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Title: Iron Acquisition and its Regulation in *Nitrosomonas europaea*

Abstract approved: _____
Daniel J. Arp

Iron is absolutely necessary for the maintenance, the defense, the differentiation and last, but by no means least, the growth and cellular division of almost all living organisms including *Nitrosomonas europaea,* an ammonia oxidizing bacterium. Given the necessity of iron, it is not surprising that *N. europaea* evolved several iron-dependent proteins to perform many essential functions. Here, we used computational and molecular techniques to study the iron transport systems and their regulation in *N. europaea*.

Despite its high iron requirement, *N. europaea* does not produce siderophores. However it can grow in media containing relatively low iron by lowering its cellular iron and heme contents and by efficiently allocating the available iron to required iron-dependent proteins. Similar to other Gram-negative bacteria, *N. europaea* is responding to iron limitation by increasing transcript levels of siderophore-dependent iron acquisition systems. In addition, transcripts of several hypothetical proteins with putative novel roles in iron acquisition were also present in higher levels when *N. europaea* is grown in iron limited conditions. Most of the genes involved in iron uptake that were mentioned above appear to be regulated by either iron alone or iron

and Ferric Uptake Regulator (Fur) protein in *N. europaea*. In addition, it appears that *N. europaea* Fur also plays a role in maintaining a balance between acquiring enough iron and allocating it to various iron-dependent proteins.

N. europaea is capable of taking up Fe^{3+}-bound ferrichrome, ferrioxamine, pyoverdine via nitABC Fe-transporter. *N. europaea* might be having alternative means for transport of Fe^{3+} or Fe^{2+} ions across the cytoplasmic membrane and also Fe^{3+}-bound to enterobactin and aerobactin siderophores.

Iron Acquisition and its Regulation in *Nitrosomonas europaea*

by
Neeraja Vajrala

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APPROVED:

Major Professor, representing Molecular and Cellular Biology

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Neeraja Vajrala, Author

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CONTRIBUTION OF AUTHORS

Daniel J. Arp contributed to study design, data interpretation and manuscript preparation of all chapters. Dr. Luis Sayavedra-Soto contributed to study design, data interpretation and manuscript preparation of all chapters. Dr. Peter J Bottomley contributed to study design and data interpretation for all chapters. Dr. Xueming Wei contributed to study design, determination of Fe, siderophore concentrations, mass spectrometry analysis of Chapter 2. Dr. Xueming Wei performed cell fractionation, membrane preparation, and heme and protein quantification, determination of citrate concentrations, protein gel electrophoresis, sample preparation for electron microscopy, data interpretation and manuscript preparation of Chapter 2. Dr. Loren Hauser contributed to organization of genes related to iron acquisition in chapter 2. Dr. Luis Sayavedra-Soto performed Southern analysis of *fur-kanP* mutant in Chapter 5. All experiments were financially supported and conducted in the laboratory of Dr. Daniel J. Arp at Oregon State University.

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IRON ACQUISITION AND ITS REGULATION IN *Nitrosomonas europaea*

CHAPTER 1.

INTRODUCTION

'Gold is for the mistress – Silver for the maid –
Copper for the craftsman cunning at his trade.'
'Good!' said the Baron, sitting in his hall,
'But Iron – Cold Iron - is master of them all.'

- Rudyard Kipling 'Cold Iron' (1902)

In the view of "iron people' this verse illustrates the fundamental importance of iron in biological systems.

1.1. Importance of Iron in Biological Systems:

Iron is a nutrient universally required by almost all living organisms. It is generally required for a large number of metabolic processes, such as photosynthesis, N_2 fixation, methanogenesis, H_2 production and consumption, respiration, the tricarboxylic acid (TCA) cycle, oxygen transport, gene regulation and DNA biosynthesis (Table 1.1)[1]. The importance of iron to living organisms is is due to the intrinsic properties of this element. Iron can exist in various oxidation states (from Fe^{-2} to Fe^{+6}), the principal ones being Fe^{2+} and Fe^{3+}[1]. Iron is also an abundant metal, being the fourth most plentiful element in the earth's crust. These properties make iron an extremely versatile prosthetic component for incorporation into proteins as a biocatalyst or electron carrier [1]. If life without iron exists, it is probably to be found among certain members of lactobacilli [2]. However, in aerobic environments, iron mostly exists in ferric state as an insoluble hydroxide (10^{-18} M at pH 7.0)[1]. This concentration is well below than what is generally required by cells (10^{-8} to 10^{-6} M) [1].

Electron transfer proteins	**Tricarboxylic acid cycle**
Cytochromes	Aconitase
Hydrogenase	Succinate dehydrogenase
Iron-sulfur proteins (Ferredoxin)	
	DNA biosynthesis
H_2O_2 and O_2 metabolism	
	Ribonucleotide reductase
Catalase	
Peroxidase	**N_2 fixation**
Oxygenase	
Superoxide dismutase	Nitrogenase

Table 1.1. Selected bacterial Iron-containing proteins.
Taken from Neilands. J. B. Ann. Rev. Nutr. 1981. 1: p. 27-46.

Thus, a plethora of microorganisms are severely restricted in iron availability. During evolution, this restriction gave an advantage to those microbes that developed skills to circumvent this nutritional limitation in several ways. Numerous studies over the ensuing 60 years have shown an impressive array of systems that microbes use to solubilize and capture iron from the environment (Table 1.2) [3].

1.2. High Affinity Iron Uptake Systems:

The first step in microbial high affinity iron assimilation is, by necessity, solubilization of insoluble iron. This solubilization can be achieved in two ways: (i) chelation: employing ferric ion chelators called siderophores as solubilizing agents; or (ii) reduction: reducing ferric iron to the relatively soluble ferrous form [3]. These systems are called high affinity systems because of their ability to scavenge iron from environments with low iron contents and require a cognate transport apparatus [3]. Siderophores are synthesized only when the cells are in a state of iron starvation [1], hence siderophores are probably not involved in primary iron mobilization in environments that contain adequate, available iron.

1.2.1. Siderophore-dependent Iron Uptake Systems:

Most microbes rely on ferric chelators called siderophores to chelate iron prior to transport [4]. Numerous bacterial proteins are involved in siderophore mediated iron uptake and considerable variation has been found in the uptake schemes used by different bacterial species [1]. Gram-negative bacteria take up Fe^{3+}-siderophore complexes via specific outer membrane (OM) receptors in a process that is driven by the cytosolic membrane (CM) potential and mediated by the energy-transducing TonB-ExbB-ExbD system. Periplasmic binding proteins shuttle ferric-siderophores

Strategy	Example
Abstain from using iron	*Lactobacillus* spp.
Reduce Fe^{3+} and transport Fe^{2+}	*Legionella* spp., *Streptococcus* sp.,
Chelation of Fe^{3+} using siderophores	Many bacterial genera
Acquire iron directly from host iron sources	
Transferrin	*Neisseria* spp., *Hemophilus influenzae*
Lactoferrin	*Helicobacter pylori*
Heme	*Vibrio* spp., *Yersinia* spp.,

Table 1.2. Strategies of bacteria for acquisition of iron.
Taken from Guerinot, M.L., Annu Rev Microbiol, 1994. 48: p. 743-72.

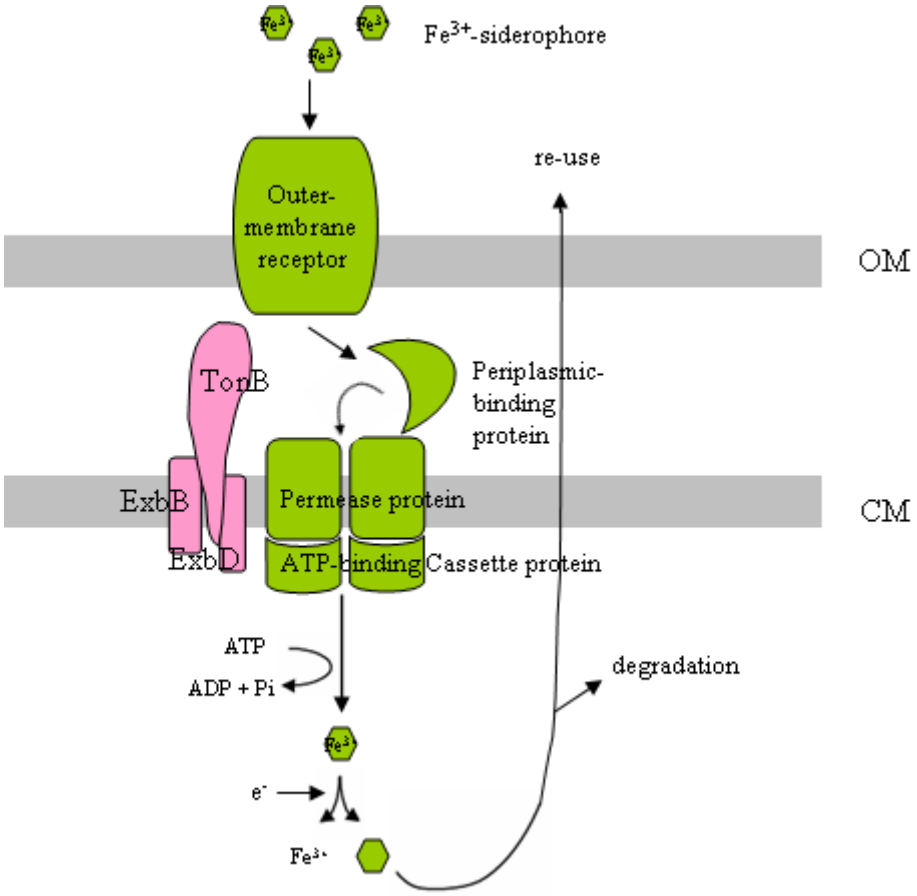

Figure 1.1. Schematic representation of siderophore-mediated iron uptake in Gram-negative bacteria. Adapted from S.C. Andrews et al., FEMS Microbiology Reviews 27 (2003) p215-237

from the OM receptors to CM ATP-binding cassette (ABC) transporters that, in turn, deliver the ferric-siderophores to the cytosol (Fig. 1.1) where the complexes are probably dissociated by reduction [4].

1.2.1.1. Siderophores:

Siderophores are defined as low-molecular weight (~600 daltons), virtually ferric specific ligands ($K_{aff} > 10^{30}$), synthesized and secreted by bacteria in response to iron limiting conditions [1]. Siderophores usually form hexadentate octahedral complexes with ferric iron and can be categorized into 3 major types: hydroxamates, catecholates and mixed-chelating types [4]. Following synthesis, siderophores are secreted across the CM [1]. Some microorganisms may not synthesize their own siderophores but can take up iron loaded exogenous siderophores [1].

1.2.1.2. Outer membrane Fe^{3+}-siderophore receptors:

Fe^{3+}-siderophore complexes are too large to pass through impermeable outer-membranes of Gram-negative bacteria. Hence their internalization process requires outer membrane receptor proteins that bind cognate Fe^{3+}-siderophores with high specificity [1]. The OM receptors are also thought to enhance the rate of Fe^{3+}-siderophore uptake, allowing bacteria to more efficiently scavenge Fe^{3+}-siderophores from their surroundings [1]. Fe^{3+}-siderophore receptor proteins, between 75 and 85 kDa in size, are expressed under iron limiting conditions [5]. Although these receptors can bind to both iron-free siderophores and their cognate Fe^{3+}-siderophores, only Fe^{3+}-siderophores are transported. Fe^{3+}-siderophore binding induces a conformational change in siderophore receptor from a non-productive state to a transport competent state [5, 6]. Subsequently, TonB interacts with an Fe^{3+}-siderophore bound receptor and

activates the transporter, resulting in transport of the Fe^{3+}-siderophore into the periplasm. The energy transduction process requires direct contact between TonB and OM receptors. A conserved hydrophobic seven-amino acid segment, the 'TonB box', at the N-terminus of TonB-dependent OM receptors has been found to be required for TonB-mediated uptake of Fe^{3+}-siderophores [7].

1.2.1.3. TonB dependent regulatory system:

Few TonB-dependent receptors are involved in both transport of Fe^{3+}-siderophores and in trans-envelope signal transduction, hence are called TonB-dependent transducers (Fig 1.2) [8]. In *E. coli*, a TonB dependent transducer *fecA* (receptor specific for Fe^{3+}-citrate transport) was found to be adjacent to two genes [8]. One of them, *fecI*, encodes a specialized sigma (σ) factor of the extracytoplasmic function (ECF) subfamily [9]. The other gene, *fecR*, encodes a transmembrane sensor protein, which was the founder of a subfamily of anti-sigma (anti-σ) factors. Interestingly, TonB-dependent transducers possess a unique N-terminal extension that interacts with anti-σ factor [6, 10] which then activates σ factor [10]. This sophisticated complex senses signals from outside the bacterial cell and transmits them across two membranes into the cytoplasm, leading to transcriptional activation of target genes [8].

