts in which mutants were screened on Fe-cit and FeNTA using the replica plating method. Mutants that did not grow or seemed to grow leaky on either iron chelate were selected for further investigation.

6.4.1.1 Transposon mutagenesis

A random transposon mutagenesis with was performed with the attempt to generate mutants defective in a compound specific for a hypothetical high or low energy respiratory pathway.

Two different attempts were made to generate *Shewanella oneidensis* MR-1 mutants. The first attempt was to mate *Shewanella oneidensis* MR-1 wt strain JG274 with the strain JG187 (*E.coli* strain WM3064 carrying the TnPhoA transposon (Manoil & Beckwith, 1985)) coupled to a kanamycine (Km) resistence. The mating of the strains followed a protocol designed by Jeff Gralnick´s lab (not published), in which cells undergo a heatshock, exconjugants are selected on LB plates containing Km. Resulting mutants were transferred onto selective containing agar plates iron-chelate using the replica plating technique. All plates contained the antibiotic kanamycine to sustain the mutation within the cells. Mutants that didn't form colonies on either Fe-NTA or on Fe-citrate or both during the replica plating process were pooled for further investigation (figure 7).

The second attempt to generate mutants was a mating of the strains JG187 with the strain JG31 (*Shewanella oneidensis* MR-1, amp sensitive). The mating protocol applied was the same as mentioned before. Exconjugants were transferred to serum bottles containing either anoxic LB amended with lactate and Fe-cit or Lactate and Fe-NTA. Both serum bottles contained the antibiotic ampicilline (Amp), to select only on not growing cells that survived the treatment. Different ratios of amp concentration and inoculum volumes were tested. The Amp-treated bottles were incubated for a week at 30°C. A ratio of 600 $\mu g \cdot ml^{-1}$ and 2 µl inoculum per 1 ml of medium led to a satisfactory amount of surviving cells after the treatment. Then mutants underwent the same replica plating technique as mentioned before and mutants that seemed to have a defect in iron reduction were pooled (figure 7). Selected mutants were grown over night in a multi well plate on LB + Km anaerobically. Using a special comb, equal amounts of this over night grown cells were transferred into micro plates containing a minimal medium containing Km and lactate as well as iron(III)-citrate or iron(III)-NTA and were incubated at 30°C in a glove bag (Coy, Michigan, USA). The color change of the medium due to iron reduction was observed and recorded by a digital camera. Afterwards the mutants

that still seemed to be defective in reducing either one iron chelate or both were further investigated.

6.4.2 DNA extraction

6.4.2.1 Shewanella:

For DNA extraction overnight cultures of all putative *Shewanella oneidensis* MR-1 mutant strains were made and DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega GmbH, Madison, Wisconsin, USA) by following the instructions of the manufacturer.

6.4.2.2 Culture N49:

Culture bottles (appr. 45 ml) were harvested by centrifugation for 20 min at 4.000 x g and 4 °C (Thermo Megafuge 1.0R, Thermo Fisher Scientific, Waltham, USA). The cell pellets also contained the iron phase and were used for DNA extraction using the FastDNA spin kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer´s instructions.

6.4.3 Sequencing of transposon insertion sites

All PCR amplifications were performed in a mastercycler ep gradient (Eppendorf, Hamburg, Germany) using the standard thermal profile of 5 min initial denaturation (94°C), 25–30 cycles of amplification (30 s/94°C, 30 s/52°C, 60 s/70°C) and 5 min of terminal extension (70°C).

The reaction mix consisted of: 5 µl 10x PCR buffer, 10 µg BSA, 5 mM DNTPs, 5 µM R1 primer, 1.25 U Takara DNA polymerase, 1-20 ng template DNA and H_2O to a final reaction volume of 50 µl.

To generate longer fragments in PCR reactions the Takara polymerase (Clontech Laboratories, Madison, Wisconsin, USA) was used. To exactly map the site of each Tn*phoA* insertion, the DNA sequence adjacent to the inserted transposon in each mutant fragment clone) was read directly from the transposon by using a Tn*phoA* specific primer (Tn*phoA*-R1 -CGCCGAAGAGAACACAGATT, Jeff Gralnick, unpublished

results). PCR amplicons were purified using “MinElute PCR Purification Kit” (Qiagen, Hilden, Germany).

Before sequencing the amplicons were desalted using DyeEx spin columns (Quiagen, Hilden, Germany). The analysis was done on an ABI 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA).

The reaction mix of the sequencing reaction contained: 1 µl Big Dye Premix, 1 µl Big Dye buffer, 5 µM R2 primer, template DNA approx. 100 ng, 1 µl H_2O.

DNA sequencing was performed by the dideoxynucleotide Sanger method (Sanger*, et al.*, 1977) by using *Taq* DNA polymerase (Fermentas, St. Leon-Rot, Deutschland) and a second primer that anneals further into the transposon (Tn*phoA* R2 – TTCCGTTCAGGACGCTACTT, Jeff Gralnick, unpublished results).

The generated sequences were checked and assembled using the DNASTAR software (Lasergene, Madison, Wisconsin, USA) and afterwards submitted to a BLAST search (nucleotide BLAST, National Center for Biotechnology Information (NCBI), Bethesda, Maryland, USA) to identify the correct site of the transposon insertion.

6.4.4 16S PCR amplification

All PCR amplifications were performed in a mastercycler ep gradient (Eppendorf, Hamburg) using the standard thermal profile (5 min initial denaturation (94 °C), 25–30 cycles of amplification (30 s / 94 °C, 30 s / 52 °C, 60 s / 70 °C) and 5 min of terminal extension (70 °C)). All standard PCR reaction mixes were prepared as described previously (Winderl*, et al.*, 2007). PCR amplicons were purified using “MinElute PCR Purification Kit” (Qiagen, Hilden).

6.4.5 Analysis of the microbial community

PCR for terminal restriction fragment length polymorphism (T-RFLP) was performed with 16S rRNA primer pairs and Ba27f(-FAM) / 907r (Weisburg*, et al.*, 1991, Muyzer*, et al.*, 1995). Amplicons were digested by using restriction enzyme MspI (Promega GmbH, Mannheim). Digested amplicons were desalted using DyeEx Spin Kit columns (Quiagen, Hilden) and size separated on an ABI 3730 DNA analyzer as described in (Lueders*, et al.*, 2006)

6.4.6 Quantitative PCR

Bacterial 16S rRNA genes were amplified using a MX3000P qPCR cycler (Stratagene, La Jolla) following the protocol described in (Kunapuli*, et al.*, 2007).

6.4.7 Cloning and sequencing

For cloning and sequencing of 16S rRNA genes, purified DNA was amplified with primer pair Ba27f / Ba1492r (Weisburg*, et al.*, 1991). Amplicons were then cloned using the pGEM-T vector system, sequenced on an ABI 3730 sequencer and the resulting data were assembled to contigs as described previously (Winderl*, et al.*, 2007). The BlastN search tool (http://ncbi.nlm.nih.gov/ BLAST) was used to identify the resulting sequences and for further phylogenetic analyses the respective sequences were fed into the ARB 16S rRNA database (Ludwig*, et al.*, 2004). Using the methods implemented in ARB (Distance matrix, maximum parsimony, maximum likelihood based phylogenetic treeing) a phylogenetic dendrogram was constructed to visualize phylogenetic relations.

6.5 Mass spectrometric methods

6.5.1 Detection of metabolites

For metabolite analysis 1ml culture were taken and centrifuged at 25,000 x g for 10 min. 150 µl of the resulting supernatant were transferred into small GC glass vials with a 200 µl insert. Metabolite analysis was carried out by LC/MS/MS on an Agilent 1200 series HPLC system coupled to an Applied Biosystems Q-Trap mass spectrometer equipped with a TurboSpray ionization source. Samples of 50 µl were injected to a LiChroCART® 125-2 Purospher® STAR RP-18e (5 µm) HPLC cartridge (Merck, Darmstadt). The column oven was set to 35 °C. A gradient of 25 % to 40 % Acetonitrile in 0.1 % acetic acid over 25 min at a flow rate of 0.3 ml min^{-1} was applied. The sample was infused into the mass spectrometer via multiple reaction monitoring (MRM) in negative mode and an entrance potential of -8 V. The declustering potential was set to -63 V and the collision energy was adjusted to -35 V.

6.5.2 Determination of CO_2 production from ^{13}C-naphthalene

To determine the amount of naphthalene mineralization during growth of N49, the culture bottles were incubated with 2 mM ^{13}C-naphthalene labelled at the C1-position. Experiments were performed in triplicates. At each time point, 2.5 ml of the liquid were withdrawn to measure biomass, Fe(II), and metabolites. The total amount of $^{13}CO_2$, as product of ^{13}C-naphthalene degradation, was determined measuring the $^{13}CO_2/^{12}CO_2$ isotope ratio in the headspace of the cultures. As the CO_2 in the headspace and the liquid phase are in isotopic equilibrium the headspace data directly reflect the isotopic values of CO_2 in the liquid sample.

