May 2013

# Analysis of Genes Involved in Anaerobic Growth in Porphyromonas Gingivalis and Shewanella Oneidensis MR-1

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# ANALYSIS OF GENES INVOLVED IN ANAEROBIC GROWTH IN *Porphyromonas gingivalis* AND *Shewanella oneidensis* MR-1

by

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A thesis Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

in Biological Sciences

at

The University of Wisconsin-Milwaukee

May 2013

ABSTRACT

Analysis of genes involved in anaerobic growth in *Porphyromonas gingivalis* and *Shewanella oneidensis* MR-1

by

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The University of Wisconsin-Milwaukee, 2013
Under the Supervision of Professor Daâd Saffarini

*Porphyromonas gingivalis* is an oral Gram-negative anaerobic bacterium implicated in periodontal disease, a polymicrobial inflammatory disease that is correlated with cardiovascular disease, diabetes and preterm birth. Therefore understanding the physiology and metabolism of *P.gingivalis* through genetic manipulation is important in identifying mechanisms to eliminate this pathogen. Although numerous genetic tools have been developed for the manipulation of other bacterial species, they either do not function in *P.gingivalis* or they have limitations. We modified a Mariner transposon pHimarEM1 system that was developed for *Flavobacterium johnsoniae* for mutagenesis of *P. gingivalis*. We introduced the *P. gingivalis fimA* promoter upstream of the transposase gene to improve the efficiency of the transposition in *P.gingivalis*. Transposon mutants of *P.gingivalis* were screened for growth or pigmentation defects and analyzed. Using this technique, strains with mutations in genes encoding cation efflux system Proteins, $Na^+$-translocating NADH-quinone reductase were identified thus demonstrating the effectiveness of this new transposon. Therefore pHimarEM1-$P_{fimA}$ can

be used to generate new mutants in *P. gingivalis* that lead to explore the metabolic pathways of *P. gingivalis*.

*Shewanella oneidensis* MR-1 is a facultative anaerobe and metal reducing bacterium that uses a large number of terminal electron acceptors for respiration. These include thiosulfate, polysulfide and sulfite. The dissimilatory sulfite reductase, Sir A is a *c* type cytochrome predicted to be a copper protein. It catalyses the six electron reduction of sulfite to sulfide and unlike other sulfite reductases, it appears to lack siroheme. Cu is essential for many respiratory enzymes such as cytochrome *c* oxidases and anaerobic nitrous oxide reductases. NosA is predicted to be a copper specific porin in the outer membrane that allows copper diffusion into the cell. In the second chapter of this thesis we hypothesized that NosA is expressed under aerobic and anaerobic conditions and is involved in biogenesis of the sulfite reductase (SirA). We also predict that additional porins may be needed for Cu entry into the cell.

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## ACKNOWLEDGEMENTS

I would like to thank my advisor Dr Daâd Saffarini for accepting me in her lab and for her constant support and guidance throughout the duration of my masters. I would also like to thank my Graduate Committee members Dr. Mark McBride and Dr Sonia Bardy for their guidance on my research.

It is my pleasure to thank Saffarini lab members Sheetal Shirodkar, Areen Banerjee, Namita Shroff, Ken Brockman, Patrick Trewitt, Rini Banerjee for their support in my research.

I am grateful and thankful to my husband Tharindu for his immense support and being a constant source of motivation in my life.

This thesis would not have been possible without my parents and my brother dedicating their time in taking care of my newborn daughter Pethmi during the last semester of my degree. I feel immense gratitude towards my parents for being there for me in every step of my life

# CHAPTER 1
# Genetic manipulation of *Porphyromonas gingivalis*

## Introduction

### Periodontal disease

Periodontitis is a bacterially induced chronic inflammatory disease that leads to destruction of tooth supporting tissue and to localized alveolar bone loss that is a hallmark of the disease (figure 1)(7).

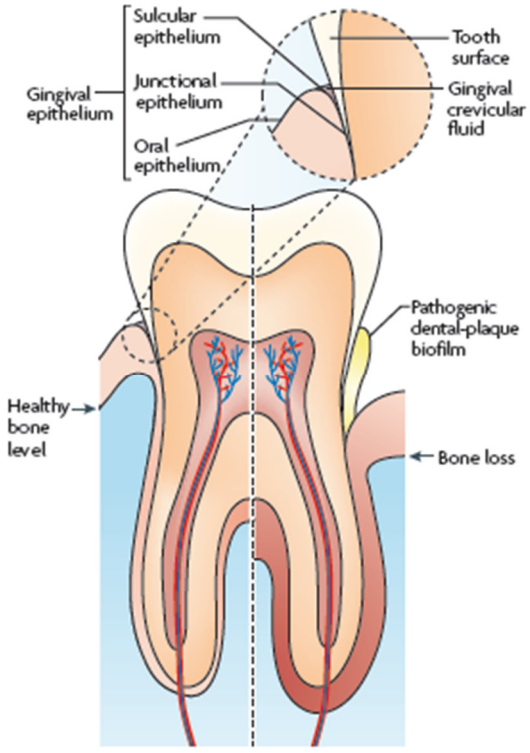


**Figure 1:** The effects of periodontitis. Healthy periodontal tissue (left) contains connective tissue and alveolar bone, which support the tooth root. In addition, the oral epithelium covers this supporting tissue, and a specialized junctional epithelium connects it to the tooth surface. The space between the epithelial surface and the tooth is called the sulcus and is filled with gingival crevicular fluid. In cases of periodontitis (right), a dental-plaque biofilm accumulates on the surface of the tooth and tooth root and causes the destruction of periodontal connective tissue and alveolar bone, resulting in the most common cause of tooth loss in the world (7).