1.2.1.4. TonB-ExbB-ExbD complex:

The TonB-ExbB-ExbD complex provides the energy required for transport of Fe^{3+}-siderophores across the outer membrane [11, 12]. TonB is anchored in the inner membrane by its N-terminal end, and it is predicted that the protein spans the periplasmic space with a proline rich region [5]. TonB is thought to directly interact with the outer membrane receptors [11]. Two other proteins, ExbB and ExbD, located

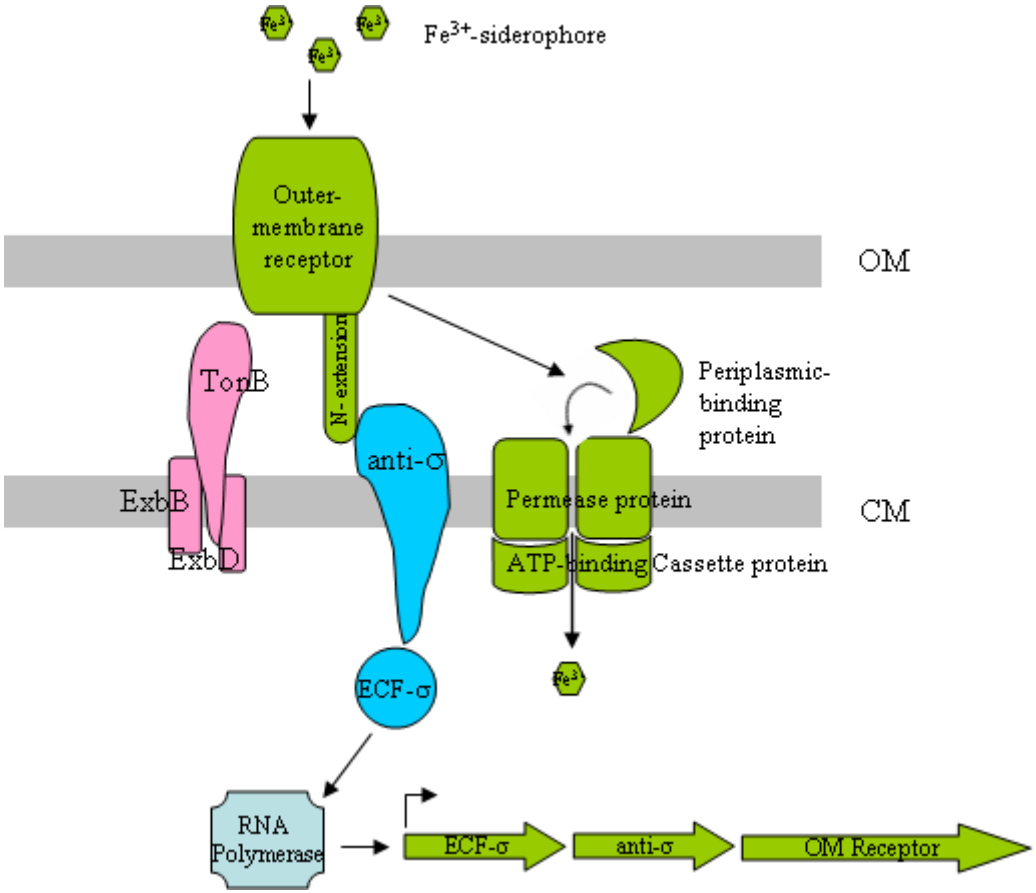

Figure 1.2. Schematic representation of TonB-dependent regulatory system.
Adapted from Braun et al., Microbiology 2003, 6: p 173-180

in the cytoplasmic membrane, form a complex with TonB. Formation of TonB-ExbB-ExbD complex is required for proper function [11]. TonB is stabilized by ExbB and in turn, ExbB is stabilized by ExbD [5]. The mechanism by which the TonB complex responds to the energized state of the cell is still unknown.

1.2.1.5. Transport across cytoplasmic membrane:

Transport of Fe^{3+}-siderophore complexes across the periplasmic space and cytoplasmic membrane is mediated by periplasmic binding proteins and associated CM transporters [13]. Each periplasmic binding protein accepts one Fe^{3+}-siderophore complex at a time but it is unclear whether the binding protein collects the Fe^{3+}-siderophore directly from the OM receptor, or whether the binding protein simply picks up the free Fe^{3+}-siderophore from the periplasm [13]. The binding protein acts as a shuttle, collecting the Fe^{3+}-siderophore released from the OM receptor and delivering it to a cognate CM transporter complex in the inner membrane. The CM transport complex requires two proteins, one to span the membrane to act as a permease and a second one that can hydrolyze ATP to provide the energy for transport. These proteins first recognize the cognate Fe^{3+}-siderophore-loaded periplasmic protein, extract and transport the Fe^{3+}-siderophore across the cytoplasmic membrane while utilizing the energy of the third ATP hydrolyzing protein component [13]. Bacteria often possess multiple OM receptors, each providing the bacterium with specificity for different siderophores but contain fewer ABC systems that are less specific. Select siderophore-mediated iron acquisition systems of *E. coli* are shown in Fig 1.3.

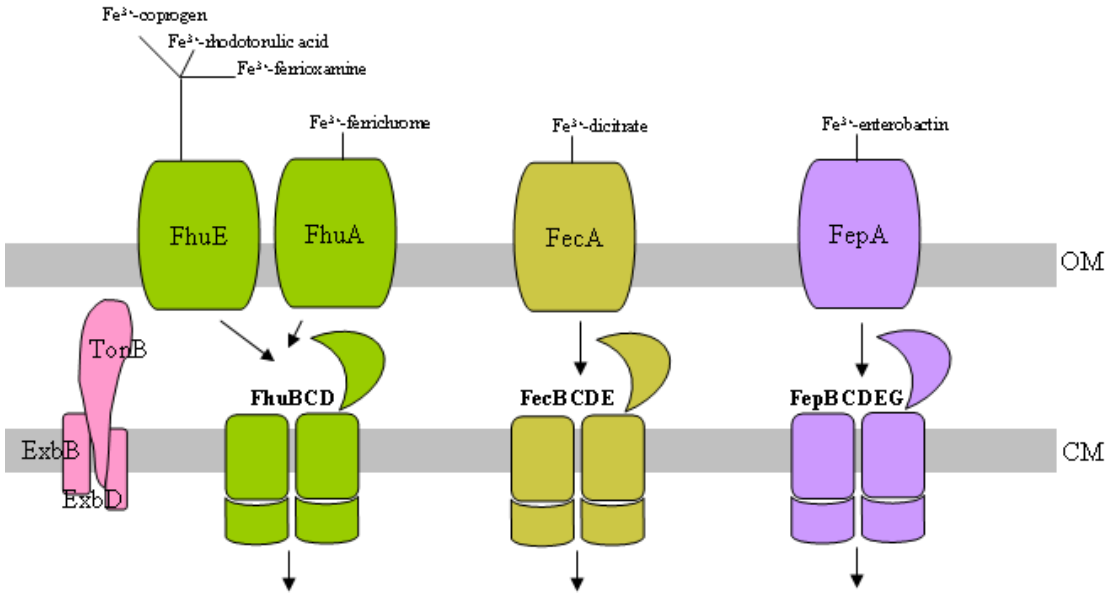

Figure 1.3. Select siderophore-mediated iron acquisition systems of *E. coli*.
Adapted from S.C. Andrews et al., FEMS Microbiology Reviews 27 (2003) 215-237

1.2.1.6. Fate of internalized Fe^{3+}-siderophores:

Once inside the cytoplasm, siderophore reductases reduce the ferric iron to Fe^{2+}, lowering its affinity for the siderophore [1]. The siderophore then can be recycled to the outside of the cell. In some cases, hydrolysis of the siderophore (i.e. enterobactin) has also been reported, which can aid in the release of bound Fe^{3+} [1].

1.2.2. Reductase-dependent Iron Uptake Systems:

Many studies have demonstrated that microbes can reduce extracellular Fe^{3+} in a variety of chelate complexes [14-16]. In yeast, membrane surface-localized reductases first generate Fe^{2+} from Fe^{3+}; multicopper oxidases then convert Fe^{2+} to Fe^{3+} which is immediately transported by a Fe^{3+}-permease (Ftr1) into the cytoplasm (Fig 1.4) [14, 17]. Another Fe^{3+} uptake pathway was proposed in *Helicobacter pylori*. In this system, flavins reduce Fe^{3+} to Fe^{2+} which is taken up through OM porin into periplasm, and finally by an inner membrane ferrous transporter (FeoB) into the cytoplasm (Fig 1.5) [18]. Iron acquisition by microbial extracellular iron reductases was shown to be catalytically efficient in rapidly mobilizing iron from environmental sources suggesting that enzymatic reduction may be a relevant pathway for the mobilization of iron [19].

1.3. Low Affinity Uptake:

Although much is known about the iron transporters used by bacteria under iron-restricted conditions, the system(s) utilized under iron sufficiency (~10 μM iron) have

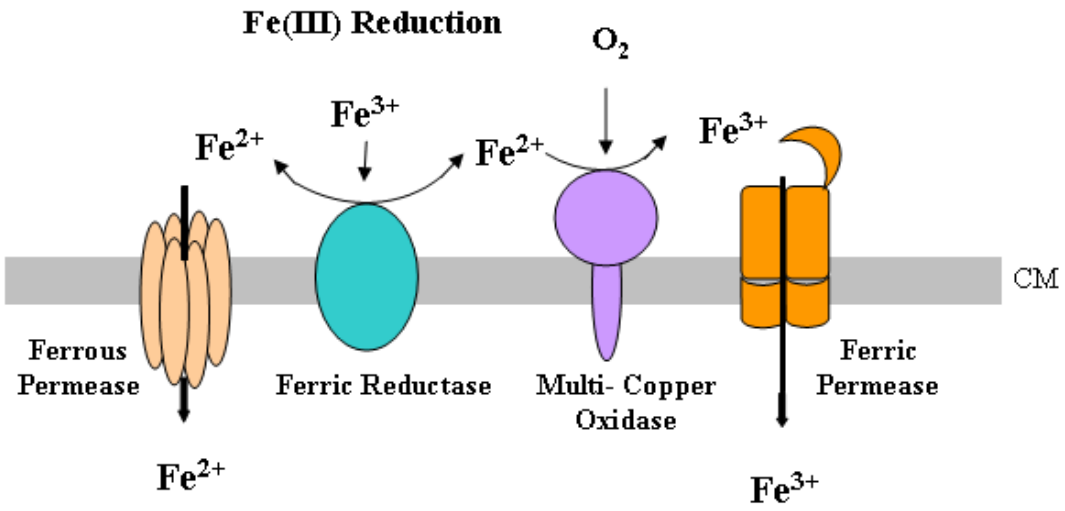

Figure 1.4. Schematic representation of reductase-mediated iron uptake in yeast plasma membrane. Adapted from Kosman et al., Molecular Microbiology (2003) **47** (5), 1185–1197

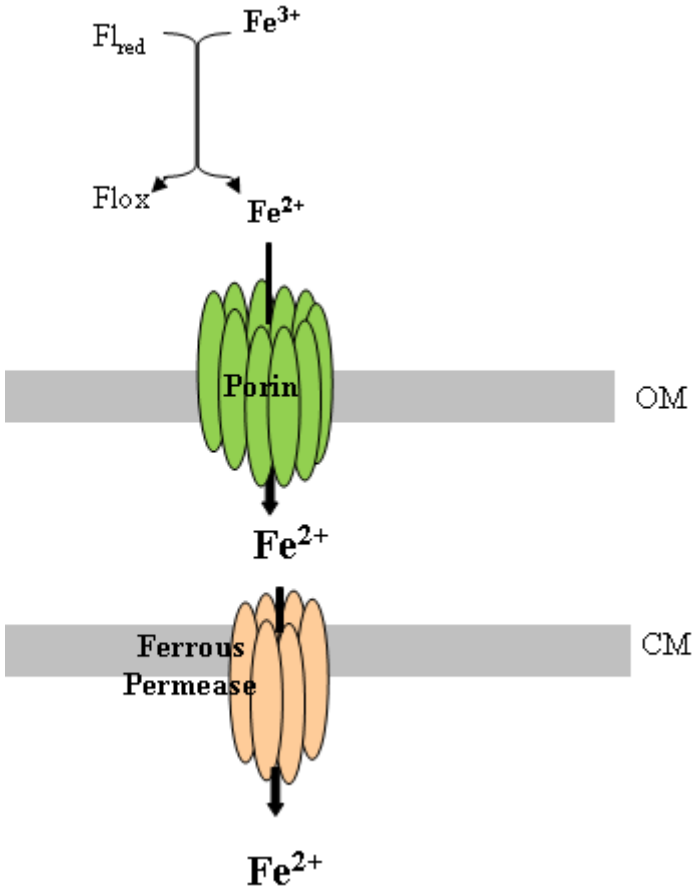

Figure 1.5. Schematic representation of reductase-mediated iron uptake in *Helicobacter pylori*. Adapted from Velayudhan. J et al., Mol Microbiol, 2000. 37 (2): p. 274-286

not been identified. Most microbes appear to have the power to utilize insoluble Fe^{3+}-oxy-hydroxide complexes which get adsorbed to their cell surface and then dissolved by release of hydroxy acids such as citrate and malate [20]. This relatively insensitive, non-specific uptake does not require any specific solubilizing and transporting compounds or membrane receptors. It is called low affinity uptake system because relatively high levels of iron are required to achieve maximal bacterial growth rates. The most compelling evidence for the presence of a low affinity system comes from mutational analysis [3]. All known components of the iron transport systems can be removed without impairing growth rate provided that higher levels of iron are supplied. Very little is known about its basic features since it is difficult to get a handle on the molecular mechanics of this system [3].