At the start and end of the experiment, three 100 µl headspace samples were taken from each culture bottle and were diluted in glass vials sealed with butyl rubber stoppers and filled with 10 ml helium. 800 µl were taken from these vials with a gas tight syringe and injected in a GC-IRMS (Finnegan MAT 253, Thermo Fisher Scientific, Waltham) consisting of a TRACE GC Ultra gas chromatograph (GC) with a split/splitless injector coupled to a Finnegan MAT 253 isotope ratio mass spectrometer (IRMS) via Finnegan GC combustion III interface. The GC was equipped with a RT-Q-Plot™ column (30 m length, 0,32 mm internal diameter, 300 °C max. temperature). The temperature was held isothermically at 230 °C and the column flow was 1.4 ml min^{-1}. Helium (grade 5.0) served as carrier gas with a constant flow rate of 1.4 ml min^{-1}. The initial oven temperature was set at 50 °C and ramped at a rate of 20 °C min^{-1} to 230 °C and held for 5 min. The $^{13}CO_2$ concentration was calculated from the carbon isotope compositions of CO_2 expressed in the δ-notation (‰) relative to the Vienna Pee Dee Belemnite standard (V-PDB):

Equation 2

$$\delta^{13}C[‰]=[(^{13}C/^{12}C_{\ sample}-\ ^{13}C/^{12}C_{\ V\text{-}PDB\ standard})/(^{13}C/^{12}C_{\ V\text{-}PDB\ standard})]x\ 1000$$

Equation 3

$$^{13}CO_2/^{12}CO_{2\ sample}=[1+\delta^{13}CO_2/1000]\ x\ ^{13}CO_2/^{12}CO_{2\ V\text{-}PDB\ standard}]$$

Equation 4

$$^{13}C_{end}[mmol]/^{12}C_{end}[mmol]/=[^{13}C_{t0}[mmol]+(\Delta^{13}C\ x\ 1)]/\ [^{12}C_{t0}[mmol]+(\Delta^{13}C\ x\ 9)]$$

The amount of naphthalene consumed was taken as equal to the accumulated $^{13}CO_2$ at the end of the experiment, because only one carbon atom of naphthalene had been labelled.

7 Results

7.1.1 Biomass Formation of S. oneidensis & G. sulfurreducens on different iron chelates with various redox potentials

G. sulfurreducens and *S. oneidensis* MR-1 readily grew with the soluble ferric complexes of Fe-citrate and Fe-NTA as terminal electron acceptors, which were completely and rapidly reduced. *G. sulfurreducens* also grew on Fe-EDTA, and *S. oneidensis* on Fe-MIDA, but not vice versa. No growth, but a slight initial ferrous iron production in cell suspension experiments could be detected with Fe-DTPA as electron acceptor ($E_{acc}^{\theta'}$ = +90 mV) for *G. sulfurreducens* but not for *S. oneidensis* MR-1. The reasons for the reluctant or absent metabolic activity for latter iron complexes remained unknown.

For both microbial strains biomass formation and electron turnover were strongly correlated for a range of ferric compounds with standard redox potentials $E_{acc}^{\theta'}$ from + 100 mV to + 450 mV (Fe-EDTA, Fe-NTA and Fe-citrate, figure 8).

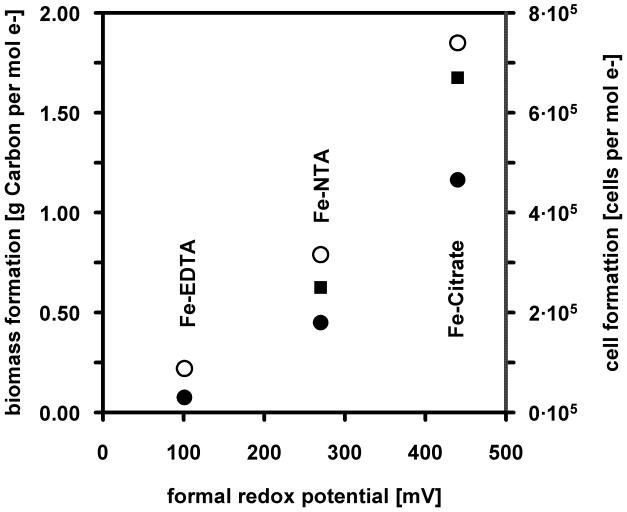

Figure 8: Adaptation of *Geobacter* and *Shewanella* biomass yields

Net biomass carbon (○) and cell production (●) per electron respired of *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 (cell production, ■) upon growth on ferric compounds with different redox potentials.

iron complex	redox potential [mV]	biomass yield [C-mol/C-mol]	expected growth rate[1] [mg C L^{-1} h^{-1}]	measured growth rate [mg C L^{-1} h^{-1}]
Fe-citrate	+440	0.61	5.81	5.93
Fe-NTA	+270	0.27	2.57	2.53
Ratio Fe-citrate:Fe-NTA		2.3		2.3

Table 1: Correlation of growth rate and biomass yield in *Geobacter sulfurreducens* growing with acetate on Fe-citrate and Fe-NTA as terminal electron acceptor.

[1]Expected growth rate with Fe-citrate is measured growth rate from Fe-NTA multiplied by 2.3 and vice versa.

7.1.2 Kinetics of Microbial Iron Reduction and Microbial Growth of S. oneidensis and G. sulfurreducens on different iron chelates

In dense cell suspensions of *G. sulfurreducens*, a constant reduction rate of ferric compounds over the first 3 h after inoculation was detected. Under these non-growth conditions, the Fe(III) respiration rates probably equaled the maximum available respiratory electron transfer capacity, or maximum reaction rate v_{max}. As depicted in figure 9, v_{max} from the dense cell suspension and also from the growth rate μ_{max} (from long-term growth experiments) increased with more positive redox potentials of the final electron acceptors. While cells with Fe-citrate as electron acceptor showed a very rapid turnover $v_{max,}$ ferric iron reduction was clearly slower using Fe-NTA and Fe-DTPA complexes (lower redox potentials). With *S. oneidensis* MR-1, the reduction rates v_{max} were higher for Fe-NTA than for Fe-citrate. The reduced amounts of the applied electron acceptors were determined after 320 h of growth. Within this time span, all applied electron acceptors were microbially reduced to more than > 90% in relation to the initial Fe(III) concentration.

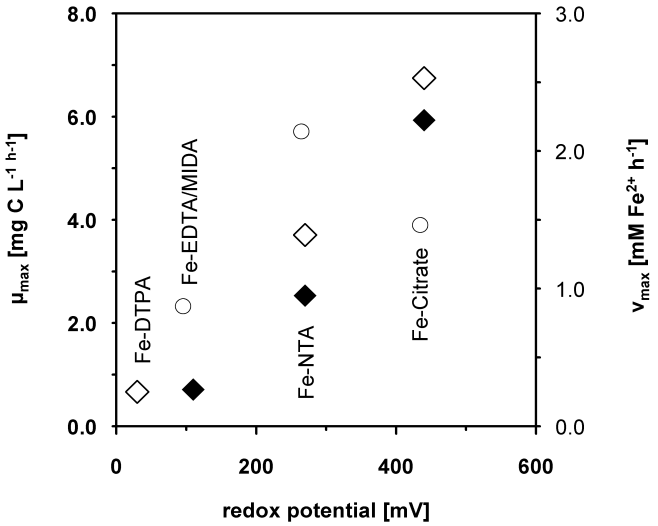

Figure 9: Kinetic parameters of iron reduction by *G. sulfurreducens* and *S. oneidensis* with ferric iron complexes of different redox potentials.

Maximum iron reduction rates in dense cell suspensions v_{max} were determined for Geobacter ◇

7.1.3 Random transposon mutagenesis

All screened mutants that could not reduce iron or not properly reduce iron compounds anymore were picked for sequence analysis of the transposon insertion site within the genome to find out which gene had been destroyed and led to the respective phenotype. Furthermore, sequence analysis was used to find out if the destroyed gene was already known to code for a compound of the iron specific respiratory pathway. Finally, five different mutants were identified to have a mutation in a gene coding for either a compound of the respiratory pathway itself or for a compound needed in biosynthesis or secretion of a respiratory part (table 2).

mutant	gene	gene function	effect /phenotype
RK 45	gspD	general secretion protein	Terminal iron reductase not properly incorporated in outer membrane du to a defect in the secretion protein
RK 52	menC	o-succinylbenzoate-CoA synthase	MenC is part of the menaquinone synthesis – therefore the mutant has a men^- phenotype
RK 100	cymA	tetraheme cytochrome C	As CymA is an integral part of the respiratory chain the mutant has a $cymA^-$ phenotype
RK 236	ispA	geranyltransferase	Geranyltransferase is an enzyme needed in the synthesis process of ubiquinone
RK 457	gspL	general secretion protein	terminal iron reductase not properly incorporated in outer membrane du to a defect in the secretion protein

Table 2: Constructed *Shewanella oneidensis* MR-1 mutants.