Periodontitis is initiated by the formation of dental plaque which is a biofilm of several bacterial species that contribute to the infection. Dental plaque results from the interactions of a highly characterized microbial community. Initial colonizers such as *Streptococcus* species bind to the salivary receptors of the tooth surface while late colonizers bind to previously bound bacteria through bridge organisms such as *Fusobacterium nucleatum* that mediate coaggregation and coadhesion (14). The 'red complex' pathogens *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* appear later in the biofilm development and have been found at sites with progressing periodontitis (1,10).

*P. gingivalis* is characterized as a 'keystone pathogen' because it promotes a larger effect on the survival and virulence of the entire microbial community than its numbers would indicate (9,11). This concept contrasts with most other inflammatory diseases where the dominant species suppresses the growth of commensal bacteria and shows high abundance at disease sites.

*P. gingivalis* is an oral Gram-negative black pigmented anaerobic bacterium that belongs to the phylum bacteroidetes. It is asacchorolytic and does not metabolize sugars such as glucose but depends on nitrogenous substrates such as peptides as a source of carbon and energy. It also requires iron for its growth (15). *P. gingivalis* has the ability to produce and secrete proteolytic enzymes, known as gingipains, that cause destruction of tissues supporting the teeth. The multiple proteases produced by *P.gingivalis* have a major impact on degrading important substrates such as collagen (a major component of periodontal connective tissue), fibronectin, fibrinogen, laminin (an extracellular matrix protein), and keratin that are found in the gingival crevice (15,10). Unlike the majority of

bacteria, *P. gingivalis* lacks a siderophore system to acquire iron and cannot synthesize heme. Hemin is thought to be acquired by proteolytic processing of hemin containing compounds. It is then stored on the surface of the cell giving rise to black pigmented *P. gingivalis* colonies on blood agar (20). Stored hemin is transported via an energy dependent process during starvation of *P.gingivalis* cells when they reside in healthy periodontal environment. Hemin is also known to regulate virulence associated activities and membrane bound hemin can scavenge oxygen to provide an anaerobic environment for *P. gingivalis* cells (20) *P. gingivalis* produces multiple virulence factors such as proteinases (e.g. gingipains), fimbriae, hemagglutinins, and capsular polysaccharides that are essential for interaction with the host and other bacterial cells (5,10).

It is now thought that the commensal microbiota and host complement are both required for *P. gingivalis* induced bone loss. The periodontium produces select innate host defense mediators (e.g. cytokines) that contribute to the clearance and killing of dental plaque bacteria. Recent studies on murine models show that *P. gingivalis*-mediated bone loss is dependent on the complement receptor C5aR activation. The *P. gingivalis* cysteine proteases (gingipains) cleave the complement component C5 and convert it to C5a that activates C5aR. This activation inhibits the TLR2 (Toll-like receptor) dependent cytokine induction and leads to suppression of the killing activity of phagocytes. This allows *P. gingivalis* to escape cytokine dependent immune clearance (17).

*P gingivalis* is not only associated with periodontitis, but also appears to be a risk factor of cardiovascular disease, diabetes, preterm labor and low birth weight (8,13). Therefore identification of unique physiological and metabolic properties of this organism is important in developing novel drug targets to eliminate this pathogen.

Although numerous genetic tools such as plasmids, transposons, and suicide vectors have been developed for the manipulation of other bacterial species, many are not suitable for genetic manipulation of *P. gingivalis*.

**Transposon mutagenesis**

Transposon mutagenesis is a very powerful technique where a transposable element is integrated randomly into different sites of the host genome. This results in mutations that allow identification of new genes and of functions of known genes. Although the transposons Tn*4351* and Tn*4400* were used in previous studies of *P.gingivalis*, their insertion was not completely random, and in many instances they resulted in multiple insertions (6). This is not ideal because it complicates the identification of disrupted genes responsible for the selected pehenotype. In this study we used a mariner based mini-*himar* transposon system (pHimar EMf) that resulted in stable random insertions. Recently, the complete genome of *P. gingivalis* strain ATCC 33277 has been sequenced (19). The transposon inserts at 'TA' sequences that are readily available throughout *P. gingivalis* genome. pHimar EMf is a derivative of pHimar EM1 (4)(Figure 2) that was successfully used to generate transposon mutants in *Flavobacterium johnsoniae*, a relative of *P. gingivalis*. In this study, we modified the above transposon by the insertion of *P. gingivalis fim*A promoter upstream of the transposase gene. Use of *fimA* promoter is expected to increase the efficiency of the transposition in contrast to the *lac* promoter present in the original construct. We used the new transposon to generate mutants of *P. gingivalis* strain ATTC 33277.