1.4. Iron Storage:

Extracellular iron is not the only source of iron available to bacteria. Many bacteria deposit intracellular reserves of iron within iron storage proteins [21]. These iron stores can then be used to enhance growth when external iron supplies are restricted. Three types of iron storage proteins are recognized in bacteria: the archetypal ferritins which are also found in eukaryotes, the haem containing bacterioferritins found only in eubacteria and the smaller Dps proteins present only in prokaryotes. Interestingly, all three types can exist in the same bacterium and multiple ferritin or bacterioferritin genes are common [1].

1.5. Iron and Redox Stress:

Although it is clearly important for bacteria to secure the iron supplies required for growth, excess iron could be toxic because it leads to oxidative damage which can

cause cell death. Most likely this damage is caused by the ability of ferrous iron to catalyze the decomposition of hydrogen peroxide, generating the highly reactive hydroxyl radical that can damage biological macromolecules [22]. The key reaction, known as Fenton reaction is shown below:

Fenton reaction: $Fe^{2+} + H_2O_2 \text{ ---> } Fe^{3+} + OH^- + OH^{\cdot}$

Hence it is essential for all microbes to strictly regulate their iron uptake.

1.6. Regulation of Iron transport:

Bacteria typically regulate their iron uptake in response to iron availability. In *E. coli* (and many other bacteria) this regulation is mediated by the ferric-uptake regulator protein (Fur) that controls the iron-dependent expression of several iron uptake genes [23]**.** Fur acts as a positive repressor, i.e. it represses transcription upon interaction with its co-repressor, Fe^{2+}, and causes de-repression in the absence of Fe^{2+} (Fig 1.6) The Fe^{2+}-Fur complex normally binds between the -35 and -10 sites at the promoters of Fur-repressed genes. Fur-binding sites were originally found to conform to a 19-bp palindromic consensus sequence known as the ‘iron box’ or ‘Fur box’ [23].

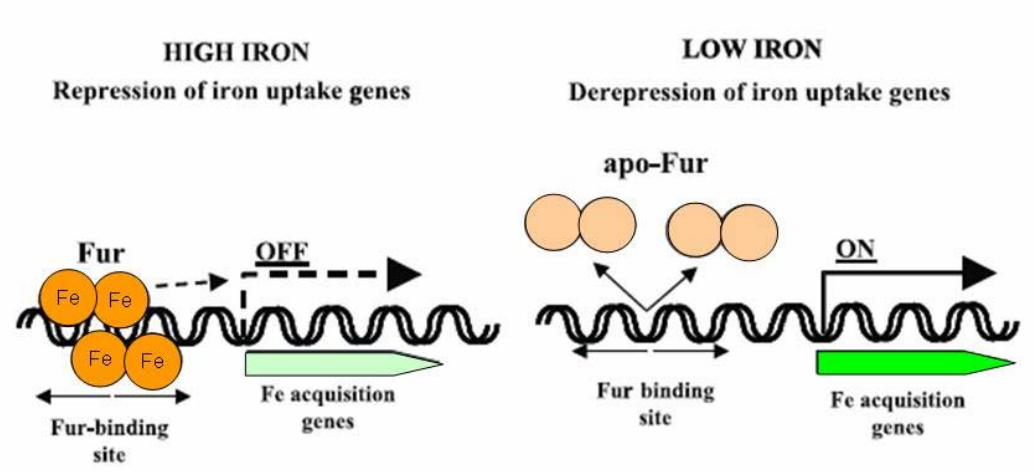

Figure 1.6. Schematic representation of Fur-mediated gene repression.
Adapted from S.C. Andrews et al., FEMS Microbiology Reviews 27 (2003) 215-237

1.7. Iron and *Nitrosomonas europaea*

Nitrosomonas europaea is an aerobic chemolithoautotroph that uses NH_3 and CO_2 for growth [24]. Mechanisms for iron transport are essential for maintaining the many cytochromes and putative heme-binding proteins involved in ammonia-oxidizing metabolism [25, 26]. In the natural niche of *N. europaea*, the biological availability of iron is low as it exists mostly in insoluble ferric form [1]. The genome of *N. europaea* has 4% (~ 90) of its genes encoding iron acquisition proteins and no genes for siderophore production [24]. *N. europaea* has the highest number of genes dedicated to iron acquisition compared to sequenced genomes of ammonia oxidizers (Table 1.3).

1.7.1. The iron acquisition genes in *N. europaea*

Of the *N. europaea* 90 iron uptake genes, 42 encode OM TonB-dependent receptors (Table 1.3), a relatively large number compared to 13, 9 and 10 genes in ammonia oxidizers such as *Nitrosomonas eutropha* [27], *Nitrosospira multiformis* [28], *Nitrosococcus oceanii* [29] (Table 1.3), 11 genes in the chemolithoautotroph *Acidithiobacillus ferroxidans* [30], or six in the heterotroph *E. coli* and 35 genes in the heterotroph *Pseudomonas aeruginosa* [1]. However, unlike *E. coli* and *P. aeruginosa*, there are no iron ABC transporter genes associated with the TonB-dependent receptor/transducer like genes in *N. europaea* [24]. In fact, there is only one complete set of genes analogous to the ABC transporter (*nitABC*) complex in *N. europaea, N. oceani* (Table 1.3) compared to 3 sets of genes in *E. coli* and 4 sets of genes in *P. aeruginosa* [1, 24, 27].

	Siderophore Biosynthesis Genes	Siderophore Receptors Homologs	TonB Homologs	ExbB/ExbD Homologs	FecI/FecR Homologs	Fe-siderophore ABC Transporter Homologs
N. europaea	--	**42**	2	2 sets & 2 orphans	**22 each**	1 complete set
N. eutropha	Incomplete - IucAC	13	3	3 sets	1 each	--
N. multiformis	Incomplete - IucBD	10	2	6 sets	1 FecR	1 complete set; 5 orphans
N. oceani	Complete - IucABCD	9	3	4 sets	--	1 complete set

Table 1.3. Comparison of siderophore-dependent iron acquistion systems in sequenced ammonia oxidizing bacterial genomes.

As with other Gram-negative bacteria [1], genes for the energy-transducing TonB-ExbBD protein complex are present in the genome. Other genes related to iron nutrition include iron-storage protein bacterioferritin (NE0863), and bacterioferritin comigratory protein (NE0722). *N. europaea* has genes to encode the many components necessary for efficient iron uptake and storage.

1.7.2. Analysis of the TonB-dependent OM siderophore receptor and transducer genes in *N. europaea*

Out of 42 OM TonB-dependent receptors genes, 22 are linked to genes encoding a *fecI/fecR* (σ-factor/anti-σ or sensor/regulator) genes [8]. The gene organization is similar to the *fecIR* and *fecABCDE* (Fe^{3+}-dicitrate) systems in *E. coli* [31], and to the *fpvIR* and *fpvA/pvd* (pyoverdin) system in *P. aeruginosa* [32]. All these 22 genes encode for a OM transducer having an N-terminal extension that has been shown to interact with the anti-σ factors in other systems [8]. The remaining 20 genes encode OM receptors lacking the N-terminal extension. These receptors may not interact directly with σ factor/anti-σ factors, though some are in proximity to OM siderophore receptor genes with σ- and anti-σ factor genes (Table 1.4) [33]. Perhaps some of these OM receptor genes are regulated by σ- and anti-σ factors associated with other OM receptor genes, which would suggest cross signaling among Ton-B dependent regulatory systems. It has to be noted that 9 OM TonB-dependent receptor genes are non-functional (truncated, interrupted, or with frame shifts). All *fecI*-type (σ factor) genes appear to be functional and only two *fecR*-type (anti-σ factor) genes are annotated as pseudogenes due to a frame shift (NE1039/40 and NE1618/9).

Table 1.4. Organization of the *N. europaea* TonB-dependent regulatory systems*.

σ factor	anti-σ	Receptor, transducer (homolog)	Putative siderophore type
NE0128	NE0127	NE0126/0125/0124	[Ferrichrome; frame shifts]
Np	Np	NE0321	u
NE0533	NE0534	NE0535 (*fhuA*)	Ferrichrome
NE0541	NE0542 <>	NE0546 (*fhuA*	Ferrichrome
NE0547	NE0548	NE0549 (*fhuA*)	Ferrichrome
NE0554	NE0555	NE0556 (*fhuA*)	Ferrichrome
NE0557	NE0558	NE0559 (*fhuA*)	Ferrichrome
Np	Np	NE0617 (*fepA*)	Catecholate /enterobactin
Np	Np	NE0627	u
Np	Np	NE0636	Vitamin B12, BtuB
Np	Np	NE0731	u
Np	Np	NE0743	
Np	Np	NE0754	[u; truncated]
Np	Np	NE0758	u
NE0818	NE0817	NE0816	u
		NE0936	[u; truncated]
NE0980	NE0979	NE0978	Ferrichrome
NE1041	NE1039/40	NE1038/1037	[u; frame shift]
NE1071	NE1070 <>	NE1063/1062	[u; frame shift &IS]
NE1079	NE1078 <>	NE1072	u
NE1086	NE1085	NE1087 (*fhuE, fpvA*)	ferric coprogen, pyoverdin
Np	Np	NE1088 (*foxA*)	(tri)hydroxamate/ferrioxamin e
Np	Np	NE1089 (*fhuA*)	Hydroxamate/ferrichrome
NE1096	NE1095	NE1094/1092 (*fhuE*)	[hydroxamate; IS]
NE1099	NE1098	NE1097 (*foxA*)	Ferrioxamine
NE1101	NE1102 <>	NE1105/1108	[u; IS]
NE1192	NE1191	NE1190	alcaligin, coprogen, pyoverdin
NE1217	NE1218	NE1220	[u; IS and truncated]
Np	Np	NE1205(*fepA*)	Catecholate/enterobactin
Np	Np	NE1531	u
Np	Np	NE1532	u
Np	Np	NE1536	u
Np	Np	NE1540 (*hugA*; *fepA*)	Heme; catecholate
NE1617	NE1618/9	NE1620/1621	[u; frame shift]
Np	Np	NE1721	u
NE1992	NE1989	NE1988/1987	[u; frame shift]
Np	Np	NE2124	u
NE2138	NE2137 <>	NE2140	u
Np	Np	NE2165 (*fcuA*)	Hydroxamate/ferrichrome
NE2435	NE2434	NE2433 (*fhuA*)	Ferrichrome
NE2486	NE2485	NE2484/2482 (*fhuE*)	[hydroxamat; IS]
Np	Np	NE2502/2503	[u; frame shift]

Table 1.4. continued

*u: unidentified; [] not a functional gene; np: no σ– anti-σ factor genes present next to the receptor genes; <> intervening genes between *fecIR* and *fecA* homologs. In grey are the areas in the chromosome where the OM siderophore receptor/transducers concentrate.

Most of the OM TonB-dependent receptors in *N. europaea* have a signal peptide (20 to 46 amino acids) at the N-terminus indicating that they are exported out of the cytoplasm [34]. Putative TonB boxes [35, 36] were identified in 17 OM siderophore receptors in *N. europaea*. In the remaining 12 functional OM siderophore receptors, no putative TonB box could be identified. Alternatives to the known amino acid consensus TonB boxes may exist in *N. europaea*.

The variety of OM TonB-dependent receptors and transducers encoded in the *N. europaea* genome is quite diverse (e.g. for ferrichrome, desferrioxamine, coprogen, pyoverdin, and for catechol/catecholate-type siderophores [24, 37]). This wide spectrum of siderophore receptor and receptor/transducers (Table 1.4) suggests that *N. europaea* can take up very diverse exogenous siderophores for its growth.