The table gives the destroyed gene and its function, as well as a description of the resulting phenotype.

7.1.4 Iron reduction behaviour of Shewanella mutant strains

To elucidate whether the mutant strains of *Shewanella oneidensis* MR-1 showed differences in the reduction of Fe-citrate and Fe-NTA the initial reduction rates in dense cell suspensions were studied over a time period of 24 hours.

According to this, three groups of mutants could be identified due to their behavior of reducing both iron chelates. The first group is built by the ispA mutant RK 236 that reduces both Fe-chelates almost as good as the wild type JG 274. The second group is built by the mutants RK 45 ($gspD^-$) and RK 457 ($gspL^-$), two mutants with defects in the type II secretion system for outer membrane proteins. This mutant type known to be defective in iron reduction, most likely due to the inappropriate secretion or location of the terminal iron reductase (DiChristina*, et al.*, 2002). These mutants showed mean reduction rates. A third group distinguishable was characterized by the reduction of poor amounts of iron(III). Members of this group were the mutants RK 52 ($menC^-$), RK 100 ($cymA^-$) and JG 299 ($mtrB^-$). The differences between the reduction rates within the groups were not significant (figure 10, 11, table 3).

Another characteristic of mutants and the wildtype strain that was readable from the reduction experiments was that the Fe-NTA reduction kinetics were generally about 50 % faster than the reduction kinetics of Fe-citrate (figure 10, 11, table 3).

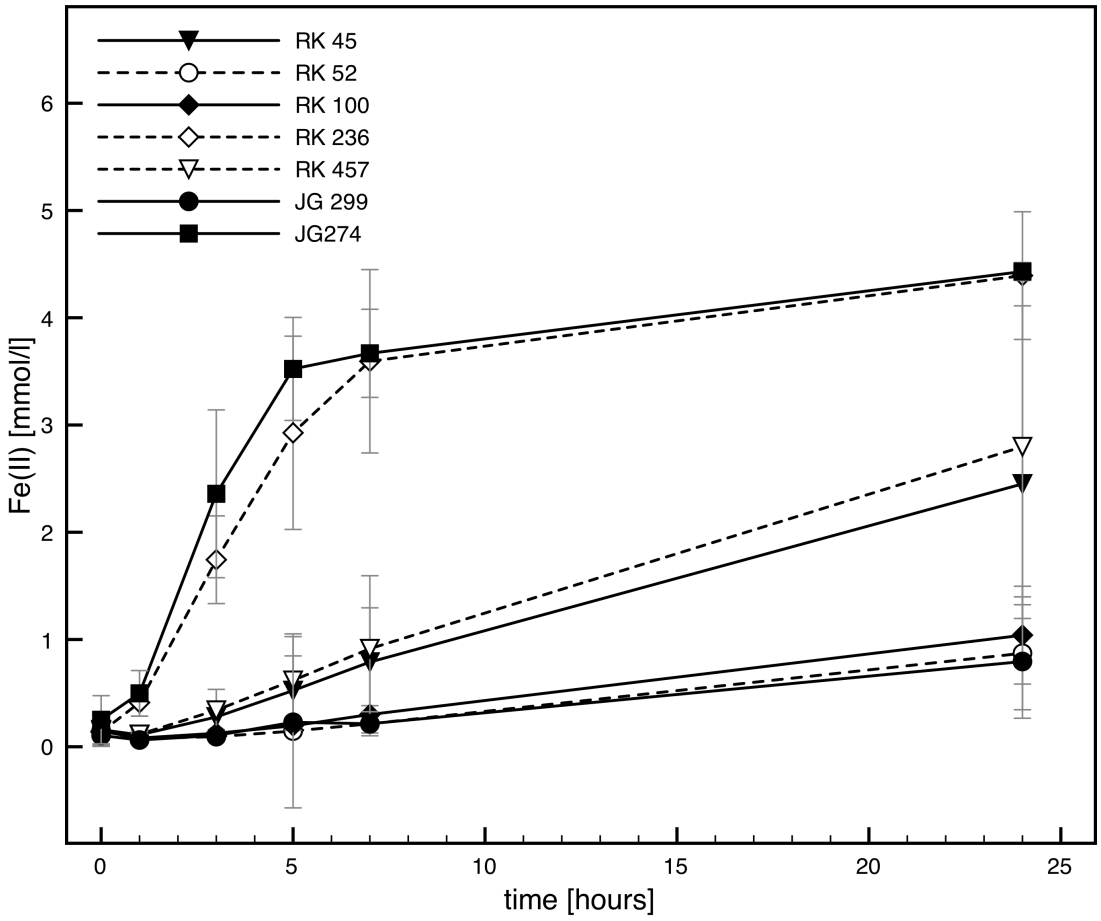

Figure 10: Fe-citrate reduction rates of *Shewanella oneidensis* strains in dense cell suspensions.

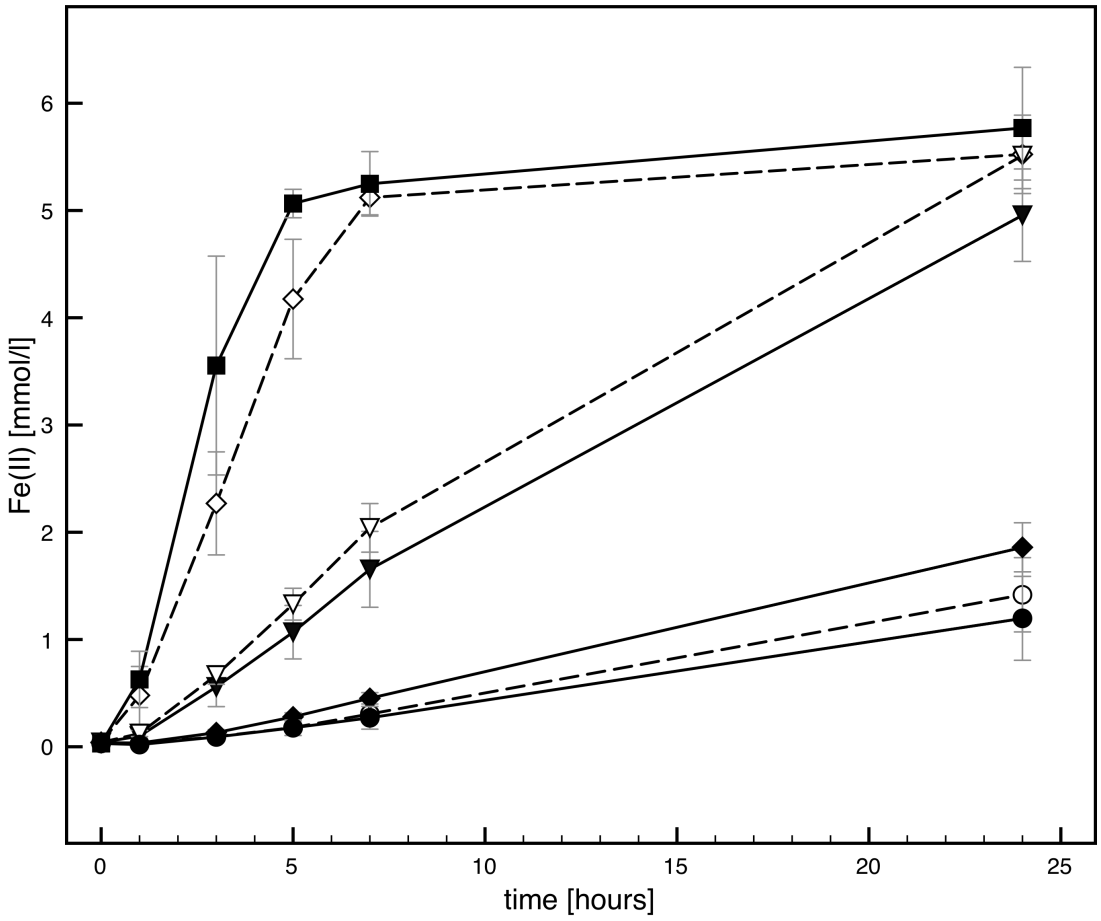

Figure 11: Fe-NTA reduction rates of *Shewanella oneidensis* strains in dense cell suspensions.

For explanation of symbols see legend in figure 10.

Mutant	Fe-cit (mmol·l^{-1}·h^{-1})	Fe-NTA (mmol·l^{-1}·h^{-1})	[1]%
RK 45	0.120	0.255	47.06
RK 52	0.030	0.045	66.67
RK 100	0.035	0.075	46.67
RK 236	0.665	0.895	74.30
RK 457	0.140	0.330	42.42
JG 299	0.020	0.045	44.44
JG 274	0.930	1.460	63.70

Table 3: **Reduction rates of tested *Shewanella* mutant strains on electron acceptors Fe-NTA and Fe-cit.**

[1]The last column indicates the percentage of which the iron reduction rates on Fe-NTA were higher than on Fe-cit

7.1.5 Growth properties of the enrichment culture N49

Repeated transfers of the naphthalene-degrading, iron-reducing enrichment culture N49 into a freshwater minimal medium led to a stable and sediment free culture. Due to the adsorber resin in the cultures and the limited solubility of naphthalene, which made a direct quantification of naphthalene impossible, degradation of the substrate was monitored by the release of $^{13}CO_2$ from ^{13}C-labelled naphthalene via GC-IRMS. The culture oxidized 7.5 ± 0.3 µmol naphthalene, as determined by the production of 75 ± 30 µmol CO_2. 404 ± 92 µmol ferrous iron were produced which accounts for an electron recovery of 89 % according to the stoichiometric equation for the complete degradation of naphthalene to CO_2 (see equation 5).