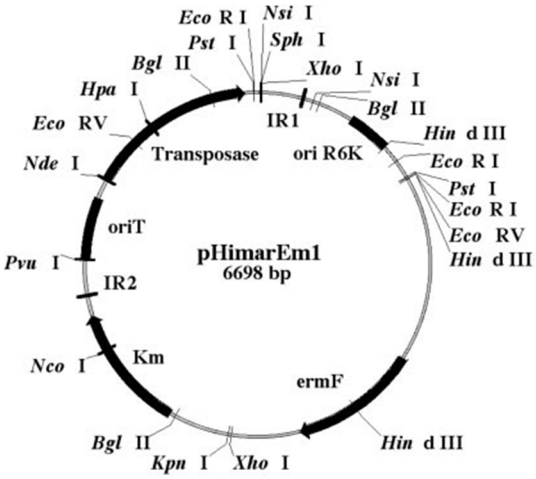


**Figure 2 :** Map of pmini*himar* EM1. Locations of oriT-Plac upstream of the transposase gene, Inverted repeats ( IR1 and IR2), Kanamycin (Km) resistance gene and Erythromycin (ermF) resistance gene are indicated (4).

## Materials and methods

### Bacterial strains and growth conditions

*P. gingivalis* ATCC 33277 was grown anaerobically using a Coy anaerobic chamber at 37ºC on TSY (Trypticase Soy Yeast) agar supplemented with menadione (1 µg/ml), hemin (10 µg/ml) and cysteine (0.5 mg/ml) or on TSY agar supplemented with 5% sheep blood. *E.coli* strain WM3064 as grown aerobically at 37ºC in Luria Bertani (LB) media supplemented with diaminopimelic acid (DAP – 0.25 mM). *E. coli* EC100D$^+$ cells were grown at 37ºC on LB. Kanamycin (25 µg/ml), and ampicilin (150 µg/ml) were added as appropriate.

### *OriT* –$P_{FimA}$ crossover PCR

A fragment that carries *oriT* (origin of transfer) was amplified from pJB3Cm6 (2) using the primers OriT F- CATGCGATCGAGGCGATTAAGTTGGGTAAC ( *Pvu*I site underlined), OriTR/fimA – CGTTTCGTAGCGTTCGTGGCTCTGCATAACCCTGCTT, and PHUSION

polymerase. A fragment that carries the *P. gingivalis fimA* promoter ($P_{fimA}$) was amplified using fimAF - GAGCCACGAACGCTACGAAACG and PfimAR-GATCCATATGGCTGATGGTGGCATTACCTTCTGTAAC (*Nde*I site underlined) using *P. gingivalis* ATCC 33277 DNA as a template. The *OriT*-$P_{fimA}$ fusion was obtained by crossover PCR of *oriT* and $P_{fimA}$ fragments using PHUSION polymerase (New England BioLabs) with the outside primers oriTF and PfimAR.

**_OriT_ –$P_{fimA}$ cloning in pUC119**

The plasmid pUC119 was digested with *Sma*I then ligated to the oriT-$P_{fimA}$ fragment using T4 DNA ligase (Fisher Scientific). The ligation mixture was digested with *Sma*I to reduce background blue colonies. The ligated DNA was precipitated with 0.3M sodium acetate and 70% ethanol, resuspended in dH2O, and then used to transform *E. coli* (EC100D+) cells by electroporation. Cells were plated in LB agar supplemented with ampicillin (150 µg/ml), 5-bromo-4-chloro-indolyl-galactopyranoside (X-gal -50 µg/ml) and Isopropyl-β-D-thio-galactoside (IPTG-80 µM). White colonies were isolated and tested for presence of plasmid that carries the desired insert using an in-gel lysis protocol. Small amounts of colonies were mixed with 10 µl of protoplastic buffer for five minutes. The mixture was added into the wells of an agarose gel containing 5µl of lysis buffer and allowed cells to lyse for 5 minutes. Samples were separated from wells at 70V and DNA bands were identified under the UV light. Colonies that appeared to carry the desired plasmid were grown in LB medium and used to purify plasmid DNA using QIAGEN QIA prep spin miniprep kit. The presence of oriT-$P_{fimA}$ in these plasmids was confirmed by PCR.

**pHimar EM1 modification**

pHimar EM1 and oriT-$P_{fimA}$ were digested with *Nde*I and *Pvu*I. Digestion of pHimarEM1 removes the *oriT* and the *lac* promoter located upstream of the transposase gene. DNA fragments were isolated from agarose gel using QIAGEN QIAEX Gel extraction kit following purification with QIAGEN spin column purification kit. The fragments were ligated using T4 DNA ligase (Fisher Scientific). Insertion of oriT-$P_{fimA}$ was confirmed by PCR and DNA sequencing. The plasmid was then transferred to *E. coli* WM3064 cells by heat shock and recombinant colonies were selected on LB agar supplemented with 25 µg/ml kanamycin and 0.25 mM DAP.

**Transposon Mutagenesis**

*E.coli* WM3064 containing pHimarEMf was grown aerobically in LB liquid at 37°C overnight and wild type *P. gingivalis* ACCT 33277 was grown on blood agar plates anaerobically for six days at 37°C as described above. *P. gingivalis* ACCT 33277 cells were scraped off the agar and mixed with *E.coli* WM3064 harboring pHimar EMf in 1:1 ratio. The cell mixture was spotted on enriched TSY agar that contains 1 µg/ml menadione, 10 µg/ml hemin, and 0.5 µg/ml cysteine. Following overnight incubation at $37^0$C in an anaerobic gas pack, the cells were scraped off the agar and plated on enriched TSY agar plates supplemented with erythromycin (10 µg/ml) to select for mutants with transposon insertions. Mutants that exhibited altered pigmentation or growth deficiency were selected for further analysis. To identify transposon-disrupted genes, chromosomal DNA was isolated using EpiCentre MasterPure DNA purification Kit. The purified DNA was digested with *Bam*H1. The insertion sequence lacks *Bam*H1 restriction sites. The digested DNA was self- ligated and then used to transform *E. coli* EC100D+.