1.7.3. Analysis of the regulatory elements for iron uptake in *N. europaea*

Three putative and distinct *fur* genes were identified in the *N. europaea* genome (NE0616, NE0730, NE1722) and probably function under different conditions with different regulatory mechanisms and range. Because of the *fecIRA*-like gene organization in *N. europaea* (see above), a regulatory pathway similar to known *fec* systems may be possible [31]. In *E. coli* and *P. aeruginosa*, Fur boxes exist upstream of the ECF σ factor (*fecI* type) genes and outer membrane receptor (*fecA* type) genes. Sequences similar to the consensus Fur Box, a 19 bp DNA sequence to which the Fur regulator protein binds [23], were identified upstream of several *N. europaea fecIR* homologue and receptor/transducer genes. For example, a putative Fur box exists upstream of *fecIR* homologues NE0127/8 (17/19 bp match), of siderophore receptor genes NE1205 (16/19 match), NE1540 (14/19), NE1089 (14/19), and of *tonB* gene

NE0354 (18/19). This indicates that in *N. europaea* a consensus Fur box exists and that these genes are regulated in a Fur-mediated fashion.

1.7.4. Analysis of Siderophore-independent Iron uptake genes:

N. europaea also has genes that may encode siderophore-independent iron uptake. A putative siderophore-independent Fe^{2+} transporter (NE0294, cytochrome C type protein) is similar to the yeast Ftr1 [14]. There are at least seven putative multicopper oxidase genes in *N. europaea*: NE0279/0315/0564/0927/1491/1543, and NE0926 which is similar to yeast FET5 [38]. Multicopper oxidases are involved in iron acquisition in bacteria [39], in yeast [40], and in *Chlamydomonas reinhardtii* [41].

1.8. Research Objectives

In *N. europaea*, resources are invested in the synthesis of Fe^{3+}-siderophore receptors rather than siderophores. Considering the low efficiency of energy generation through NH_3, this organism appears to have adopted a strategy whereby energy is invested in producing a plethora of siderophore receptors, rather than in producing siderophores. The large number of iron acquisition mechanisms, both siderophore-dependent and independent, reflects the importance of iron to the metabolism of *N. europaea*. Iron-containing proteins are encoded by 2% of the genes in the genome of *N. europaea*. The high number of genes for iron acquisition helps ensure that these iron-containing proteins have the iron necessary to carry out their functions in the lithotrophic life style of *N. europaea* [25]. The main objectives of this research are to study the iron acquisition systems and their regulation in *N. europaea* using molecular tools.

Chapter 2 describes the physiological responses of *N. europaea* under iron stress. This study showed that *N. europaea* was able to grow in medium with relatively low

iron by adjusting its physiology. The study also showed that *N. europaea* responded to Fe limitation by elevated production of OM siderophore receptors potentially useful for iron uptake. In addition, growth of *N. europaea* was increased by the addition of siderophores to Fe-limited medium, which demonstrates that *N. europaea* can use heterologous siderophores for iron acquisition. These results demonstrate the functionality of some of the siderophore receptor genes in *N. europaea*.

Chapter 3 describes transcriptional profiling of *N. europaea* under iron-limitation conditions. This study enabled us to gain a better understanding of the metabolic changes occurring in response to this stress. The study also enabled us in identifying several new potential iron acquisition systems that are unique to *N. europaea* or to ammonia oxidizing bacteria or present just in *N. europaea* and other organisms but not other ammonia oxidizers.

Chapter 4 describes characterization of the *N. europaea nitABC* iron transporter. The study confirmed that the *N. europaea nitABC* operon is involved in iron acquisition and provided further evidence that the *nitABC* transporter is involved in transport of iron associated with ferrioxamine, ferrichrome and pyoverdine siderophores.

Chapter 5 describes the role of a *fur* homolog in iron metabolism and gene regulation in *N. europaea.* This study enabled us to identify an Fe-sensing *fur* homolog in *N. europaea*. Construction of a *fur* promoter knock-out mutant (*fur-kanP*) strain enabled us to study the effect Fur on the expression of Fe-regulated genes and the physiology of *N. europaea.*

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IRON NUTRITION AND PHYSIOLOGICAL RESPONSES TO IRON STRESS IN *Nitrosomonas europaea*

CHAPTER 2

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2.1 Abstract

Nitrosomona europaea, as an ammonia-oxidizing bacterium, has a high Fe requirement. Under Fe-limiting conditions (0.2 μM), *N. europaea* was able to assimilate up to 70% of the available Fe in the medium even though it is unable to produce siderophores. Addition of exogenous siderophores to Fe-limited medium increased growth (final cell mass). Fe-limited cells had lower heme and cellular Fe contents, reduced membrane layers, and lower NH_3 and NH_2OH dependent O_2 consumption activities than Fe-replete cells. Fe acquisition-related proteins, such as a number of TonB-dependent Fe-siderophore receptors for ferrichrome and enterobactin and diffusion protein OmpC, were expressed to higher levels under Fe limitation, providing biochemical evidence for adaptation of *N. europaea* to Fe-limited conditions.

2.2. Introduction

Ammonia-oxidizing bacteria, such as *N. europaea*, are important participants in the global N cycle because they transform ammonia (NH_3) to nitrite (NO_2^-), the first step in nitrification. As lithotrophs, these bacteria derive all their energy and reductant necessary for growth from the oxidation of NH_3. As autotrophs, these bacteria can meet their C requirements solely using carbon dioxide (CO_2). *N. europaea* is a member of the β-proteobacteria and has a single circular chromosome of 2,812,094 base pairs with 2,460 protein-encoding genes [1]. In *N. europaea*, the genes encoding transporters of inorganic ions are plentiful, but those encoding transporters of organic compounds are scant, consistent with the life style of this bacterium.

Iron (Fe) is an essential cofactor for the transfer of electrons in many enzymes participating in energy-generating pathways. However, biological availability of Fe is typically low because in nature Fe exists mostly in the form of insoluble oxy-hydroxide complexes, and in the cell Fe is present mostly in iron-binding proteins [2]. To cope with the low biological Fe supply, bacteria have evolved high-affinity Fe transport systems for scavenging Fe from the environment, ways to store Fe intracellularly, and mechanisms to down-regulate certain high Fe-containing proteins to economize Fe needs [2].

In bacteria there are two common mechanisms for efficient Fe acquisition [3]: a) direct uptake through ferrous (Fe^{2+}) uptake systems or through heme and hemoprotein uptake systems, and b) a more prevalent mechanism, the production and excretion of siderophores that bind free ferric iron (Fe^{3+}) or sequester Fe^{3+} from other Fe^{3+}-binding proteins [4-6]. The uptake of Fe^{3+}-loaded siderophores in Gram-negative bacteria

requires a sophisticated transport system. Outer membrane (OM) receptors capture Fe^{3+}-loaded siderophores and translocate them into the periplasmic space. This process requires energy (proton-motive force) that is mediated by the TonB/ExbBD complex located in the cytoplasmic membrane. Fe-loaded siderophores are then transported across the periplasm by periplasmic-binding proteins, and finally into the cytoplasm by ATP-dependent membrane transporters [5, 6].

With the exception of genes for the synthesis of citrate, *N. europaea* notably lacks genes for siderophore production [1]. In contrast, up to 4% of the *N. europaea* genes code for Fe-related transport systems, including the uptake of Fe^{3+}-loaded siderophores. There are 22 sets of genes organized similarly to the genes for ferric citrate uptake system: *fecI/fecR/fecA* (genes for σ-factor/anti σ factor/TonB-dependent OM Fe-siderophore transducer); nine of the 22 *fecA* homologues in this group appear to be nonfunctional owing to frame shift, truncation, or interruption by transposons. There are twenty additional Fe-siderophore OM receptor and transducer genes that have no cognate σ-factor/ anti σ-factor genes; four genes of this group appear nonfunctional [1]. Congruent with the high number of genes related to Fe acquisition, over 2% of the genes code for heme synthesis and heme-containing proteins and for proteins with Fe-S centers, reflecting the importance of Fe to the energy metabolism of *N. europaea* [7]. How *N. europaea* acquires sufficient Fe for its metabolism without the production and excretion of siderophores is intriguing. We hypothesize that *N. europaea* has multiple avenues for Fe acquisition, including ferric and possibly ferrous uptake systems and the uptake of siderophores produced by other organisms. Despite the importance of Fe to ammonia-oxidizing bacteria, Fe nutrition has not been

studied in this group of bacteria. In this work we determined the Fe requirements for *N. europaea* and its physiological response to Fe stress.

2.3. Materials and methods

2.3.1. Bacterial culture

N. europaea (ATCC 19178) was cultured as described [8, 9] and harvested in mid to late exponential phase. The standard (Fe-replete) medium contained 10 μM Fe^{3+} complexed with EDTA. Medium made from reagent grade chemicals without any addition of Fe salts still contained about 0.2 μM Fe (Fe-limited medium). The Fe-free medium was made by treatment with the metal chelator Chelex (Sigma, St. Louis, MO). To this medium, Fe and other removed metals (e.g. Mg, Mn and Cu) were added as necessary. All glassware was soaked in 1.0% HNO_3 overnight and rinsed thoroughly with double deionized water. Because medium containing 0.1 μM Fe could not provide sufficient cell material for analyses, medium containing 0.2 μM Fe was adopted for all Fe-limiting experiments. Nitrite (NO_2^-) concentration was determined colorimetrically with the Griess reagent (sulfanilamide and N-naphthylethylenediamine) [10]. The accumulation of NO_2^- is consistently proportional to the increase in cell mass during growth.

2.3.2. Determination of Fe, siderophore and citrate concentrations

Fe content was determined by the ferrozine assay following digestion of cells or cellular fractions in 5% HNO_3 overnight at 100°C [11]. Concentrations lower than 10 μM Fe were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) as described [12]. The quantification of total siderophore content in culture

supernatants was done as described [13]. Citrate was determined using the Citric Acid Bioanalysis Kit (R-Biopharm, Darmstadt, Germany).

2.3.3. Electron microscopy

Washed *N. europaea* cells were fixed in glutaraldehyde and formaldehyde, and post-fixed in OsO_4. After the bacterial cells were embedded and sectioned, the grids were stained with uranyl acetate and Pb citrate or as previously described [14]. The thin sections of cells were examined on a Philips CM-12 STEM transmission electron microscope, operated at 60 kV.

2.3.4. Enzyme activity determinations

Whole cell NH_3-dependent and hydroxylamine-dependent O_2 uptake activities were measured as described [15]. HAO *in vitro* activity was determined by the hydroxylamine-dependent 2,6-dichlorophenol-indophenol (dcPIP; the electron acceptor) reduction assay [16]. Malate dehydrogenase activity in membrane-free extracts was determined by monitoring NADH oxidation in the presence of oxaloacetic acid. NADH concentration changes were monitored on a Beckman DU640 Spectrophotometer using the kinetics mode at 340 nm and the extinction coefficient 6.22 $l \bullet mmol^{-1} \bullet cm^{-1}$ [17].

2.3.5. Cell fractionation, membrane preparation, and heme and protein quantification

Cell membranes were prepared by breaking cells via sonication at 30% energy output for 10 sec on Ultrasonic Processor XL202 sonicator (Heat Systems-Ultrasonics, Inc.,

Farmingdale, NY), followed by ultracentrifugation at 40,000 rpm (rotor SW501) for 1 h on a Beckman L8-70 Ultracentrifuge (Beckman Instrument, Inc., Fullerton, CA). Crude membrane preparations were washed by thorough homogenization in TRIS buffer (0.1 M; pH 7.8) containing 1 M KCl, collected again by ultracentrifugation and resuspended in TRIS buffer (50 mM; pH 7.8). Simultaneously with the membrane preparation, soluble fractions were collected for heme content determination, and for protein content and peptide composition analysis. Heme content was quantified after extraction with pyridine as described [18]. Protein content was estimated by the Micro BCA Protein Assay Kit (Pierce, Rockford, IL).

2.3.6. Protein gel electrophoresis and mass spectrometry analysis

Peptide composition was analyzed using polyacrylamide gel electrophoresis (PAGE) as described [19]. For protein identification by tandem mass spectrometry (MS/MS), selected peptide bands (stained with Coomassie Brilliant Blue R 250) were excised and destained in water followed by 50% acetonitrile-50 mM bicarbonate. Gel slices were dehydrated with 100% acetonitrile and dried under vacuum. Gel slices were rehydrated with trypsin solution (Promega, Madison, WI) and digested at 37°C for 16 h. After digestion, peptides were extracted three times with 250 µl 0.1% trifluoroacetic acid-60% acetonitrile. The extracts were pooled for mass spectrometric analysis. The data were acquired using data-dependent HPLC-MS/MS. Peptide extracts were run on a Waters Q-TOF (Time of Flight) Ultima Global (Milford, MA). The peptides were eluted from a PicoFrit column (New Objective Inc., Cambridge, MA) with a slow increase in concentration of acetonitrile in 0.1% formic acid. As the peptides eluted from the column, the mass and charge state was determined by the data

system, and instrument conditions were adjusted to fragment the peptide "parent" ion. The data in the MS/MS spectra were searched using Mascot (Matrix Science, London, UK) against a general protein database and the *N. europaea* sequence database [1], and the theoretical MS/MS spectra were compared to the experimental spectra (significance threshold was 0.03 and Mascot score of 25).