Equation 5

$C_{10}H_8 + 48\ Fe^{(III)}(OH)_3 \rightarrow + 10\ HCO_3^- + 48\ Fe^{2+} + 28\ H_2O + 86\ OH^-$

No decrease of naphthalene or increase of Fe(II) could be detected in the autoclaved controls as well as the controls without electron acceptor. The turnover of naphthalene was coupled to growth as depicted by the increasing cell numbers (figure 12). Culture N49 did also grow after pasteurization indicating that the organisms responsible for naphthalene degradation produce spores. Spores were also observed in microscopic analysis although it could not be proven that the respective organisms are identical with the naphthalene degraders, because the culture was not pure after pasteurization (data not shown).

7.1.6 Metabolites produced by culture N49 during growth on naphthalene

LC/MS/MS (Liquid Chromatography / Tandem Mass Spectrometry) analysis of culture supernatants revealed two different metabolites with the corresponding masses of 144/116 (naphthol) and 172/128 (2-naphthoic acid). Naphthol was found in all experimental setups, even in sterile control bottles and the concentrations did not correlate with growth (data not shown). In contrast, 2-naphthoic acid (2-NA) was found only in bottles that showed microbial growth. Concomitant with increasing biomass, 2-naphthoic acid concentrations increased and decreased in the stationary phase indicatig consumption (figure 13).

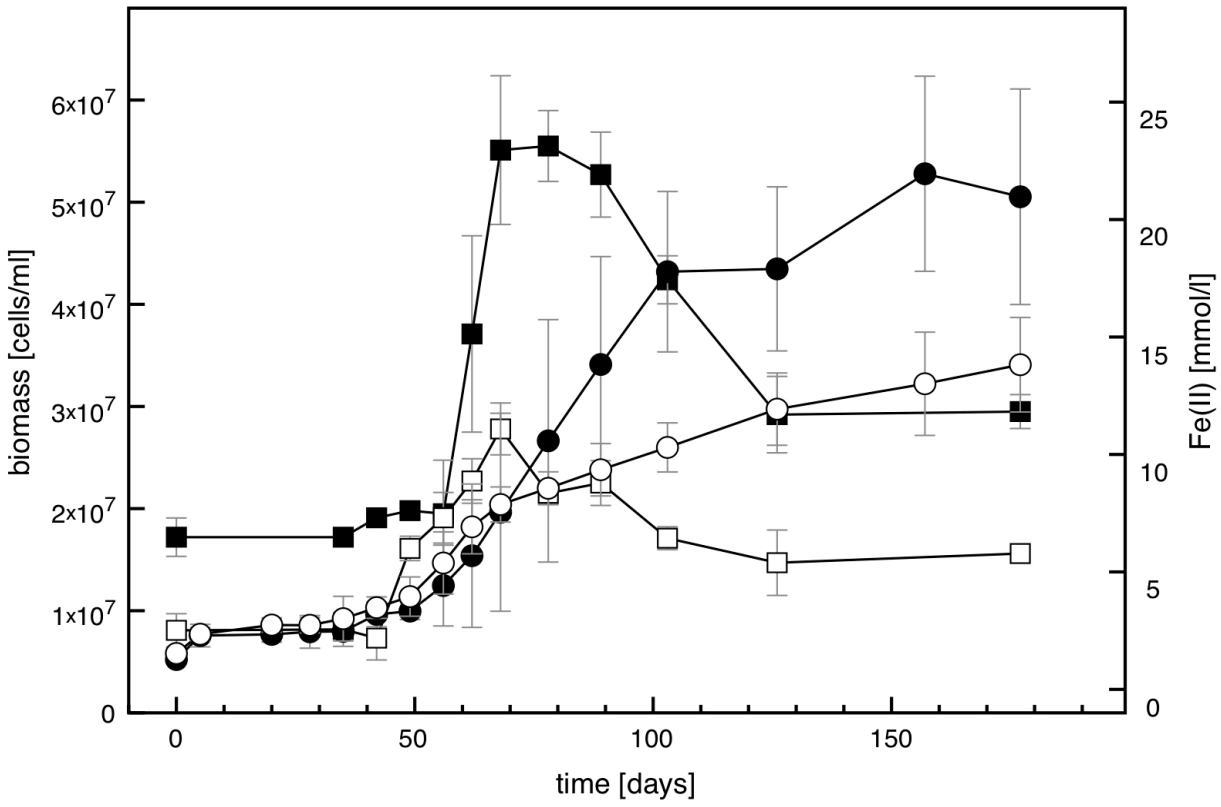

Figure 12: Growth of culture N49 with ^{13}C-naphthalene as electron and carbon source and ferrihydrite as electron acceptor.

Full symbols: untreated cultures, open symbols: pasteurized cultures. Squares symbolize biomass (cells·ml^{-1}) and circles Fe(II)-concentration (mmol·l^{-1}). Error bars depict standard deviations of three replicate incubations.

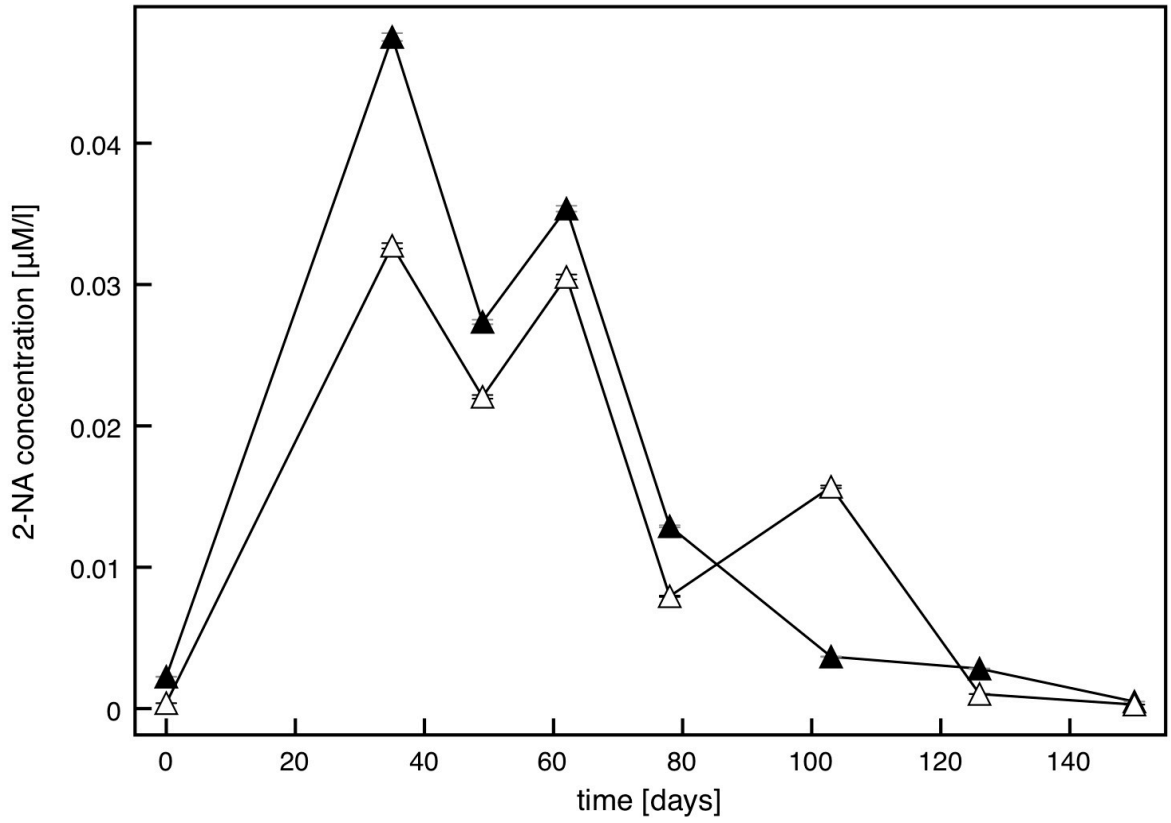

Figure 13: 2-NA concentration during growth of N49 on naphthalene

2-naphthoic acid (2-NA) concentrations during growth of untreated (full triangles) and pasteurized N49 cultures (open triangles).