Transformed cells were plated on LB agar supplemented with 25 μg/ml kanamycin. Plasmid DNA was purified from kanamycin resistant colonies and analyzed on agarose gels to determine the size of purified plasmids. Sites of transposon insertion were identified by DNA sequencing using Phimar EM1- primer 615 (TCGGGTATCGCTCTTGAAGGG) (3,4). Sequences were analyzed with Mac Vector software and comparisons and predictions of sequences were made using BLAST algorithm. *P. gingivalis* ATCC 33277 genome information was obtained from http://www.genome.jp/kegg-bin.

## Results and Discussion

### Generation of pHimar EMf

In an effort to identify *P. gingivalis* genes that are important for growth, we initially used pHimar EM1 for generation of transposon mutants. Expression of the transposase gene, which is carried on this plasmid, is driven by the *E. coli lac* promoter. Transposition was not efficient, and very few colonies were obtained following mating with *E. coli*. This inefficiency is thought to be due to poor expression of the transposase gene. *P. gingivalis* promoters differ from those of *E. coli*, and *E. coli* promoters do not generally function in *P. gingivalis* (12).

To overcome the problems encountered in using pHimar Em1, I replaced the *lac* promoter present upstream of the transposase on this plasmid with the *P. gingivalis* 33277 *fimA* promoter (21). This promoter drives the expression of *fimA* that encodes the structural protein of the major fimbriae that are considered a major virulence factor of *P. gingivalis*. FimA facilitates the adherence to oral tissues and also to surrounding bacterial cells and contribute to intracellular invasion. Replacement of the *lac* promoter in pHimar

EM1was accomplished by removal of the *oriT*/$P_{lac}$ by digestion with PvuI and NdeI and ligation of the resulting plasmid DNA to *oriT*/$P_{fimA}$. This resulted in the generation of pHimar EMf, and the insertion of $P_{fimA}$ upstream of the transposase was confirmed by DNA sequencing (Figure 3).

TTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTAATTCTTGAAGACGAA
AGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTA
GAGCTTATCGGCCAGCCTCGCAGAGCAGGATTCCCGTTGAGCACCGCCAGGTGCGAA
TAAGGGACAGTGAAGAAGGAACACCCGCTCGCGGGTGGGCCTACTTCACCTATCCTG
CCCGGCTGACGCCGTTGGATACACCAAGGAAAGTCTACACGAACCCTTTGGCAAAAT
CCTGTATATCGTGCGAAAAAGGATGGATATACCGAAAAAATCGCTATAATGACCCCG
AAGCAGGGTTATGCAGAGCCACGAACGCTACGAAACGTGGACGATAGCAGAGAAAA
CTTTCCCGAAAGCGATAGAGCCGACAGCTAATGCGGCCATAATAGACCTTCGTGCCG
GTAGGTATCCGCAGGCTCTGGCTCGCCTCGAAGCACGCAAGAGCGAACCCAAGCTAT
GGATGTTGTTGGGCTTGGCATATGCCTACAGCGAAAAATGGGCTGAAGCCGAGAGCT
ATCTTACTCGCGCTGCGCAGCAAGGCCAGCCCGGAGCACAACACAATCTGAACGAA
CTGCGACGCTATATGCAAGACAATCTCTAAATGGGAAAAGATTAGATTTTTAGAAAA
CAATATTCACTTTTAAAACAAAAACGAGATGAAAAAAACAAAGTTTTTCTTGTTGGG
ACTTGCTGCTCTTGCTATGACAGCTTGTAACAAAGACAACGA

**Figure 3:** DNA sequence of oriT-P*fimA* insert (sequencing result) obtained by crossover PCR. Sequence highlighted in yellow color represents *fimA* promoter region and the sequence highlighted in grey color represents the oriT region of pHimar EM1.

pHimar Emf was successfully used to generate *P. gingivalis* mutants with a transposition efficiency of $1x10^{-9}$.

**Isolation of *P. gingivalis* mutants deficient in growth or pigmentation**

The transposon *himar*EM2 was transferred from *E. coli* into *P. gingivalis* by conjugation. Several conditions for the mating procedure were used to determine conditions that lead to high transposition efficiency. The use of *P. gingivalis* grown in liquid enriched TSY medium did not result in efficient transfer of pHimar EM2 into these cells. The reasons for this are not known. Therefore, we used cells grown on solid media

(enriched TSY or blood agar), and our experiments indicate that use of *P. gingivalis* cells grown on blood agar results in the highest transposition efficiency.