2.4. Results

2.4.1. Fe requirements and physiological responses to Fe limitation

N. europaea was grown in media containing a wide range of Fe concentrations to define its Fe requirements. The maximum cell mass attained at 0.1 and 0.2 μM Fe was about 30% and 60% respectively of the cell mass attained in standard medium containing 10 μM Fe (Fig. 2.1A). *N. europaea* acquires Fe at these low Fe concentrations, even though it does not have the genes necessary for siderophore synthesis. An Fe concentration below 1 μM is generally considered a low Fe condition, and Fe at 0.1 μM, an Fe-deficient condition [20]. Concentrations ranging from 10 to 250 μM Fe resulted in normal growth of *N. europaea* as judged by culture optical densities. Growth was inhibited with 1200 μM Fe, probably due to Fe toxicity and consequent oxidative damage to the cells [21].

The growth rates and maximum cell masses obtained for *N. europaea* in media initially made with 10 μM non-complexed ferric iron ($FeCl_3$, Fe^{3+}), or Fe^{3+}-EDTA, or ferrous iron ($FeSO_4$, Fe^{2+}), were similar. Addition of up to 40 μM α, α'-dipyridyl (Sigma), a strong Fe^{2+} chelator commonly used to create Fe deficient conditions [22], did not inhibit *N. europaea* growth in medium containing 0.2 μM Fe. This result

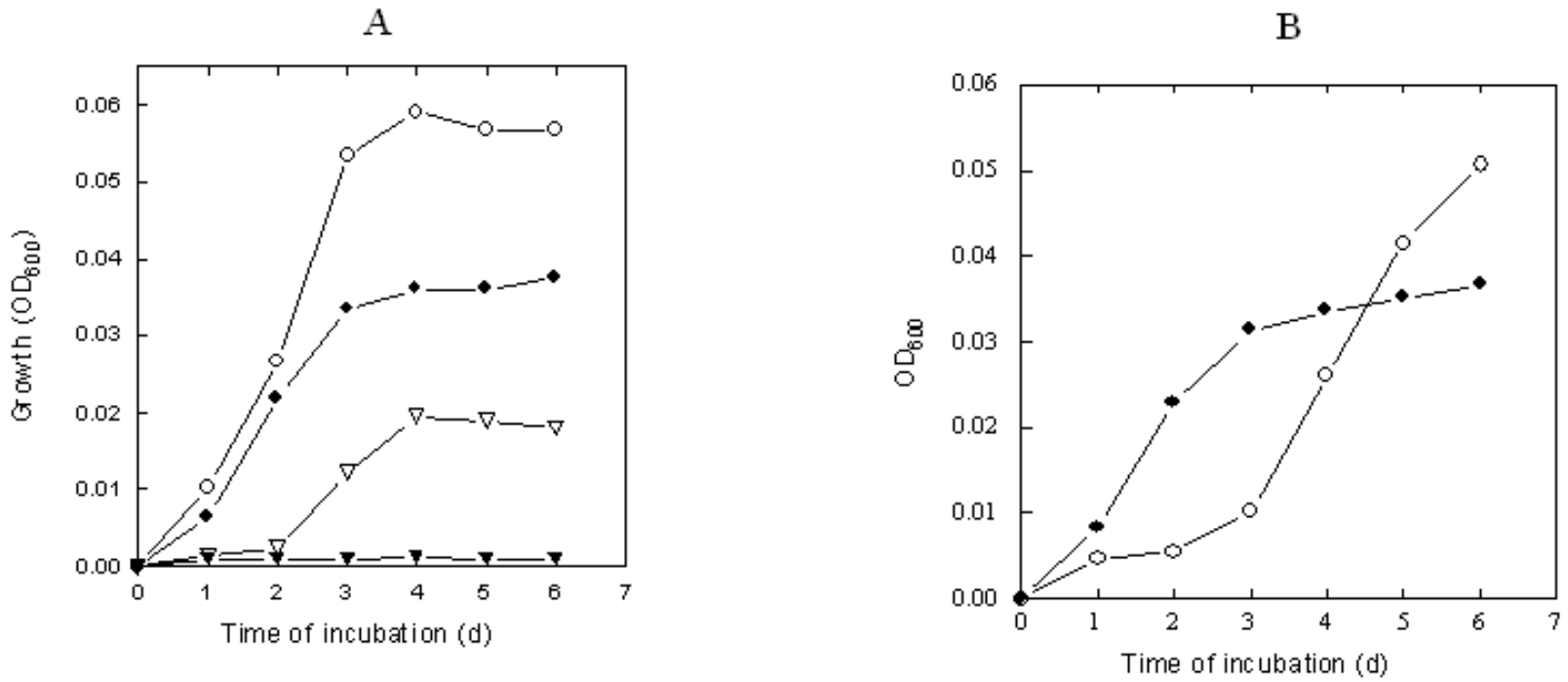

Fig. 2.1 *N. europaea* growth as affected by various Fe concentrations and by the siderophore desferal. **A**. Growth (optical density) in media containing 10 (o), 0.2 (●), 0.1 (▽), and < 0.01 (⓿) µM Fe. The 10 µM Fe culture was inoculated with Fe-replete cells, the three other cultures were inoculated with cells grown in Fe-limited medium. **B**. Effect of desferal on the growth of *N. europaea* in Fe-limited medium. Growth curves shown are in Fe-limited (0.2 µM) medium without desferal (●) and Fe-limited medium containing 5.0 µM desferal (o). The data presented are the average of duplicates with <10%variation.

indicates that *N. europaea* can use Fe^{3+}, the predominant form of Fe in the medium under aerobic condition at near neutral pH, rather than rely on trace amounts of Fe^{2+} for growth. However, this result does not rule out the possibility of *N. europaea* taking up Fe^{2+}.

Fe concentrations below 1.0 μM often trigger siderophore synthesis in other bacteria [23]. Early stationary phase culture supernatants were collected from *N. europaea* and analyzed for siderophore content. As expected, no siderophores were detected in the media under Fe-limiting or Fe-replete conditions. As a positive control, the same method was successfully applied to the detection and quantification of siderophores in an *Azotobacter vinelandii* Fe-limiting culture (data not shown).

Since *N. europaea* has the necessary genes to produce citrate, we considered citrate a candidate Fe siderophore. Citrate was detected in culture filtrates from both Fe-limited and Fe-replete cultures. Culture filtrates from Fe-sufficient cultures had a higher concentration of citrate (~5 μM) than those from low-Fe cultures (~3 μM). Citrate concentrations were approximately proportional to the cell densities of the two cultures.

2.4.2. Promotion of *N. europaea* growth by exogenous siderophores

Addition of the siderophore desferal (Sigma) to the Fe-limited medium led initially to a slower growth rate (Fig. 2.1B). However, after 3 days, the growth rate increased, and by the fifth day cell densities in the desferal-containing medium exceeded those in the medium without desferal (Fig. 2.1B). Addition of 20 μM desferal had a similar

effect as 5 μM. Siderophore ferrichrome (10 μM) (Sigma) increased *N. europaea* growth (final cell mass) in Fe-limited medium to a similar extent as desferal but without a long lag phase (data not shown). Addition of citric acid to either Fe-limited or Fe-replete media did not lead to significant increases in growth (data not shown). The increases in cell densities in the presence of desferal and ferrichrome suggest that *N. europaea* has the potential to utilize external siderophores for Fe uptake under Fe limitation

2.4.3. Physiological and ultrastructural changes of *N. europaea* upon Fe limitation

Medium with 0.2 μM Fe yielded about 60% cell mass of the Fe-replete medium and affected cell appearance and physiology. The color of cells harvested from Fe-limited media was lighter and the heme content of the Fe-limited cells was over three-fold lower than that of Fe-replete cells (Table 2.1). Furthermore, Fe-limited cells had nearly ten-fold less Fe than Fe-replete cells (Table 2.1). About 70% of the Fe initially in the medium was taken up and incorporated into Fe-limited cells during growth (Table 2.1). Analyses of the spent media and cellular Fe contents by ICP-MS confirmed this result. Heme-bound Fe accounted for about 85% of Fe in the soluble fraction of Fe-limited cells and 59% in Fe-replete cells (Table 2.1). Fe limitation influenced both the amount of soluble cytochromes produced and the proportion of Fe distributed to cytochromes.

As indicators of the overall cell activity, NH_3- and NH_2OH-dependent O_2 uptake rates and *in vitro* HAO and malate dehydrogenase activities were measured. The NH_3- and

Table 2.1. Heme C and Fe content* in the cells' soluble (membrane-free) fraction, and the total cell Fe content* in *N. europaea* grown in Fe-limited (0.2 μM) and Fe-replete (10 μM) media.

Heme and Fe content in	Cell cultured in medium with	
	Fe, 0.2 μM	Fe, 10 μM
Soluble fraction:		
Heme C (nmol/mg protein)	2.9 ± 0.7	8.8 ± 2.1
Heme C (nmol/unit OD_{600}•ml)	0.42 ± 0.1	0.91 ± 0.21
Fe (nmol/mg protein)	3.4 ± 0.4	15 ± 3.0
Whole cell:		
Fe (nmol/mg protein)	13.6 ± 2.2	136 ± 9.8
Fe (nmol/ml culture, or μM)**	0.14 ± 0.2	1.95 ± 0.1
Cellular Fe concentration (mM)***	1.88 ± 0.3	16.3 ± 1.2

* Fe was extracted with 5% HNO_3 (digesting the soluble fraction or cells) at 100°C overnight, extraction was repeated once.

** The amount of Fe taken up by cells divided by the culture medium volume for comparison with the Fe concentrations in the original growth medium.

*** Fe concentration within the cells. According to Schmidt et al. [24], 6×10^8 molecules per cell equals 1 M in one *N. europaea* cell volume (i.e. 1 μm^3). With the cell density in the Fe-replete culture of ~1.2×10^8/ml, cellular Fe concentration is 1.95×10^{-9} (moles/ml)$\times6.023\times10^{23}$ (molecules/mole)/(1.2×10^8 (cells/ml)$\times6\times10^8$ (molecules/cell))= 16.3×10^{-3} M = 16.3 mM. Cell density of Fe-limited culture is ~0.72×10^8/ml, resulting in a cellular [Fe] of 1.88 mM.

NH_2OH-dependent O_2 uptake (AMO and HAO) activities in whole cells were about two-fold lower in Fe-limited cells than in Fe-replete cells (Table 2.2). HAO activity in cell-free extracts was also lower in cells from Fe-limited medium than cells from normal medium. Malate dehydrogenase was chosen as a representative of non Fe-containing enzymes. In contrast to the decrease of NH_3- and NH_2OH-dependent O_2 uptake activities, specific malate dehydrogenase activity of Fe-limited cells was about four-fold higher than that of Fe-replete cells (Table 2.2).

Nitrosomonas cells are characterized by extensive membrane invaginations [25]. AMO is located in the cell's inner membranes [26]. Many other Fe-containing proteins such as cytochromes and electron transport enzymes are also localized in the membranes. We hypothesized that Fe would be an important factor affecting *N. europaea* membrane content and structure. To test this, Fe-limited and Fe-replete cells were fixed, stained, and sectioned for electron microscopic examination. The internalized membranes of the cells from Fe-limited medium had fewer layers and were less regularly stacked than cells cultured in Fe-replete medium (Fig. 2.2). Many of the Fe-limited cells were irregular in shape (images not shown).