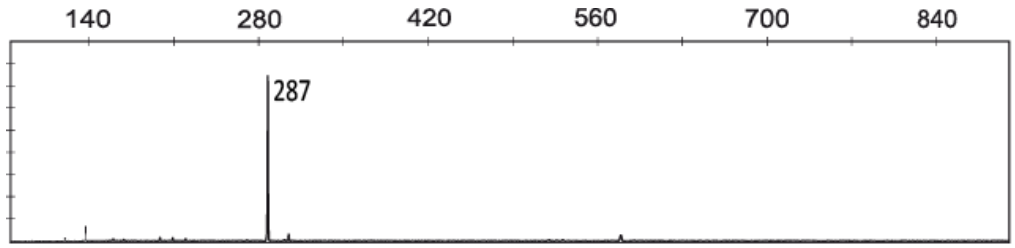

Figure 14: Electropherogram of N49 grown on napthalene

Electropherogram of t-RFLP analysis with culture N49 grown on naphthalene as sole carbon source. Numbers give the length of the TRF in base pairs.

7.1.7 Electron donors and acceptors utilized by culture N49

Different polycyclic aromatic hydrocarbons as well as nonaromatic compounds were tested as alternative carbon sources for N49 with ferrihydrite as electron acceptor. The culture was able to grow with 1-methylnaphthalene, 2-methylnaphthalene, 1-naphthoic acid, 2-naphthoic acid. The microbial community composition remained the same for all substrates, indicating that the same organisms utilized the substrates. No growth was observed on 1-naphthol, 2-naphthol, anthracene, phenanthrene, indane, and indene. Furthermore, sulfate, elemental sulfur (S^0), and nitrate were tested as alternative electron acceptors for N49. However, only S^0 led to a measurable increase in biomass (table 4).

Trait	N49	Major TRF observed
naphthalene	+	287 bp
2-methyl naphthalene	+	287 bp
1-methyl naphthalene	+	287 bp
2-naphthoic acid	+	287 bp
1-naphthoic acid	+	287 bp
2-naphthol	-	-
1-naphthol	-	-
anthracene	-	-
phenanthrene	-	-
indane	-	-
indene	-	-
S^0	+	-
sulfate	-	-
nitrate	-	-

Table 4: Tested alternative substrates and electron acceptors for the enrichment culture N49

7.1.8 Analysis of the microbial community composition of culture N49

To identify the microorganisms involved in naphthalene degradation of the enrichment culture N49, T-RFLP (Terminal Restriction Fragment Length Polymorphism) and comparative sequence analysis of the 16S rRNA genes of bacteria were performed. The electropherogram of naphthalene grown cells showed a single dominant bacterial TRF of 287 bp (figure 14) indicating that the naphthalene degrading culture is mainly composed of one type of organism. T-RFLP analyses of liquid and solid phase of the cultures were identical (data not shown). To identify the dominant microorganism in the culture, 16S rRNA gene PCR amplicons were cloned and sequenced. Only one dominant type of sequences was present for bacterial (n= 85) rRNA genes, respectively. ARB analysis revealed that the closest phylogenetic relative (≥ 99 % 16S sequence similarity) was the dominant organism of the iron-reducing, benzene-degrading culture BF (figure 15) (Kunapuli*, et al.*, 2007, Kunapuli*, et al.*, 2010). In culture BF, the Gram-positive microorganism constituted more than 90% of the microbial community and was responsible for the degradation of benzene. The next cultivated and described relative in the data bank was *Thermincola carboxydiphila* within the Gram-positive *Peptococcaceae* with 92 % sequence similarity (Sokolova*, et al.*, 2005).

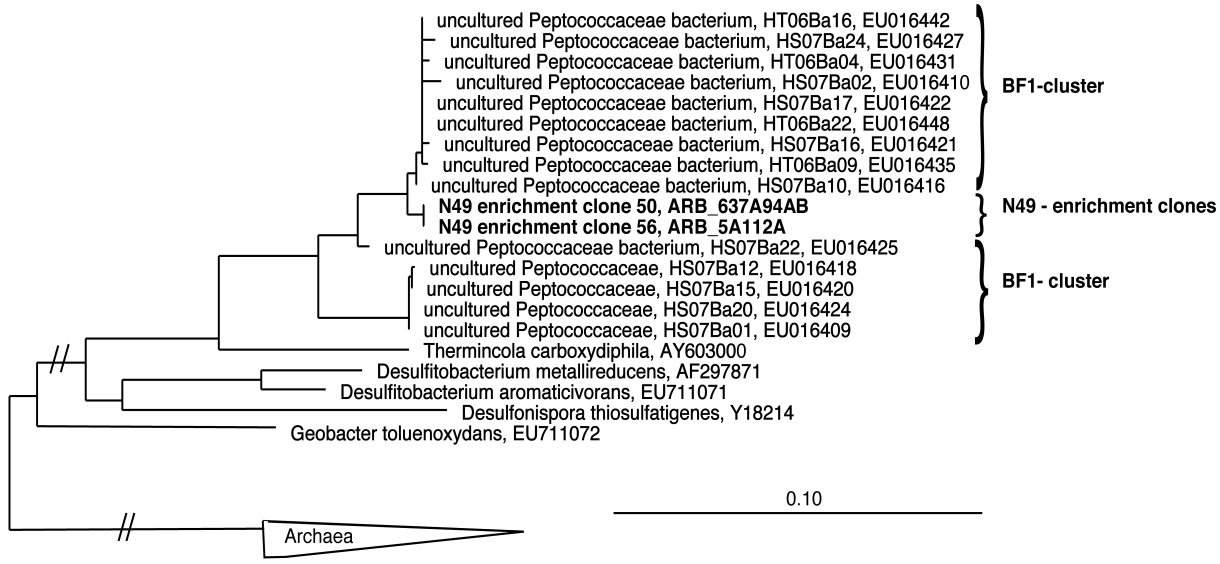

Figure 15: Phylogenetic dendrogram including culture N49

Phylogenetic dendrogram comprising clones from culture N49 designed with the ARB-software (using the Fitch algorithm implemented in ARB). The scale bar represents 0.10 changes per nucleotide.

8 Discussion

8.1.1 Biomass formation as a function of the redox potential difference

Growth experiments with *Geobacter sulfurreducens* and *Shewanella oneidensis* using electron acceptors of different redox potentials indicated a clear correlation between the total available Gibb's free energy of the metabolic reaction and the biomass produced by the microorganisms. This clear biomass-energy correlation demonstrates the metabolic versatility of iron-reducing bacteria. The formal potentials of the electron accepting soluble iron complexes used spanned from +101 mV to +440 mV, and the microorganisms adapted their biomass formation to the available energy gain in the order Fe-citrate > Fe-NTA > Fe-EDTA/EGTA, which is in accordance with the respective formal potential sequence.

Considering the redox potential range of the different forms of ferric iron in the natural environment, this seems to be a very beneficiary metabolic adaptation. It would guarantee that an iron-reducing microorganism can exploit a maximum of the available free energy of a redox reaction no matter what electron acceptor occasionally is met and would therefore avoid the waste of energy in form of heat release.

In earlier studies, 0.17 g C per mol e^- have been assessed for the growth of *Shewanella oneidensis* on ferrihydrite and 0.20 g carbon per mol e^- for the growth on Fe-citrate (Kostka*, et al.*, 2002), although complete reduction was not achieved in this experiments which makes it difficult to determine the absolute yield. In another study, under nitrogen limiting conditions with acetate as electron donor, a biomass formation of 0.40 g C per mol e^- has been determined for *Geobacter sulfurreducens* (Bazylinski*, et al.*, 2000). The data obtained in this study for the growth of *G. sulfurreducens* with Fe-citrate as electron acceptor were 4.6 times higher (1.85 g C per mol e^-). Moreover, one study reported similar carbon fixation values between 0.18 to 4.07 g C per mol e^- for *Shewanella putrefaciens* growing on lactate as electron donor and fumarate as acceptor (0.33-7.54 g cell protein multiplied by 0.54 as the average percentage of carbon per protein (Myers & Myers, 1994). Yet none of

the latter studies systematically investigated the correlation between yield and the redox potential of the electron acceptor.