Following conjugation, *P. gingivalis* mutants were selected on blood agar plates. Mutants that exhibited reduced red pigmentation or slower growth (i.e. smaller colonies) were isolated for further analysis. *P. gingivalis* 33277 grown on blood agar plates is almost black in color due to the accumulation of heme on the surface of the colonies. We isolated mutants that had either reduced or no black pigmentation in an attempt to identify genes that are involved in heme uptake. In addition, we targeted the isolation of mutants that form small colonies compared to the wild type, since these mutants are expected to have insertions in genes important for metabolism and growth of the bacterium.

**Identification of transposon insertion sites in *P. gingivalis* mutants**

The use of pHimar EMf allows rapid identification of transposon insertion sites in the genome of mutants of interest. The insertion sequence contains an R6K origin of replication, and therefore, digestion and self-ligation of chromosomal DNA results in a plasmid that can be maintained in *E. coli* strains that express the Pir protein (3). In addition, the sequence of pHimar EMf is known, and primers to sequence DNA adjacent to the transposon insertion have been designed (4). Furthermore, the presence of the transposase gene outside the insertion element ensures the generation of stable mutants. We have isolated several *P. gingivalis* mutants, and used protocols described above to identify the disrupted genes. Our analysis identified genes that are predicted to encode glycosyl transferases, cation efflux system proteins, $Na^+$translocating NADH-quinone

reductase and von Willebrand factor A protein. A list of identified genes and their predicted functions is given in Table 1.

**Table 1** Examples of genes disrupted in *P.gingivalis* strains obtained using pHimar EMf. Gene loci and the known or predicted functions of these disrupted genes are indicated

| Gene Locus | Known or predicted function |
|---|---|
| PGN 0995 | TPR Domain Proteins |
| PGN 0996 | TPR Domain Proteins |
| PGN 0427 | glycosyl hydrolase; K07405 alpha-amylase |
| PGN 2014 | Cation Efflux System Protein/ HlyD secretion protein – haemolysin translocator |
| PGN 0114 | Na(+)-translocating NADH-quinone reductase subunit |
| PGN 0361 | glycosyl transferase family 2; K07011 |
| PGN 1812 | polyphosphate kinase |
| PGN 2081 | 1-deoxy-D-xylulose-5-phosphate synthase |
| PGN 1468 | lipoyl synthase; K03644 lipoic acid synthetase |
| PGN 0361 | glycosyl transferase family 2 |
| PGN 0067 | probable conserved transmembrane protein found in conjugate transposon TraE |
| PGN 1396 | anaerobic ribonucleoside triphosphate reductase |
| PGN 0959 | transcriptional regulator |
| PGN 1926 | transposase in ISPg1 |
| PGN 0531 | von Willebrand factor A |
| PGN 0539 | metallo-beta-lactamase superfamily protein |
| PGN 0787 | conserved hypothetical protein |
| PGN 0788 | peptidyl-dipeptidase |
| PGN 0618 | aspartate-semialdehyde dehydrogenase |
| PGN 0619 | conserved hypothetical protein |
| PGN 1613 | ATP-dependent DNA helicase RecG |
| PGN 1612 | probable beta-phosphoglucomutase |
| PGN 0359 | ABC transporter permease protein Suf D |

Previous studies done on mutants defective in putative glycosyl transferases in *P.gingivalis* exhibited defective biosynthesis of the polysaccharide portions of lipopolysaccharide, decreased gingipain activities, strong autoaggregation, and increased biofilm formation thus showing a great impact on *P.gingivalis* virulence (22).PGN 2014 cation efflux pump protein contains a HlyD domain that is largely present in bacterial haemolysin translocator HlyD proteins and RND family efflux transporter that is predicted to function in nodulation, acriflavin resistance, heavy metal efflux and multidrug resistance proteins. It has been reported that a RND pump translocator in *V. cholera* is involved in bacterial colonization in the host, and also in the ability of *P. aeruginosa* to infect cell cultures, hence it is involved in colonization and virulence (16).

*nrqB* encodes a subunit of the $Na^+$-translocating quinol oxidoreductase, which is a component of complex I in the *P.gingivalis* electron transport chain and is required for the optimum growth of the organism (18).

Von Willebrand factor A domain is found in the blood coagulation protein Von Willebrand factor that is involved in different cellular functions including adhesion, haemostasis, signaling and immune defense in intergrins.

As described above, the modified pHimar EMf mutagenesis system not only successfully allows the generation of stable mutants but is also effective in easy analysis to locate the insertion site in the disrupted genes. Although this system meets the expectations it is essential to investigate if the insertions introduced by this system are single or multiple by performing a southern transfer. These promising preliminary results can lead to the use of this mutagenesis system to aid in locating important genes involved in the basic metabolism and physiology of *P.gingivalis*.

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# CHAPTER 2

## Role of NosA in sulfite reduction and copper transport in *Shewanella oneidensis* MR-1

## Introduction

Prokaryotic and Eukaryotic organisms generate energy via respiration by moving electrons down a potential energy gradient toward acceptors with more positive redox potential. In aerobic respiration, oxygen is used as the terminal electron acceptor whereas in anaerobic respiration uses different exogenous electron acceptors other than oxygen. Examples include nitrite, sulfate, sulfite, and metal oxides. Many bacteria and archaea carry out anaerobic respiration reflecting the flexibility of energy-generation that allows their survival in diverse habitats. Anaerobic respiration also plays a major role in biogeochemical cycling of elements and thus has an ecological and economic significance. Applications of the reduction process of anaerobic respiration include bioremediation of environmental pollutants (radionuclides, toxic elemental waste), and in biotechnology for energy generation through microbial fuel cells. For example, U(VI) which is a major source of nuclear reactions becomes insoluble U(IV) when reduced. This prevents the movement of uranium through groundwater. Similarly, soluble forms of Cr(VI) tend to precipitate when reduced to Cr(III) which aids in the environmental cleanup processes (1).