2.4.4. Expression of membrane receptor proteins under Fe-limiting condition.

Membrane fractions of cells grown in Fe-limited and Fe-replete media were isolated and their protein compositions compared by SDS-PAGE. Several proteins were expressed about ten fold higher in cells grown in Fe-limited than in Fe-replete media (gels not shown). The comparison was also made between the proteins of the soluble fractions of the Fe-replete cells and Fe-limited cells, but no major differences were

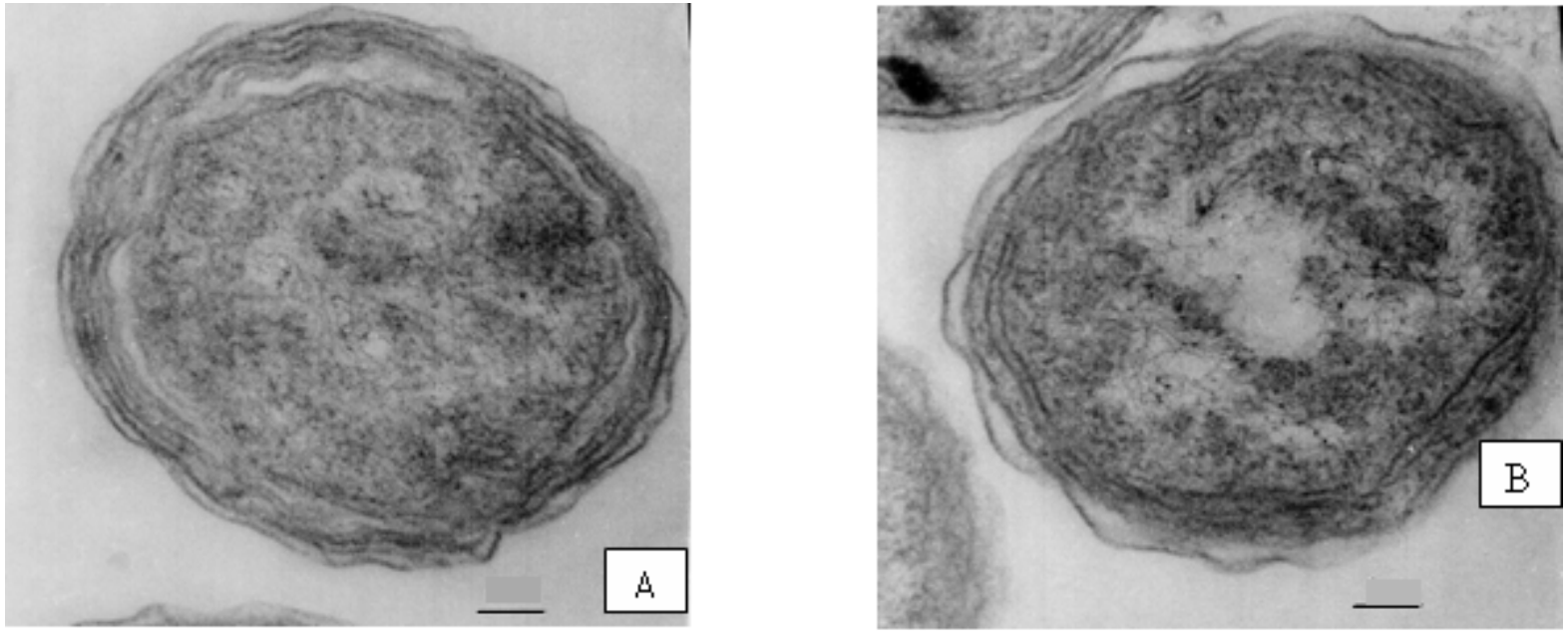

Fig. 2.2. Transmission electron microscopic images of sections of *N. europaea* cells grown in (A) 10 μM Fe medium and (B) 0.2 μM Fe medium. Note the internalized membranes or invaginations. Transmission electron microscope images were taken at *72,500*× magnification. Images shown are representative of dozens examined. The line in the picture represents a length of 0.1 μm (micron).

Table 2.2. Enzyme-catalyzed activities of *N. europaea* grown under Fe-limited (0.2 μM) and Fe-replete (10 μM) conditions.

Activity*	Cell cultured in medium with	
	0.2 μM Fe	10 μM Fe
NH_3-dependent O_2 consumption (AMO)		
Whole cell	670 ± 52	1151 ± 112
NH_2OH-dependent O_2 consumption (HAO)		
Whole cell	282 ± 20	607 ± 47
HAO**		
Cell-free extract	173 ± 9	518 ± 35
Malate dehydrogenase		
Membrane-free extract	708 ± 66	168 ± 10

*All activities are in units of nmoles•(min •mg protein)$^{-1}$.

**HAO activity in membrane-free extracts was determined as hydroxylamine-dependent dichlorophenolindophenol (dcPIP) reduction.

observed. The membrane proteins differentially expressed were excised from the gels and subjected to HPLC/MS/MS analysis. Of the top 12 proteins identified by tandem MS, seven are clearly related to Fe acquisition (Table 2.3). Six of the proteins that were highly up-regulated under the Fe-limiting condition are outer memebrane siderophore receptors. Three are putative receptors for catechol-type siderophores, one is for ferrichrome, one for heme, and one is for unidentified siderophores. In addition, an OM protein OmpC (a general diffusion Gram-negative porin), a multicopper oxidase, and a type II secretion pathway protein were also identified among the highly expressed proteins in Fe-limited cells (Table 2.3). Only one siderophore receptor, encoded by NE1532, was detected uniquely in Fe-replete cells, albeit a much larger amount of total membrane protein was required for isolating enough samples for MS/MS detection.

2.5. Discussion

Despite its lack of siderophore production, *N. europaea* can grow moderately well at low Fe concentrations (0.2 μM) (Fig. 2.1A). In order to grow at 0.2 μM Fe, many other microorganisms rely on siderophores for Fe uptake. For instance, in *E. coli* [23] and *Fusarium venenatum* [27] siderophore synthesis was turned on when Fe was below 1 μM. In some species that require high Fe availability, siderophore synthesis is turned on even when Fe was well above 1 μM. For example, when Fe level was around 5 μM, *Azotobacter vinelandii* and *Magnetospirillum magneticum* AMB-1 started to produce siderophores [28, 29]. *Pseudomonas* species usually become Fe-deficient at 1-2 μM [30, 31], even though most of them can produce siderophores.

Table 2.3. Major membrane proteins identified by LC tandem mass spectrometry that are differentially expressed in cells grown in Fe-replete and Fe-limited media.

Gene	Putative function and homologue (Blast E value)
NE0731	OM Fe siderophore receptor (possibly for catechol) (*P. aeruginosa*, e^{-157})
NE2124	OM Fe siderophore receptor (possibly catechol) (*Dechloromonas aromatica*, 0; *Ralstonia eutropha*, $3e^{-83}$)
NE1540	OM heme receptor, HugA (*Plesiomonas shigelloides*, $5e^{-118}$)
NE1089*	Similar to *Bordetella parapertussis* BfrB (0); OM ferrichrome receptor, FhuA (e^{-118})
NE1531	OM receptor proteins mostly for Fe transport (*Azotobacter vinelandii,* $2e^{-160}$)
NE1532**	OM receptor proteins mostly for Fe transport (*A. vinelandii,* $4e^{-152}$)
NE1205	OM receptor for ferrienterobactin-like (catechol) siderophore, FepA (0)
NE2563*	OM protein, general diffusion Gram-negative porins, OmpC ($6e^{-18}$)
NE0315*	Multicopper oxidase type 2, MnxG (*Bacillus* sp. strain SG-1, $3e^{-52}$)
NE1604*	Bacterial general (type II) secretion pathway protein D (secretin) (e^{-116})
NE1548*	Acyl-CoA dehydrogenases, CaiA ($5e^{-50}$)
NE2206	PpiC-type peptidyl-prolyl cis-trans isomerase, *ppiD* (e^{-113}); also annotated as SurA, parvulin-like peptidyl-prolyl isomerase

* Uniquely identified in membranes of Fe-limited cells; ** Uniquely identified in membranes of Fe-replete cells. Others are found in the protein bands of both Fe-limited and Fe-replete cell membranes but are at least ten-fold higher in Fe-limited cells.

N. europaea has a high cellular Fe and heme content (Table 2.1) relative to *E. coli*. This is consistent with *N. europaea*'s many heme proteins that include HAO, heme/copper type cytochrome oxidase, cytochrome peroxidase, and other cytochromes (e.g. c554, c_m552, p460) [7, 32]. *N. europaea* cultured in Fe-limited medium had a cellular Fe concentration 19-fold higher (1.88 mM) than the level reported for *E. coli* (0.1 mM) cultured in minimal medium (~0.1 μM Fe) [33]. *N. europaea* grown in Fe-replete medium had a cellular Fe concentration 80-fold higher (16.3 mM) than that for *E. coli* cells (0.2 mM) grown in LB medium (~2.0 μM Fe) [33]. The heme C content (0.9 nmol/unit OD_{600}•ml) in the soluble fraction of *N. europaea* from Fe-replete medium (Table 2.1) was much greater than ~0.1 (nmol/unit OD_{600}•ml) detected in *E. coli* grown in full Fe medium [34]. This high heme content is evident in the distinct reddish-brown color of *N. europaea* cells, compared to the pale yellowish color of *E. coli* cells.

Differences in internal membrane content and structure were apparent between Fe-limited and Fe-replete cells. An early study showed that *Nitrosomonas* cell membranes contain a large proportion of Fe-containing proteins (A-type cytochromes and electron transport enzymes) [25]. Cellular Fe content of Fe-limited cells was much lower than that of Fe-replete cells (Table 2.1). This lower Fe content probably reflects lower Fe storage in addition to the lower heme content. However, the cell density in Fe-limited medium was not affected to the same degree as the Fe and heme contents. These results suggest that when Fe supply is limited, *N. europaea* allocates a greater proportion of total Fe to the most critical molecules such as hemes. The lower specific activities of AMO and HAO in Fe-limited cells than in Fe-replete cells (Table

2.2) might be due to the lowered available heme in Fe-limited cells (Table 2.1) because HAO requires 24 hemes per enzyme for function [35]. The reason for the elevated malate dehydrogenase activity in Fe-limited cells is not clear at present, but this result indicated that not all metabolic activities were down-regulated under Fe limitation.

Other studies have showed the increased production of certain OM proteins upon Fe deficiency in other bacteria (see e.g. [36, 37]), but the availability of the genome sequence and HPLC/MS/MS enabled us to reliably determine the identities of the proteins differentially expressed under Fe-limited and Fe-replete conditions. *N. europaea* appears to respond to Fe limitation by making more proteins that can potentially participate in Fe-uptake (Table 2.3). The six membrane proteins include OM receptors for several different types of siderophores. This result provides biochemical evidence for the possible role of the genes and their protein products in Fe acquisition and hence in the growth and survival of *N. europaea* under Fe-limiting conditions.

Negative regulation of the expression of Fe uptake systems by ferric uptake regulator (Fur), Fur box, and Fe levels is well characterized in other species such as *E. coli* and *P. aeruginosa* [38-40]. Three putative *fur* genes are identified in the *N. europaea* genome [1]. Of the 29 intact OM siderophore receptor/transducer genes in the genome, three genes (NE1089/1205/1540) are preceded by sequences similar to the consensus Fur box. These three genes were among those highly induced in Fe-limited cells (Table 2.3). A putative Fur box is also present upstream of NE2563 (porin OmpC). Based on the MS/MS results and the genome information, a negative

regulatory mechanism involving Fur is likely the reason for the elevated expression of the siderophore receptors under Fe limitation. The HPLC/MS/MS results are also interesting in that only six OM siderophore receptors were highly expressed in cells grown in siderophore-free, Fe-limited medium (Table 2.3). All of them are encoded by genes that do not have cognate σ-factor/anti σ-factor genes [1], and none belongs to the OM siderophore transducer family which has an N-terminal extension that interacts with the anti σ-factor to effect the regulatory pathways [41]. This result suggests that the expression of the majority of the 29 genes is likely dependent on the induction by their respective siderophores.

The increased production of the OM siderophore receptors upon lack of Fe and the ability of *N. europaea* to use common siderophores desferal and ferrichrome for its Fe acquisition supports our hypothesis that *N. europaea* can obtain Fe in its natural habitats by competing for Fe^{3+}-loaded siderophores made by other organisms. *N. europaea* carries out aerobic oxidation of NH_3 most efficiently at neutral to slightly alkaline pH. Natural environments with these properties are often Fe-limited owing to the extremely low solubility of ferric iron (10^{-18} M at pH 7), the predominant form of Fe in the presence of O_2 at neutral to alkaline pH; hence, siderophores are necessary for Fe acquisition. The genome makeup of *N. europaea* suggests an Fe acquisition strategy that is adapted to both low energy yield and high Fe demand associated with NH_3 oxidation. Siderophore biosyntheses may require as many as a dozen genes (see e.g. [40]) and can be a significant draw on the carbon and energy budget of a bacterium. In *N. europaea*, limited resources are not invested in the synthesis of siderophores but in many siderophore receptors whose production is negatively

regulated by Fe levels. This strategy is effective, provided that there are siderophores in the environment. In the natural growth environments of *N. europaea*, other common soil microorganisms produce siderophores [42-45]. Some studies suggest that soil pore water might contain siderophores at concentrations up to 240 μM [46], and there is evidence that these siderophores are utilized by other bacteria [47] some of which are non-siderophore-producers [48].