In our experiments, additional utilization of citrate as carbon or electron source during growth with ferric citrate as electron acceptor by *Geobacter* or *Shewanella* cannot be fully excluded (Ahrendt*, et al.*, 2007). In control experiments, *Geobacter* and *Shewanella* grew also with sodium citrate as substrate (data not shown) raising the question if the citrate released from the reduction of ferric citrate served as additional electron donor in the respective experiments. Thus, on the first glance, a possible utilization of citrate as additional electron and/or carbon source might influence our data sets. However, as we show in the following discussion it is not relevant for the biomass/electron yield calculations if the electrons for anaerobic iron respiration and electron transport phosphorylation originate from the added acetate, lactate, or a potentially additional citrate. First, there is no substrate level phosphorylation step involved in citrate oxidation in the Krebs cycle. Thus, all energy gain of both compounds by the organism will be conserved in the respiratory chain only. Second, we did not measure substrate depletion but ferrous iron production. Therefore, all calculations of biomass production were related to the electrons respired, no matter if the electron for respiration originated from acetate or citrate oxidation. Per electron, there is exactly the same energy in the oxidation of acetate or citrate because for both substrates the electrons are channelled into the respiratory chain via NADH. The source of electrons is therefore of no primary importance for the yield calculations. Third, the production of biomass with both acetate and citrate as carbon source will always be facilitated by the production of acetate and subsequently pyruvate. Thus, once a citrate molecule is oxidized to CO_2 and acetate in the Krebs cycle, the same amount of ATP has to be invested to build biomass, no matter if the acetate was taken up directly or produced by citrate oxidation. The only difference in energy demand for biomass production between acetate and citrate might occur in the production of certain amino acids such as glutamic acid, aspartic acid, etc. which derive directly from precursors occurring in the TCA-cycle such as oxalacetate or α-ketogluterate. Here, a little more ATP has to be invested for protein production in the case of acetate as carbon source because the Krebs cycle has to be refilled from acetate to produce oxalacetate or α-

ketogluterate in a heterotrophic CO_2-fixation reaction. This would not be the case with citrate as carbon source. However, this difference can be assumed to be very small for the total energy demand of biomass production and is therefore negligible for our calculations.

Our findings show that iron-reducing microorganisms are able to adapt their energy gain from respiration to the redox potential of the electron acceptor. Although this has been implicitly assumed since a long time the absolute proof has been missing so far. In fact, such as study is only possible with an electron acceptor such as soluble iron compounds, which differ in their redox potential with different complexing ligands, whereas the biochemistry of the respiratory chain remains the same. With electron acceptors such as oxygen, nitrate or sulphate, one could only vary the redox potential by changing the concentration. However, as it can be easily calculated by the Nernst equation, within physiological concentrations of electron acceptors, significant changes of the Gibbs free energy of the reaction do not arise.

8.1.2 Dependency of the growth kinetics on redox potential in Geobacter sulfurreducens

A clear correlation between the redox potential of the electron acceptor, the maximum growth rate μ_{max} and the reaction kinetic v_{max} in dense cell suspensions was observed for *G. sulfurreducens* cultivation. Both parameters were linearly dependent on the maximum available catabolic free energy (figure 9). The general growth rate (μ) is proportional to the change of biomass over time (dx/dt) normalised to the absolute biomass (X) (eq. 6). On the other hand, the change of biomass is directly linked to the change in substrate concentration (dc_{Sub}/dt) via the biomass yield coefficient (Y) (eq. 7). Introducing equation 7) into 2) shows that the growth rate (μ) is directly proportional to the yield coefficient (Y) (eq. 8). Therefore, higher growth rates at more positive redox potentials of the electron acceptor might derive from the higher yield coefficient (Y).

Equation 6

$$\mu = dx/dt * 1/X$$

Equation 7

$$dx/dt = -Y\ dc_{Sub}/dt$$

Equation 8

$$\mu = -Y\ dc_{Sub}/dt * 1/X$$

As the biomass yield (Y) for *G. sulfurreducens* growth on e.g. acetate as substrate is 2.3 times higher with Fe-citrate than with Fe-NTA as electron acceptor, this yield difference directly implies that the growth rate has to be also at least 2.3 times higher, which indeed is the case (table 1).

Both parameters, yield and growth rate, have been measured independently. However, the clear correlation displayed in table 1 reveals that they are closely interrelated which perfectly suits equation 8. Therefore, we postulate that the entire change of the growth kinetics is determined solely by the yield, which in turn is dependent on the redox potential of the electron acceptor. This demonstrates the impact of the thermodynamics of the metabolic reaction on both the metabolic efficiency as well as the kinetics. From the iron-reducing microorganisms perspective this denotes that a more efficient harvesting of the possible energy gain results in faster growth.

Data from other authors using different solid iron oxides, AQDS or U(VI) (Roden & Zachara, 1996, Lloyd*, et al.*, 2003) also suggested that reduction rates for *G. sulfurreducens* are dependent on the redox potential of the electron acceptor. This is in line with our own results from dense cell suspension experiments demonstrating that the thermodynamics (the redox potential) of the metabolic reaction have a major impact also on the kinetics of the reaction in terms of v_{max}. This indicates that the higher energy gain in case of electron acceptors of higher formal redox potential not only leads to higher biomass yields but also to a competitive kinetic advantage of the organisms.

A slightly different picture of the reaction kinetics appeared for *S. oneidensis*. Although for growth with Fe-citrate as electron acceptor the same increase in

biomass for the higher redox potential was observed in comparison to the low formal potential iron-complexes, the highest activity was observed with Fe-NTA as electron acceptor (figure 9). A similar sequence was observed in (Dobbin*, et al.*, 1995) using outer-membrane cytochrome extracts of *Shewanella* (Wang*, et al.*, 2008). Here, the reaction rates followed the order Fe-EDTA > Fe-NTA > Fe-citrate. We speculate that the higher activities using Fe-NTA can be correlated to the increased permeability of the outer membrane of *Shewanella oneidensis* towards Fe-NTA (Schuetz*, et al.*, 2009), which may allow additional reduction reactions within the periplasm.

However, a *Geobacter*-type dependence of the reaction rate in cell suspension experiments on different electron acceptors was already observed for *Shewanella putrefaciens* (Fe-citrate: 1.23 mM e^- per h, Fe-NTA: 0.31) (Haas & Dichristina, 2002). The use of substrate-level phosphorylation (SLP) in *Shewanella* has been reported (Hunt *et al.*, 2010). However, our data suggest an adaptation of the energy gain to the redox potential of the electron acceptor, which would be impossible if *Shewanell*a would derive its energy exclusively from SLP. Further respiratory energy conservation steps beyond SLP are required to achieve this adaptation.

8.1.3 Molecular mechanisms of adapting energy gains to the redox potential

The major question arising from our observations is how the microorganisms can perform the adaptation to the redox potential on a molecular level. In iron-reducing bacteria, up to 111 multiheme cytochrome c type proteins can be involved in the electron transport systems from the NADH-dehydrogenase and the inner membrane quinone pools towards the extracellular or periplasmic electron acceptor (Methe*, et al.*, 2003, Shi*, et al.*, 2007). Individual hemes of some cytochromes are arranged in chains to yield a directional electron transfer while cyclic multihemes can also store electrons to some extent (Harada*, et al.*, 2002). Additionally, the ppcA protein has been suggested as a molecular valve, having influence on the direction of the electron flow and on energy transduction (Pessanha*, et al.*, 2006). Furthermore, redox-sensing domains containing cytochromes have been discovered (Londer*, et al.*, 2006), as well as redox-linked conformational changes in cytochromes (Morgado*, et*

al., 2007). In addition, there is an indication that redox-sensitive outer membrane cytochromes (OmCs) influence the arrangement of other omCs (Kim*, et al.*, 2005). Furthermore, a recent study demonstrated the impact of deletions of outer membrane cytochromes on the efficiency of iron reduction (Voordeckers*, et al.*, 2010). When OmCs were knocked out, the amount of electrons transferred per unit of biomass produced was reduced - the yield increased.

From all this observations we derive two hypotheses how different energy conservations might be regulated.

1) The simplest assumption would be a regulation on the gene expression level. If microbes would sense a higher redox potential of ferric iron species in the environment they might express additional cytoplasmic membrane complexes that could perform an electron transport step where protons are transported across the membrane. Thus, more protons per electron respired would be pumped across the membrane and thus the energetic yield increases. However, Myers and Myers (Myers & Myers, 1994) showed with 2-dimensional gel electrophoresis that protein expression did not significantly change between cells grown on Fe-NTA and Fe-citrate which contradicts this hypothesis. In fact, in the many mutagenesis experiments performed over the past decade there were no other cytoplasmic membrane proteins discovered to be involved in electron transport in iron-reducing microorganisms than NADH-oxidoreductase and quinole oxidases (CymA for *Shewanella* (Myers & Myers, 1997) and MacA for *Geobacter* (Butler, et al., 2004).

2) A second hypothesis, which we propose here, is a proton bifurcation mechanism. The proton bifurcation should be located at the menaquinole oxidase where the menaquinole is oxidised to menaquinone in a two electron oxidation step. If the redox potential of the terminal electron acceptor is high enough (e.g., Fe-citrate), this oxidation step would be accompanied by a release of two protons into the periplasmic space conserving energy for ATP synthesis. However, if the redox potential is not high enough (e.g. Fe-EDTA) to allow for pumping of two protons per two electrons, we propose that one proton is slipping into the cytoplasm. The produced semiquinone is much more acidic than the menaquinole (Jiang*, et al.*, 2009) and can release the second proton to the periplasmic space. Thus, only one proton is pumped across the membrane instead of two depending on the

thermodynamic energy gain for the respective electron acceptor. Therefore, the release of one proton to the alkaline cytoplasm would enable the pumping of one proton across the membrane into the periplasm on the expense of less energy conservation and reduced reaction kinetics. This proposed proton bifurcation finds its parallel in electron bifurcation mechanisms described recently (Herrmann*, et al.*, 2008, Thauer*, et al.*, 2008)

8.1.4 Iron reduction behaviour of S. oneidensis mutant strains

Dense cell suspensions of *Shewanella oneidensis* MR-1 mutants and the wildtype were investigated with regard to the reduction behaviour of Fe-citrate compared to Fe-NTA.