*Geobacter* and s*hewanella* species are well known metal reducers that have the ability to transfer electrons to electrode surfaces that can be used for electricity generation (2). Further developments of these processes will lead to applications in wastewater treatments combined with energy production. Therefore identifying and analyzing the metabolic pathways of these species is important, if we are to manipulate

and develop these organisms for such applications. This study focuses on sulfite reduction during anaerobic respiration of *Shewanella oneidensis*.

*Shewanella oneidensis* MR-1 is a facultative anaerobic Gram-negative and metal reducing bacterium and belongs to group of gamma Proteobacteria (4). *S. oneidensis* MR-1 has the ability to use a number of different terminal electron acceptors such as $O_2$, thiosulfate ($S_2O_3^{-2}$), tetrathionate ($S_4O_6^{-2}$), sulfite ($SO_3^{-2}$), elemental sulfur, insoluble metal oxides, fumarate, dimethyl sulfoxide(DMSO), trimethylamine N-oxide (TMAO), nitrate and nitrite (9). There are several terminal reductases and 42 *c* cytochromes that are predicted in the MR-1 genome that are responsible for this respiratory versatility in this organism (8).

**The role of copper in respiration**

Cu is essential for many respiratory enzymes such as cytochrome *c* oxidases and anaerobic nitrous oxide reductases. Free copper is toxic, and therefore Cu specific chaperones and porins are needed to take Cu into the periplasm (12). There may be copper specific or non-specific porin proteins in the outer membrane that allow copper diffusion into the cell and are not yet identified in MR-1. It is known that the octahaem SirA (sulfite reductase), *c* type cytochrome catalyses dissimilatory sulfite reduction in *Shewanella oneidensis* MR-1 (11). SirA is predicted to be a heme-copper protein and Cu may be involved in its activity. Sir A represents a novel class of enzyme that appears to be common to all *Shewanella* species that reduce sulfite (10). SirA is located in a ten-gene cluster (Figure 4), and homologs of SirGCDJKLM are found in other different species *Pseudomonas syringae* pv. tomato T1, *Wolinella succinogenes*,

*Thermodesulfovibrio yellowstonii* DSM 11347, *Vibrio fischeri* ES114, *Salmonella typhimurium*, *Escherichia coli* and *Pseudomonas aeruginosa* PAO1 respectively (10). NosDFY encodes an ATP-binding cassette (ABC) copper transporter and NosL encodes a copper chaperone. Sir JKLM homologs NosLDFY plays a major role in transporting copper to Cu-S nitrous oxide reductase (NosZ) thus involved in maturation of nitrous oxide reductase (6). The copper specific porin NosA from *Pseudomonas stutzeri*, is thought to form a pore through which copper can enter the periplasm before being delivered to the copper-containing $N_2O$ reductase (7). NosA belongs to the family of proteobacterial TonB-dependent outer membrane receptor/transporters and is predicted to bind and translocate copper ions. Also *nos* genes are transcribed constitutively at a low level in *Pseudomonas stutzeri* (6). Therefore in this study we hypothesize that the *S. oneidensis* NosA is expressed under aerobic and anaerobic conditions and it is involved in biogenesis of the sulfite reductase (SirA). We also predict the presence of other porins that may be involved in copper diffusion in MR-1.

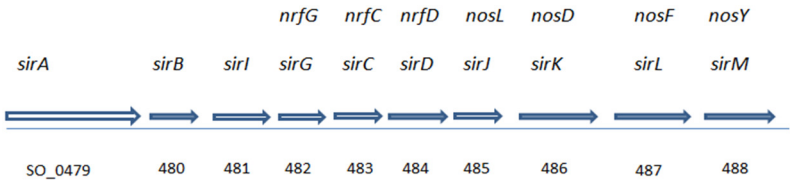


**Figure 4:** sir A gene cluster .sirA belongs to a ten gene cluster including sirB,I,G,C,D,J,K,LM in MR-1 and homologs of these genes are indicated as nrfG,C,D, nosL,D,F,Y in other species (10).

## Materials and methods

### Bacterial strains and growth conditions

Aerobic cultures of *Shewanella oneidensis* MR1, and *E. coli* strains were grown in LB at 30ºC and 37ºC respectively. Anaerobic cultures of *S. oneidensis* strains were grown in basal medium (pH 7.4) supplemented with 50 mM lactate and 0.02% casamino acids. DMSO and fumarate were added as electron acceptors at 10 mM. Growth and reduction of sulfite (10 mM) was performed anaerobically in a Coy anaerobic chamber in biometer flask. The side arm of the flasks contained 10 ml of 40% KOH to trap $H_2S$ (5).