In the experiments without added siderophores, it seems unlikely that the highly expressed siderophore receptors are involved in Fe uptake in pure cultures since no siderophore was detected except low levels of citrate (3 to 5 μM). It should be pointed out that these citrate concentrations are at the lower limit level of detection for the method used. However, given that the *N. europaea* genome has no genes with high similarity to the known *fecA*, and that the addition of citrate to Fe-limited cultures did not increase growth significantly, the role, if any, of citrate in Fe acquisition for *N. europaea* seems limited. Nonetheless, cultures grew moderately well in Fe-limited medium. This observation raises the possibility of a siderophore-independent Fe uptake pathway in *N. europaea*. One possibility, the uptake of Fe^{2+}, seems unlikely under oxic and neutral pH conditions where Fe^{2+} contributions would be negligible. Furthermore, dipyridyl, an Fe^{2+} chelator, did not inhibit *N. europaea* growth. However, *N. europaea* has most of the genes for potential siderophore-independent Fe^{3+} uptake. In yeast, membrane surface-localized reductases first generate Fe^{2+} from Fe^{3+}; multicopper oxidases then convert Fe^{2+} to Fe^{3+} which is immediately transported by Ftr1 into the cytoplasm [49, 50]. While *N. europaea* genome only has genes with low similarity to the characterized ferric reductase genes, it has a gene (NE0294) that

matches well to the genes for a high affinity Fe^{3+} transporter and has at least seven putative multicopper oxidase genes [1]. The product of NE0294 is a cytochrome C type protein that is similar to the yeast high-affinity Fe^{3+} transporter Ftr1. NE0926, one of the multicopper oxidase genes, is similar to the yeast FET5 necessary for Fe uptake [51]. Multicopper oxidases are essential for Fe acquisition in *P. aeruginosa* when the source is Fe^{2+} [52], and for Fe^{3+}/Fe^{2+} uptake in *Chlamydomonas reinhardtii* [53]. Another Fe^{3+} uptake pathway was proposed in *Helicobacter pylori* [54]. In this system, flavins reduce Fe^{3+} to Fe^{2+} which is taken up through OM porin into periplasm, and finally by FeoB (inner membrane ferrous transporter) into cytoplasm. However, *N. europaea* genome has only one gene (NE1286) with low similarity to the known *feoB*. Nonetheless, a porin (OmpC, encoded by NE2563) and a multicopper oxidase (MnxG, by NE0315) were among the highly-expressed proteins under Fe-limited conditions (Table 2.3). Further studies are needed on the potential siderophore-independent Fe uptake systems in *N. europaea*, especially on OmpC, FeoB, and multicopper oxidases.

In summary, this study showed that *N. europaea* was able to grow in medium with relatively low Fe by adjusting its physiology, and it responded to Fe limitation by elevated production of OM siderophore receptors potentially useful for Fe uptake. In addition, growth of *N. europaea* was increased by the addition of siderophores to Fe-limited medium, which demonstrates that *N. europaea* can use heterologous siderophores for Fe acquisition. The ability to respond to the changing Fe availability would enable *N. europaea* to compete successfully for limited Fe supply in its

habitats. Further genetic and functional studies on *N. europaea* Fe uptake systems are warranted.

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TRANSCRIPTIONAL PROFILING OF *Nitrosomonas europaea* UNDER IRON LIMITATION

CHAPTER 3

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Microbiology
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3.1. Abstract:

The effects of iron limitation on the transcriptome of *Nitrosomonas europaea* (ATCC 19178) was studied using whole genome expression microarrays. By comparing the transcriptomes of cells grown in iron-replete medium versus iron-limited medium, 247 genes were identified as differentially expressed. Genes with higher transcript levels included those with confirmed or assigned roles in iron acquisition. Genes with lower transcript levels included those encoding proteins containing iron or that use heme as a cofactor. This strategy enabled us to identify several potential iron acquisition systems and gain a better understanding of the metabolic changes occurring in *N. europaea* in response to iron limitation.

3.2. Introduction

Iron is an essential micronutrient for most microorganisms and plays a central role in many redox enzymes that function in electron transport chains of intermediary metabolism. All microorganisms require iron, with exceptions such as *Lactobacilli* [1] which utilize manganese and cobalt as biocatalysts in place of iron. Although iron is abundant in nature, most microorganisms face the problem of obtaining enough iron from the environment because of extremely low solubility of Fe^{3+} (10^{-18} M) at pH 7. Microbes counter the difficulties posed by iron availability in a number of ways. One common mechanism involves the solubilization of extracellular iron by low molecular weight chelating agents called siderophores, followed by their internalization via specific transporters. Another widespread approach is the extracellular enzymatic reduction of iron by iron reductases, with subsequent transport via specific permeases [2, 3].

Iron acquisition in *Nitrosomonas europaea* (ATCC 19718), a chemolithoautotrophic ammonia oxidizing bacterium that depends heavily on iron for metabolism of ammonia, is particularly interesting since it lacks genes for siderophore production, and has genes with only low similarity to known iron reductases, yet grows relatively well in medium containing low Fe (0.2 μM) [4].

N. europaea derives all its energy and reductant for growth from the oxidation of ammonia to nitrite and fixes CO_2 to meet the cell's demand for carbon. With some additional mineral salts for growth, they manage to build all essential cell components such as nucleotides, amino acids, fatty acids and all key elements in translation, transcription, and other processes [5]. To metabolize ammonia, *N. europaea* uses an

array of heme proteins that include hydroxylamine oxidoreductase, heme/copper type cytochrome oxidases, cytochromes c554, c_m552, p460, and others all of which must have iron to function [6, 7]. This is consistent with the fact that the cellular iron concentration of *N. europaea* is several fold higher than that of *Escherichia coli* [4]. *N. europaea* cannot synthesize its own siderophores, yet it is capable of acquiring iron from media containing low iron concentrations [4, 5]. Many other bacteria including *E. coli* [8] *and Azotobacter vinelandii* [9] rely on siderophore synthesis to grow below 1 μM iron concentrations. *N. europaea* is able to utilize iron bound siderophores [4, 10] that are known to be produced by other organisms. However, the mechanism by which *N. europaea* acquires sufficient iron for growth from Fe-limited medium in the absence of siderophores is not yet understood.

Here we use transcriptional profiling to study the iron acquisition systems of *N. europaea.* Microarray studies have investigated the effects of iron restriction in many different organisms, including *E. coli* [11], *Pseudomonas aeruginosa* [12], *Campylobacter jejuni* [13] and *Neisseria meningitidis* [14]. A common feature in all these studies is up regulation of transcripts of siderophore dependent iron acquisition genes as the products of these genes are essential for survival of the bacteria under iron limiting conditions. Higher transcript levels of siderophore dependent iron acquisition homologs were observed in our study as well, thus emphasizing the importance of their regulation during iron restriction. Transcripts of a large number of energy metabolism gene homologs, mainly encoding Fe-containing proteins, were found to be at lower levels under iron limitation in our study. In addition, several genes with potential roles in iron acquisition were detected, some of which have not

been previously reported. Together, the data reveal new insights into iron acquisition and provide an explanation for how *N. europaea* adjusts its physiology under iron limiting conditions.

3.3. Materials and Methods

3.3.1. Bacterial culture and treatments

Nitrosomonas europaea (ATCC 19178) was grown in batch cultures with 25mM $(NH_4)_2SO_4$ (50mM NH_4^+) and 3.8mM Na_2CO_3 as described (Ensign et al., 1993; Stein & Arp, 1998). The standard (iron-replete) medium contained 10 μM Fe^{3+} complexed with EDTA. Medium made from reagent grade chemicals without any addition of iron salts still contained about 0.2 μM iron (iron-limited medium) and has been used in previous studies relating to *N. europaea* iron metabolism [4, 10]. All glassware used for the experiments was soaked in 1.0% HNO_3 overnight and rinsed thoroughly with double deionized water. *N. europaea* cells grown in iron-limited medium had pale-yellowish color when compared to reddish-brown colored cells grown in iron-replete medium, thus providing an easy visual inspectoin that the cells grown in iron limited medium were in fact iron limited. Cells from three independent cultures (three iron-replete [300ml culture at 0.04 OD] and three iron-limited cultures [500ml culture at 0.025 OD]) were harvested at mid exponential phase by centrifugation. Total cellular RNA was extracted using 1ml of Trizol (Ambion Inc., Austin, TX) following the manufacturer's instructions. The extracted RNA was purified with an RNeasy Mini kit (Qiagen Inc., Valencia, CA.) and treated with RNase-free DNase I (Qiagen) to digest residual chromosomal DNA. The concentration of purified RNA was determined by using a Nanodrop spectrophotometer (Nanodrop Technologies, Rockland, DE) and

RNA quality was checked by the A260/A280 ratio and RNA 6000 Nano LabChip kit on the Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Palo Alto, CA.). Intact, good quality RNA (>9.0 RIN score) was used for the experiments.

3.3.2. DNA microarray construction and microarray experiment.

The NimbleChip 4-plex Made-to-Order array for *N. europaea* was manufactured by Roche NimbleGen Systems, Inc. based on the published genome sequence (AL954747) (14). Identified genes in *N. europaea* are represented on the array by the probe sets with 14 pairs of 60-mer perfect match/mismatch oligo probes. Microarray analyses were performed in triplicate (three Fe-replete and Fe-limited), using the same amounts of RNA samples extracted from independent experiments. cDNA synthesis, labeling, hybridization, scanning and data normalization were performed at Roche Nimblegen Core Facility, Iceland. DNASTAR ArrayStar v2.1 software was used to analyze normalized gene expression data obtained from Roche Nimblegen's NimbleScan software. NimbleScan normalizes expression data using quantile normalization. Gene calls are generated using the Robust Multichip Average (RMA) algorithm. Differentially regulated genes were detected using a 2.0 fold change as a minimum for up- or down regulation. Student's t-Test with a cutoff p-value of 0.05 was used to compare the means of gene expression values for two individual replicates or for two groups of replicates for a given gene. Biological functions of up regulated or down regulated hypothetical genes were searched by BLAST.

3.4. Results

3.4.1. Global analysis of *N. europaea* iron-regulated gene expression

We investigated the changes in the transcriptome of iron-limited *N. europaea* cells compared to iron-replete cells. Of 2,461 *N. europaea* genes, 125 displayed statistically significant changes (≥2.0 fold) in transcript levels of 3 independent experiments (Fig 3.1). Additionally, 122 genes displayed statistically significant changes (≥2.0 fold) of transcript levels in at least two out of three independent experiments. Transcript levels of 2008 genes were considered to be unchanged. The latter group comprised genes with no statistically significant changes and those with changes < 2.0 fold. Significant (≥4.0 fold) changes in transcript levels were detected for 44 genes, of which 32 were up regulated and 12 were down regulated (Table 3.1). The functional classes most frequently represented among these genes were inorganic ion transport and metabolism, followed by energy production and conservation followed by hypothetical, unclassified, unknown genes (Fig 3.2). Gene names, descriptions and functional classes were taken from http://genome.ornl.gov/microbial/neur/.

3.4.1.1. Differential expression of genes related to inorganic ion transport and metabolism

The major response of *N. europaea* to iron limitation was in transcript levels of genes involved in inorganic ion transport and metabolism. Transcript levels of 80 genes belonging to this category changed at least 2.0 fold under iron limited. The majority of these transcripts i.e., 66, were present at higher levels while 14 were present at lower levels.

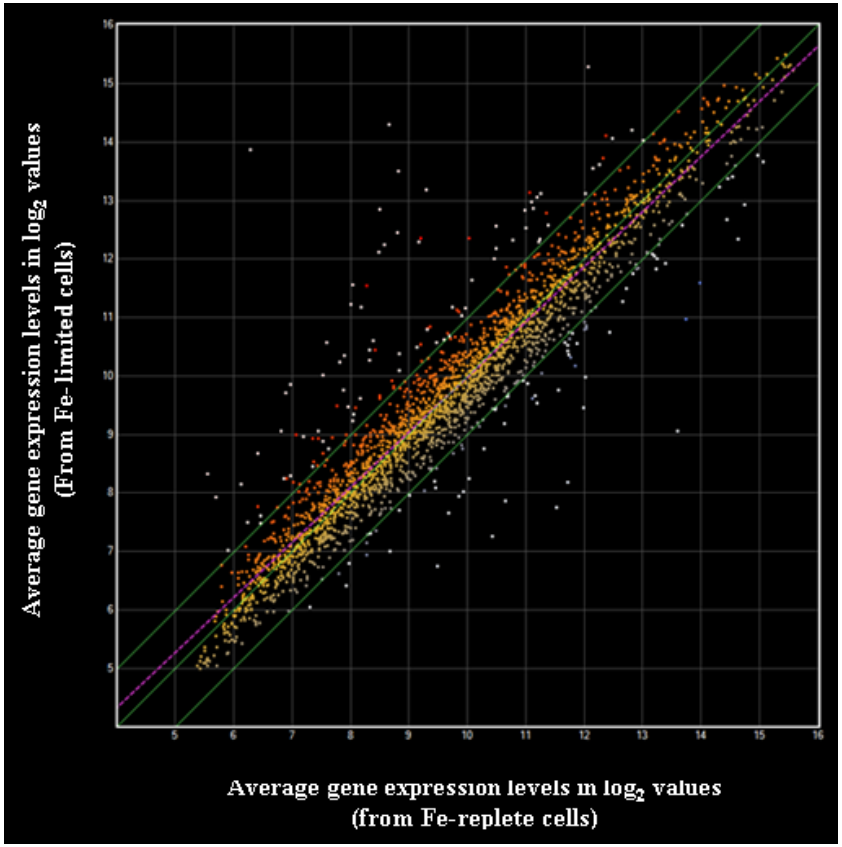

Figure 3.1. Scatter plot view of *N. europaea* gene expression levels in response to iron limitation. The plot shows average gene intensity data from iron-replete and iron-limited cells. The diagonal dotted line indicates no change in expression for both conditions. The area between the upper and lower diagonal lines indicates no significant (< 2.0 fold) change under both conditions. The area above the upper diagonal line indicates genes expressed significantly higher (> 2.0 fold) under iron-limited condition, and the area below the lower diagonal line indicates genes expressed significantly higher under iron-replete conditions. Intensity values are the average of three independent experiments.