The general outcome of the iron reduction assays was the observation that mutants and wildtype all showed in average 50 % higher reduction kinetics on Fe-NTA than compared to Fe-citrate. This confirms the previous findings in this thesis showing *Shewanella oneidensis* MR-1 having different reduction kinetics when grown on Fe-citrate and Fe-NTA compared to *Geobacter sulfurreducens*. Interestingly, both strains were able to gain more biomass out of the reduction of Fe-citrate compared to Fe-NTA. As Voordeckers and Kim (Kim*, et al.*, 2005, Voordeckers*, et al.*, 2010) investigated, the reduction behaviour of different OmC mutants of *Geobacter sulfurreducens* the authors stated that deletion of the omc-part of the respiratory chain leads to the diffusion of soluble iron compounds into the periplasm, which results in decreasing reduction rates. The data of this study shows the same effect in case of the gsp-mutants (RK 45, RK457) and the mtrB mutant (JG 299) because the gsp system is necessary for the proper localization of the compounds OmcA and OmcB (DiChristina*, et al.*, 2002, Myers & Myers, 2002, Shi*, et al.*, 2008). Defects of the secretory system or within mrtB might result in phenotype missing the compounds OmcA and/or OmcB or in a possible mislocation of either one or both (e.g. the compounds facing inward or being located within the periplasm, one of the compounds missing etc). But this does not explain the higher reduction rates of mutants and wildtype *Shewanella oneidensis* MR-1 strains on Fe-NTA in general. Hence studies from Gescher (Gescher*, et al.*, 2008) with an *E.coli* strain genetically

modified and turned into an iron reducer showed the comparison of protoplasts and intact cells towards the reduction behavior of Fe-NTA, Fe-citrate and AQDS. The authors found the reduction rates of Fe-NTA in intact cells being 60 % higher for Fe-NTA. They therefore stated that the outer membrane might be a diffusion barrier to Fe-citrate and AQDS, what is consistent with our results of 50 % higher iron reduction rates on Fe-NTA.

Besides this theory, another work has been published about the reduction kinetics of the outer membrane of cytochromes OmcA and MtrC (OmcB) on different iron chelates. In this study, the kinetics for a given chelate turned out to be in the order Fe-EDTA > Fe-NTA > Fe-citrate. A different reduction behaviour thought to be dependent on the reorganization energy of the respective iron chelate (Wang*, et al.*, 2008). Together, these results emphasize and confirm previous results of this thesis, iron reduction kinetics of *Shewanella oneidensis* MR-1 on Fe-NTA being higher than on Fe-cit, which speaks for the existence of interspecific differences between *S. oneidensis* and *G. sulfurreducens*. Furthermore, it could be shown that these differences seem to be independent from those genes, which were destroyed in the mutants tested in this work, because mutants and wildtype followed the same trend. Additionally, this work is able to support previous studies, which showed the importance of the compounds CymA, menaquinone, mtrB and the proper localization of the teriminal reductase for iron reduction in *Shewanella*. However, no new and yet unknown component of the respiratory complex could be found within the performed mutagenesis, which leads to the assumption, that the working hypothesis was not correct and that the organism might have another strategy for conserving more energy out of the reaction with Fe-citrate such as an electron bifurcation. To elucidate this, remains a topic of future research.

8.1.5 Key microbial members of enrichment culture N49

Based on T-RFLP and 16S rRNA gene sequence analyses and the results of the quantitative PCR, the enrichment consisted mainly of bacteria belonging to the family *Peptococcaceae* within the phylum *Firmicutes*. The closest relative in the data bank (≥ 99 % 16S sequence similarity) was the dominant benzene-degrading organism from the iron-reducing enrichment culture BF (Kunapuli*, et al.*, 2007, Kunapuli*, et al.*, 2010). Considering the high phylogenetic similarity (99 %) of the major keyplayers in cultures N49 and BF, which is beyond the generally accepted 16S sequence similarity of 97 % for the species level, we propose that the naphthalene degrader N49 and the benzene degrader BF depict two different strains of an iron-reducing candidate species that is specialized on the degradation of non-substituted aromatic hydrocarbons. Considering the close phylogenetic proximity of the two keyplayers in N49 and BF it would be interesting to know if N49 was able to grow on monoaromatic hydrocarbons. However, this has not been tested yet.

The nearest cultivated and described relative in the culture collections (92 % 16S sequence similarity) is the organism *Thermincola carboxydiphila* which was isolated from a hot spring of the Baikal Lake region (Sokolova*, et al.*, 2005). However, the phylogenetic distance of N49 and BF to the genus *Thermincola* is larger than the commonly observed 16S sequence similarity within one genus. The two organisms N49 and BF might therefore constitute a novel species within a novel yet undescribed candidate genus. The discovery of strains N49 and BF supports recent findings that Gram-positive organisms might play a much larger role in the degradation of aromatic hydrocarbons than anticipated before. The vast majority of anaerobic aromatic hydrocarbon degraders isolated so far belong to the beta- and delta-proteobacteria. Most of the strict anaerobes are sulfate reducers of the genus *Desulfobacterium* (Galushko*, et al.*, 1999, Musat*, et al.*, 2009) and a few *Geobacteraceae* (Lovley, et al., 1993, Coates, et al., 2001, Kunapuli, et al., 2010). The lately isolated Gram-positive aromatics degraders belong to such different genera as *Desulfitobacterium* (Kunapuli, et al., 2010) *Desulfotomaculum* (Tasaki, et al., 1991, Morasch, et al., 2004) and the family of the *Peptococcaceae* (Kunapuli*, et al.*, 2007).

As revealed by the pasteurisation test, strain N49 is spore-forming which is not uncommon within the Gram-positive organisms of the *Peptococcaceae*. We speculate that spore formation might reveal an important physiological advantage in contaminated aquifers. Recent studies revealed that aquifers are not as static habitats as previously anticipated and surprisingly strong spatial fluctuations of contaminant plumes can occur (Anneser*, et al.*, 2008, Anneser*, et al.*, 2010, Jobelius*, et al.*, 2010). As most of the biomass is attached to the sediments it might happen that the environmental conditions for microbes change drastically exposing them to hostile conditions like molecular oxygen, high sulphide concentrations or high concentrations of aromatic hydrocarbons. Spore formation would allow degrader populations to sustain such periods and proceed with growth when the geochemical parameters return to more suitable conditions.

Dissimilatory iron reduction is found in many phylogenetic groups of bacteria or archaea (Weber*, et al.*, 2006). Usually, these organisms can reduce different iron minerals and basically every iron-reducing microorganism can reduce humic substances or analogues such as AQDS. Although there are slight variations between organisms, the general principle of the respiratory chain seems similar and the organisms are comprehensively termed metal-reducing. It was not specifically tested, what iron minerals or quinone analogues culture N49 could utilize but always added small amounts of AQDS to enhance electron shuttling. The reduction rates for AQDS or pure ferric iron might therefore differ substantially for culture N49.

8.1.6 Pathway of anaerobic naphthalene degradation

Several studies have shown that the central intermediate 2-naphthoic acid was accumulating in growth media with naphthalene as carbon source (Zhang & Young, 1997, Meckenstock*, et al.*, 2000, Zhang*, et al.*, 2000). Experiments with ^{13}C-bicarbonate buffered medium showed that the ^{13}C-label was incorporated into the carboxyl group of the metabolite 2-naphthoic acid, which suggests a carboxylation of naphthalene as initial reaction mechanism (Zhang & Young, 1997, Meckenstock*, et al.*, 2000, Zhang*, et al.*, 2000). However, one has to admit that metabolites accumulate over long cultivation times and are detected in trace amounts only. They

can only be taken as hints for putative degradation pathways. In our experiments, LC/MS/MS analysis revealed 2-naphthoic acid as intermediate with increasing concentrations during growth and a decline when cultures entered the stationary phase. Furthermore, substrate utilization tests showed that culture N49 was able to grow on 2-naphthoic acid. Therefore, it is likely that 2-naphthoic acid is a real central metabolite in the naphthalene degradation pathway of strain N49. Methylation was also discussed as initial reaction mechanism for naphthalene activation (Safinowski & Meckenstock, 2006). The authors performed growth experiments with deuterated naphthalene as sole carbon source and found ring-deuterated metabolites such as naphthyl-2-methyl-succinic acid and naphthyl-2-methylene-succinic acid. These compounds are known to be key intermediates of the 2-methyl-naphthalene degradation pathway (Annweiler*, et al.*, 2000, Annweiler*, et al.*, 2002, Selesi*, et al.*, 2010, Bergmann*, et al.*, 2011). Culture N49 was also tested for naphthyl-2-methyl-succinic acid and naphthyl-2-methylene-succinic acid but none of these metabolites could be detected although the culture could grow with 2-methylnaphtalene. Culture N49 can also grow with 1-methylnaphthalene. As the two currently known anaerobic degradation pathways of naphthalene and 2-methylnaphthalene proceed via 2-naphthoic acid it will be biochemically very interesting to analyze how the organism performs the activation and degradation of 1-methylnaphthalene. However, metabolite analysis of a 1-methylnaphthalene grown culture has not been performed so far. Alternatives are a fumarate addition reaction like in the well studied 2-methylnaphthalene degradation pathway of strain N47 or a carboxylation to 1-methyl-2-naphthoic acid similar to naphthalene degradation (Safinowski & Meckenstock, 2004, Selesi*, et al.*, 2010).