### Generation of nosA mutants

1 kb DNA fragments upstream and downstream of *nosA* were generated by PCR using the primers listed in Table 1.1 and Phusion polymerase. Each fragment was ligated into the high copy number plasmid pUC118 individually and religated using the BamH1 restriction sites that were engineered in the internal primers. The ligated 2Kb fragment was cloned into the SmaI site of the suicide plasmid pER21 (10). Recombinant plasmids were transferred from *E. coli* β2155 to wild type *S. oneidensis* by conjugation. Mutants were selected on 5% sucrose and confirmed by PCR using outside primers of the 2Kb fragment. *psrA*/*nosA* double mutant was generated as using the previously made Δ*psrA* as the background as described above.

### Transposon mutagenesis in Δ nosA background

Deletion of nosA did not affect sulfite reduction due to the presence of additional porins that allow copper diffusion into the cell. To identify other porins SR1514 (Δ*nosA*) was conjugated with *E.coli* cells containing pMini*Himar* RB1 plasmid for 4 hours. Transconjugants were plated on basal medium containing 10 mM DMSO, 15 μM $CuCl_2$,

0.02% Casamino acid, and 50 mM lactate. Wild type cells are sensitive to this copper concentration. Copper resistant mutants are expected to have disrupted genes that are involved in active copper import across the outer membrane or that encode additional porins that allow copper diffusion into the periplasm.

Cu resistant mutants with transposon insertions were analyzed by anaerobic growth assays with regular basal media (0.6 µM $CuCl_2$) and with 1 µM $CuCl_2$.

**$H_2S$ detection assay**

$H_2S$ detection agar medium containing basal media, 1% agar, 10mM sodium sulfite, 0.015% ferrous sulfate, 0.02% casamino acid and 50mM lactate was used to test the mutants for sulfite reduction. Glass vials contacting 5ml of the above agar medium were stab-inoculated with cultures grown on LB agar plates. Formation of a black precipitate (FeS) was recorded at 24 hour intervals for 2-3 days (10).

To measure sulfite reduction by the wild type and mutant strains, cells were grown anaerobically in 100 ml basal media in a Coy anaerobic chamber in biometer flasks. The basal medium was supplemented with 50 mM lactate, 0.02% casamino acids, and 10 mM sulfite as an electron acceptor. The side arm of the flasks contained 10 ml of 40% KOH to trap $H_2S$ (5). MR-1 and mutant cells were grown overnight in basal medium and 500 µl of each culture was used as inoculum. The flasks were sealed and incubated in the anaerobic chamber for up to 96 hrs.

The amount of $H_2S$ that was produced was measured using the methylene blue assay (3). 500µl of the KOH trap was removed and mixed with 25 ml of $dH_20$ in the chamber. 1 ml of mixed diamine reagent (20 g *N, N*-dimethyl-*p*-phenylenediamine sulfate

and 30 g $FeCl_3 \cdot 6H_20$ in 500 ml of cool 50% hydrochloric acid) was added to the mixture. After allowing the color to develop for 20 minutes the optical density (OD) was measured at 670 nm. Hydrogen sulfide concentrations were calculated using sodium sulfide as a standard.

**Results and discussion**

Qualitative $H_2S$ detection assay was performed to determine the sulfite reduction in *nos*A mutant (Figure 5). Compared to MR-1(wild type), *nosA* mutant shows reduced $H_2S$ accumulation indicating reduced sulfite reduction with different Cu concentrations. Also this experiment indicated increased sulfite reduction with increasing Cu concentrations in the media except 50 µM Cu. This may be the lethal effect of higher Cu concentration on cells. Higher amounts of copper accumulation inside cells could be toxic to cells and cause cell damage by formation of hydroxyl radicals. According to the qualitative $H_2S$ detection assay (figure 5), *nosA* deletion mutant (Δ*nosA*) shows less formation of black precipitate indicating less sulfite reduction compared to wild type (MR-1). These results suggest that NosA is involved in sulfite reduction and copper diffusion into the cell. However Δ*nosA* mutants did not show complete elimination of black precipitate which indicates other porins that allow copper into the cell that being used in sulfite reduction.

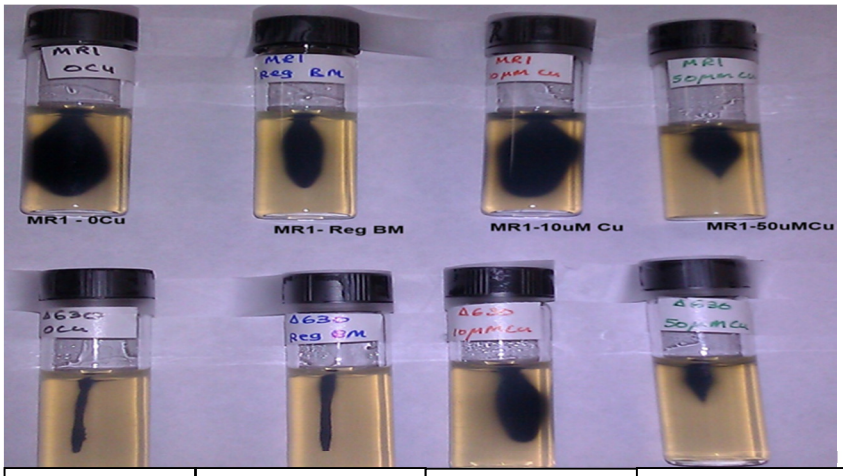


**Figure 5:** Qualitative sulfite reduction assay. Cells were incubated in basal media containing 10 mM sodium sulfite and 0.015% $FeSO_4$ with different Cu concentrations. Formation of black precipitate indicates sulfite reduction. Sulfite reduction is reduced in Δ*nos*A compared to MR-1.

Quantitative sulfite reduction assay was performed to calculate the amount of $H_2S$ being produced during the sulfite reduction. Compared to the wild type, *nosA* mutant did not show a significant difference in sulfite reduction in the quantitative assay. This could be due to the formation of the intermediate thiosulfate in the biometer flask during the experiment. Thiosulfate can be chemically produced from $SO_3$ and $H_2S$ before it is completely reduced to $H_2S$. Sulfite reduction was reduced significantly when *psrA* was deleted in the Δ*nosA* background. *psrA* is responsible for thiosulfate reduction where thiosulfate gets reduced to $H_2S$. In the absence of *psrA* and *nosA* , the production of $H_2S$ is only through sulfite reduction (figure 6). Although the production of $H_2S$ by *psrA* deletion mutatnt (Δ*psrA*) has not reported, it is important as another comparison in this experiment.

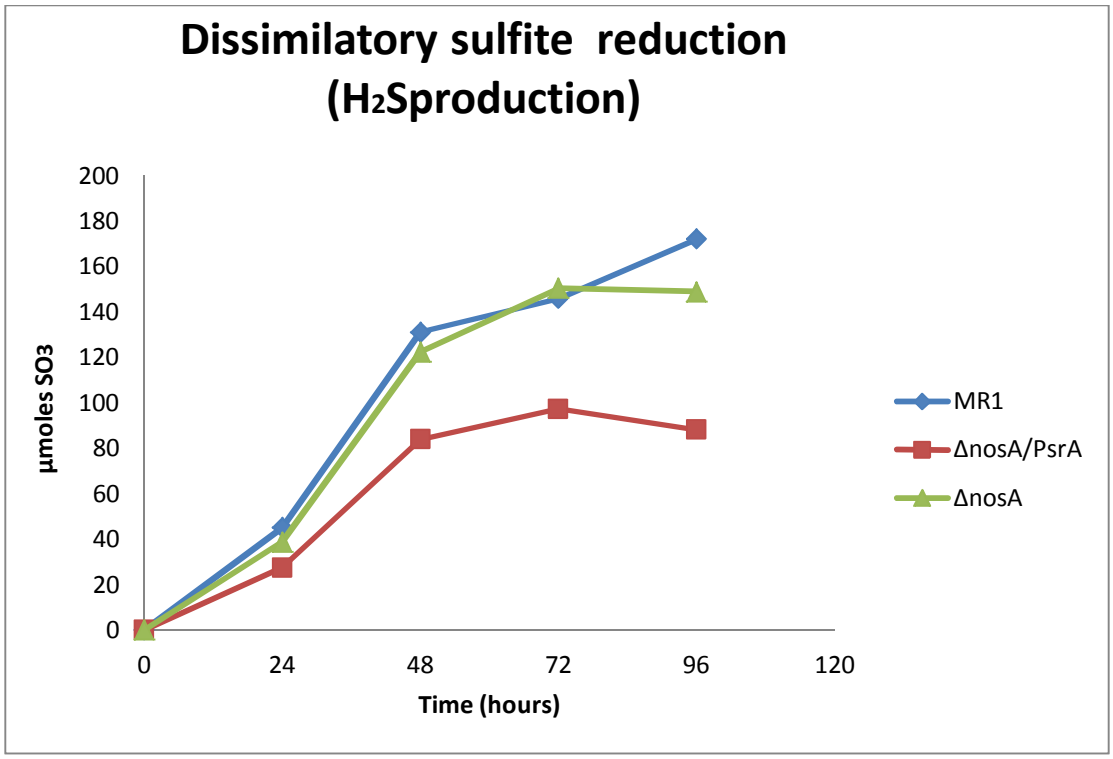


**Figure 6:** Graph of dissimilatory sulfite reduction. Dissimilatory sulfite reduction ($H_2S$ production) in basal media without added copper.Cells were incubated anaerobically in biometer flasks and $H_2S$ was trapped in the sidearm using KOH.
Unlike in qualitative stab assay, the difference in sulfite reduction between Δ*nos*A and in MR-1 is not significant in the quantitative sulfite reduction assay. But sulfite reduction is significantly decreased in the double mutant of Δ*nosA*/*psrA*.

## Conclusions

Sulfite reduction is decreased in Δ*nos*A compared to MR-1. NosA may act as a copper porin similar to its function in *P. stutzeri*. However, it may not be the only porin that allows copper into the cell. Furthermore complementation of *nosA* is essential to analyze if the phenotype can be restored when complemented. Also the promoter expression of *nosA* under aerobic and anaerobic conditions needs to be analyzed.Although *nos*A deletion reduced sulfite reduction it did eliminate considerable amount of sulfite reduction in the stab vials in the qualitative sulfite assay. This leads to the next hypothesis we made, that there may be other porin proteins that are involved in copper diffusion in MR-1. Himar mutagenesis was performed using Δ*nos*A background

to isolate other genes that are involved in Cu diffusion. 15 µM $CuCl_2$ was used to screen for the *himar* mutants deficient in copper uptake. Results of preliminary *himar* mutagenesis studies did not identify any significant genes that may be responsible for copper uptake by *S. oneidensis*.

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