The current enrichment culture N49 indicates that anaerobic PAH-degradation can be performed also by iron-reducing microorganisms. It furthermore highlights that Gram-positive microorganisms might play a more important role in anaerobic aromatics degradation than anticipated

9 General conclusions and outlook

As already mentioned before, the degradation of polycyclic aromatic hydrocarbons and the dissimilatory reduction of ferric iron are parts of a still quite young research field. The molecular backgrounds behind these processes are still puzzles to be solved. Concerning this thesis the molecular mechanisms behind the respiratory processes in iron reducing organisms were investigated. *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* served as model organisms to solve the questions 1) if iron reducers can adapt the production of biomass to the respective energy provided by the redox gradient of electron donor and electron acceptor and 2) how this could be achieved by the organism itself. Furthermore, topic of microbial iron reduction and the topic of PAH degradation were connected through the investigation of the iron reducing, naphthalene degrading enrichment culture N49.

The results of this study considerably show that iron-reducing microorganisms like *Shewanella* and *Geobacter* can adapt to the ”redox range“ offered by iron oxides by producing more biomass the more energy is available. This mechanism may allow a fine-tuning of the metabolic machinery of iron-reducing bacteria according to the available electron acceptor. However, the biomass yield is not necessarily congruent with the reduction kinetics on the respective electron acceptor, which indicates that interspecific differences might exist. Hypothetic high- and low-energetic pathways, to achieve the adapting biomass yields, seems, based on the present results, rather unlikely but can not be fully excluded. If this adaptation takes place via a kind of proton bifurcation at functional protein sites or even by modification of the ATPases (Dimroth & Christoph von Ballmoos, 2006) remains a matter of speculation. Nevertheless, this ability of adaptation allows the microorganisms to use different iron species occurring at a range of redox potentials in the environment as terminal electron acceptors. Maybe the numerous cytochromes that have been found in genome sequences and proteomic studies are responsible for optimal electron flow at specific redox potentials and reflect this versatility of the electron transport chain.

This versatility fits to an ecology which implies the dissimilatory reduction of soluble, poorly soluble or insoluble electron acceptors with a large range of redox potentials, and it enables *Geobacter* to maintain at least a minimum of metabolic activity on almost all kinds of ferric iron and many other metallic electron acceptors. The mechanism of adaptation described in our study might also have implications for microbial fuel cells, in which iron-reducing microorganism grow on solid anodes.
The detailed functions behind the adaptive biomass yield remain cloudy, but this study gave an insight in its existence and is therefore a starting point for new ideas and investigations. Nevertheless, the results show the metabolic versatility and adaptability of the investigated bacteria, but more experiments are needed to clarify this topic.
The analysis of culture N49 ties microbial iron reduction to microbial naphthalene degradation. To the best of our knowledge N49 is the first description of a highly enriched iron reducing, naphthalene degrading culture. The fact, that the keyplayers of this culture belong to the genus *Peptococcus* and the close relatedness to the benzene degrading culture BF, were the reasons to suggest a new candidate species and genus. The *Peptococcaceae* might therefore play a much greater role in PAH degradation than expected before. Additionally also the fact that the involved organism grows on 1-methylnaphthalene as well, leaves room to speculate how the organism achieves the breakdown of naphthalene in detail. One more hint in the direction to a carboxylation as initial activation mechanism in anaerobic naphthalene degradation, is the finding of 2-NA being a metabolite.
This thesis presents a “missing link” between dissimilatory iron reduction an anaerobic naphthalene degradation and therefore combines both processes to leave open questions about first activation step in naphthalene degradation, other organisms involved and about the mechanisms behind the adaptable energy gain of bacteria reducing iron compounds. However, this thesis is able to provide results that can elucidate these questions of open debate a bit further so that one day the whole puzzle might be set together completely.

10 References

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11 Clarification

11.1 Adaptation of *Geobacter* and *Shewanella* to the redox range of iron chelates:

In this part of the thesis all experiments with *Shewanella oneidensis MR-1* were done by the PhD candidate. The methods section about these experiments was written by her as well and she took part in discussing the results.

11.2 Random transposon mutagenesis of *Shewanella oneidensis*-MR1

The method of the random transposon mutagenesis and screen was taught and supervised by PD Dr. Jeffrey Gralnick, St. Paul, Minnesota, USA, the practical work was done by the PhD candiate herself.
Furthermore, all other experiments were done by the canditate, as well as the writing of this chapter. All results were evaluated and discussed together with Prof. Dr. Rainer Meckenstock.
As mentioned in the methods section, the final part of the sequencing was done by the Genom Analyse Zentrum (GAC), a facility of the Helmholtz Zentrum München.

11.3 Analysis of the enrichment culture N49

All experiments were carried out by the PhD canditate herself the results were discussed and evaluated with Prof. Dr. Rainer Meckenstock. The publication was written on her own and discussed with Prof. Dr. Rainer Meckenstock. The publication has been accepted by FEMS Microbiology Ecology.

The candidate got advise and help in the setup of the GC-IRMS experiments by Dr. Martin Elsner and his group members Sandra Reinnicke and Martina Höche. The help consisted of setting up the GC-IRMS machine, and teaching the PhD canditate how to use the instrument herself.
Concerning the molecular work, the PhD canditate got help and advice on the quantitative PCR by Dr. Tillmann Lüders and his PhD student Dörte Dibbern.
The last part of the sequencing, as mentioned in the methods section, was done by the Genom Analyse Zentrum (GAC), a facility of the Helmholtz Zentrum München.
Furthermore, culture N49 has not been enriched by the student.
As far as the metabolite analysis is concerned, the candidate got advice in the setup, handling and of the LC-MSMS as well as the evaluation of the data by Dr. Housna Mouttakki.

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An dieser Stelle möchte ich anschließend erwähnen, dass die Kollegen am IGÖ einfach wundervoll sind. Ich danke Euch nicht nur für die tolle Atmosphäre sondern vor allem für die motivierenden Worte und ideenreichen Gespräche, für Hilfestellungen und Einweisungen an Gerätschaften. 2010 habe ich mehrere Rechner durch mein „Karma“ zu Fall gebracht – Danke Michael Stöckl, daß Du mich nicht gefressen hast!

Ein persönliches Dankeschön geht an Eva, die meine Aufmerksamkeit auf das IGÖ lenkte, ohne sie hätte ich nicht gewusst, dass diese Stelle spontan wieder frei geworden war. Außerdem bist Du seit dem Studium eine gute Freundin – Danke für alles. Weitere persönliche Dankeschöns gehen and Marc, Muna, Tine, Betti, Robert, Claudia, Franz, Marco, Thorben, Agnies, Sylvie, Housna, Martina, Brigitta, Ramon, Sviatlana, Frederick, Dörte und Maria – meine engsten Leidensgenossen, Laborkollegen, Officemates und Freunde. Ohne Euch hätte ich das nicht geschafft ☺

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13 Appendix

13.1 Curriculum Vitae

R I T A K L E E M A N N

Alter	32
Geburtsort	Bremerhaven
Wohnort	München

AUSBILDUNG

04/2011 bis heute	**Biomerieux Industries / AES Chemunex GmbH** Regional Sales Manager Region Süddeutschland
10/2006 – 03/2011	**Helmholtz Zentrum München & TU München** Institut für Grundwasserökologie Doktorandin bei Prof. Dr. Rainer Meckenstock
10/2000 - 01/2006	**Universität Konstanz** Studium der Biologie Abschluss: Diplom
10/1999 - 09/2000	**Universität Bremen** Studium Lehramt Biologie und Sport
05/1999	**Gymnasium Schulzentrum Alwin Lonke Strasse, Bremen** Abschluss: Abitur

13.2 Erklärung nach Anlage 5 der Promotionsordnung

Ich erkläre an Eides statt, dass ich die der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Energetics und Physiology of microbial iron reduction

in München am Lehrstuhl für Grundwasserökologie unter der Anleitung und Betreuung durch Prof. Dr. Rainer Meckenstoc ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 5 angegebenen Hilfsmittel benutzt habe.

Ich habe die Dissertation in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

Die vollständige Dissertation wurde in .... veröffentlicht. Die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München hat der Vorveröffentlichung zugestimmt.

Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

Die Promotionsordnung der Technischen Universität München ist mir bekannt.

München, den ....
Unterschrift