	>2-fold	>2- to 4-fold	>4- to 8-fold	>8- to 16- fold	>16- to 32-fold	>32-fold
Genes with increased transcript levels	134 (5.4 %)	102 (4.1 %)	16 (0.65 %)	12 (0.49 %)	2 (0.08%)	2 (0.08%)
Genes with decreased transcript levels	113 (4.6 %)	101 (4.1 %)	8 (0.32 %)	3 (0.12%)	1 (0.04 %)	0 (0.0 %)
Total	247 (10%)	203 (8.2 %)	24 (0.97 %)	15 (0.61 %)	3 (0.12 %)	2 (0.08%)

Table 3.1. Number of genes whose transcript levels changed >2.0 fold in Fe-limited cells compared with Fe-replete cells. Percentrages in parentheses refer to the 2,461 *Nitrosomonas europaea* genes arrayed.

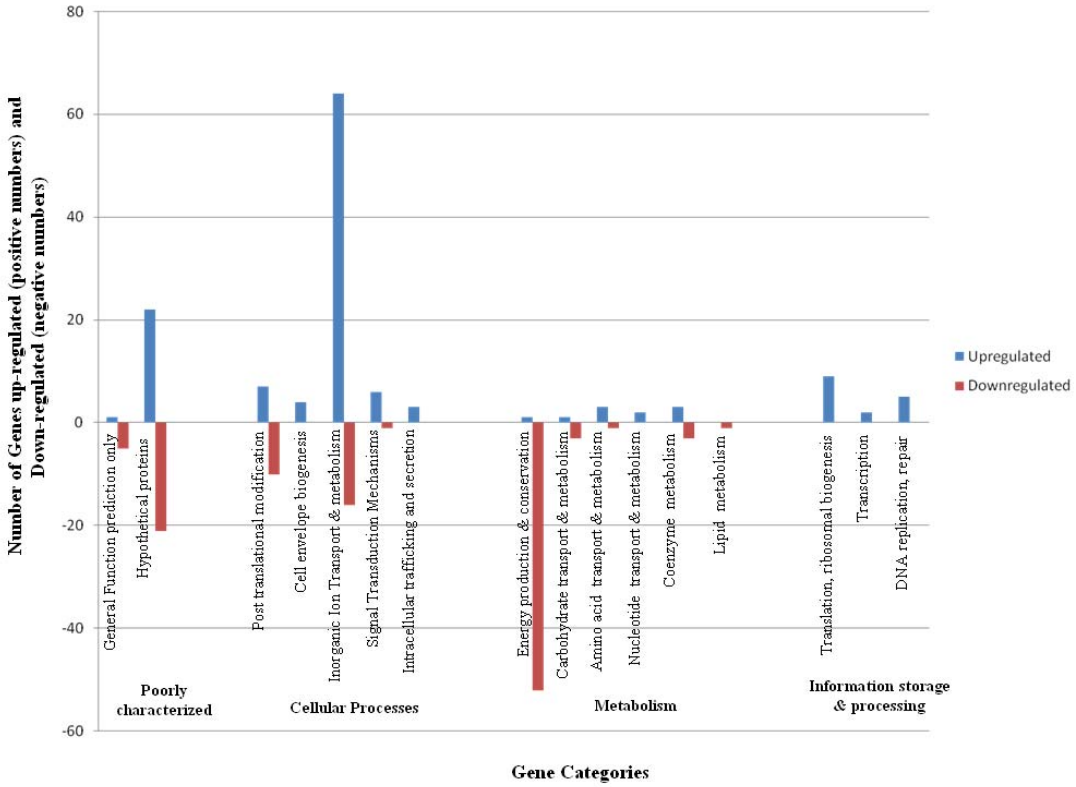

Figure 3.2. Functional classification of genes: Gene categories of *N. europaea* that changed expression levels in response to Fe limitation. Genes upregulated (blue bars) and downregulated (red bars) are those that passed the filtering criteria of 2 or greater change in expression level (iron-limited/iron-deplete ratio) and had a *P* value of <0.05. *P* values for changes in expression were assessed by Student's *t* test with ArrayStar software.

3.4.1.1.1. Siderophore dependent iron acquisition system genes

Like in other bacteria, transcripts of siderophore mediated iron acquisition system homologs were present at higher levels under iron limitation conditions in *N. europaea*. Transcripts of 9 TonB-dependent receptor homologs representing several iron-siderophore types including ferrichrome, ferrioxamine, enterobactin and cobalamin were present at higher levels (Table 3.2). Transcripts of 11 anti-σ (transmembrane sensor; FecR) homologs and 11 ECF-σ (extracyotoplasmic sigma factor; FecI) homologs were are present at higher levels (Table 3.2). Transcripts of the iron-siderophore ABC transporter complex and TonB-ExbB-ExbD complex, which are thought to be essential for iron-siderophore uptake, were also present at higher levels (Table 3.2). These results are consistent with other Gram-negative bacteria like *E. coli* [11], *P. aeruginosa* [12] and *N. meningitidis* [14] where many siderophore mediated iron acquisition system genes had higher transcript levels under iron limitation conditions.

Iron limitation also lead to down regulation of a relatively small number of siderophore mediated iron uptake gene homologs. Transcript levels of 3 TonB-dependent receptor homologs (NE1531, NE1532 and NE1536) and 1 FecR (NE0534; transmembrane sensor) homolog were lower under iron limitation (Table 3.2). Interestingly, each of these homologs has a glycoside hydrolase homolog (NE0537, NE1530 and NE1537) in its near vicinity. Perhaps these genes are not involved in iron acquisition, given the lower transcript levels under iron limitation and the contiguous glycoside hydrolase genes that also have lower transcript levels (Table 3.2).

Table 3.2. Genes associated with iron uptake and metabolism (>2.0 fold)

Gene[1]	Description[4,5]	Relative Fold Change[2,3]
~~NE0124/5/6~~	~~TonB-dependent receptor - pseudo gene~~	
NE0127	putative transmembrane sensor - fecR	+3.1
NE0128	probable sigma-70 factor, ECF subfamily - fecI	+2.1*
NE0153	GTP-binding protein - feob?	+2.6*
NE0230	hypothetical protein - multiple pdz domains??	+9.7*
NE0231	hypothetical protein - laminin beta chain domain??	+8.9*
NE0314	hypothetical protein - Gluc/Leu/Phe/Val dehydrogenase??	+2.9
NE0315	possible multicopper oxidase	+2.9
NE0316	hypothetical protein - DUF89??	+2.5
NE0352	Biopolymer transport protein ExbD/TolR	+3.3
NE0353	MotA/TolQ/ExbB proton channel family	+3.6
NE0354	possible TonB protein	+2.7*
NE0508	hypothetical protein	+12.8
NE0509	SCO1/SenC	+26.1
NE0510	hypothetical protein	+9.4
NE0534	putative transmembrane sensor	-2.2
NE0535	*TonB-dependent receptor protein*	*-1.6*
NE0536	BNR Repeat - glycoside hydrolase??	-2.6
NE0543	putative transmembrane protein??	+3.8*
NE0544	putative transmembrane anchor protein??	+7.1
NE0636	TonB-dependent receptor protein - cobalamin type??	+2.3*
NE0726	hypothetical protein - lipocalin domain??	+50.4
NE0727	dihydroorotase	+8.8
NE0730	Ferric uptake regulator family	+15.0
NE0731	TonB-dependent receptor protein - catecholate type??	+11.6

Table 3.2. continued

NE0863	Bacterioferritin	-2.0*
NE0978	*TonB dependent receptor*	*+1.4*
NE0979	putative transmembrane sensor	+2.2*
NE0980	putative sigma-70 factor, ECF subfamily	+2.1*
NE1029	Bacterial extracellular solute-binding protein, family 1	+2.6
NE1030	iron transport system permease protein	+2.9
NE1031	putative iron transport system ATP-binding protein	+2.5
~~NE1062/63~~	~~TonB dependent receptor pseudo gene~~	
NE1070	putative transmembrane sensor	+2.2*
NE1071	probable sigma-70 factor, ECF subfamily	+2.0*
NE1078	putative transmembrane sensor	+5.4
NE1079	*probable sigma-70 factor, ECF subfamily*	*+1.4*
NE1080	*outer membrane efflux protein*	*+1.3*
NE1081	probable fusion ABC transporter - iron-siderophore type???	+2.2
NE1082	hypothetical protein - ABC export system, membrane fusion protein	+2.4
NE1083	hypothetical protein - putative iron uptake protein	+4.7
NE1084	hypothetical protein - piuB	+4.4
NE1085	putative transmembrane sensor	+5.6
NE1086	probable sigma-70 factor, ECF subfamily	+3.9
NE1087	*TonB dependent receptor protein*	*+1.9*
NE1088	TonB-dependent receptor protein - ferrioxamine type	+3.7*
NE1089	TonB-dependent receptor protein - ferrichrome type??	+12.5
NE1095	*putative transmembrane sensor*	*+1.8*
NE1096	Sigma factor, ECF subfamily	+3.4
NE1097	TonB-dependent receptor protein - ferrioxamine type	+4.8
NE1098	putative transmembrane sensor	+5.7
NE1099	Sigma factor, ECF subfamily	+4.3
NE1101	Sigma factor, ECF subfamily	+2.6*
NE1102	putative transmembrane sensor	+2.4*
~~NE1105/08~~	~~TonB dependent receptor - pseudo gene~~	
NE1205	TonB-dependent receptor protein - catecholate type??	+2.5*

Table 3.2. continued

NE1217	putative sigma-70 factor, ECF subfamily	+2.2*
NE1218	putative FecR protein	+2.4
~~NE1220~~	~~TonB-dependent Receptor, pseudo gene~~	
NE1530	*putative signal peptide protein - glycoside hydrolase??*	*-1.9*
NE1531	TonB-dependent receptor protein	-4.1
NE1532	TonB-dependent receptor protein	-2.2*
NE1536	TonB-dependent receptor protein	-2.1*
NE1537	BNR repeat - glycoside hydrolase??	-2.8
NE1538	hypothetical protein - metallopeptidase??	+2.6*
NE1539	hypothetical protein - ferrochelatase??	+20.5
NE1540	TonB-dependent receptor protein - heme type??	+195.0
NE1543	Multicopper oxidase type 1	+ 7.5
NE1984	putative transmembrane protein - piuB??	+2.8*
NE1985	hypothetical protein - transmembrane lipoprotein??	+6.9
NE1986	hypothetical protein - membrane protein??	+4.7
~~NE1987/88~~	~~TonB-dependent receptor - pseudo gene~~	
NE1989	*putative transmembrane sensor*	*+1.1*
NE1992	*Sigma Factor ECF subfamily*	*+1.7*
NE2038	Myeloperoxidase, thyroid peroxidase, cyclooxygenase catalytic domain - **oxidase/peroxidase**	+8.1
NE2039	hypothetical protein	+2.8
NE2124	TonB-dependent receptor protein - catecholate type??	+12.7
NE2125	putative hydroxylase - hydroxylase PiuC	+2.2
NE2126	FMN-dependent alpha-hydroxy acid dehydrogenase??	+3.6*
NE2138	Sigma factor, ECF subfamily	+2.0*
NE2323	GTP-binding protein Era - feoB	+2.3
NE2432	hypothetical protein - piuB	+2.3
NE2433	TonB-dependent receptor protein - ferrichrome type??	+2.6
NE2434	putative transmembrane sensor	+4.8
NE2435	putative FecI	+2.5*

Table 3.2. continued

~~NE2482/84~~	~~TonB dependent receptor - pseudo gene~~	
NE2485	putative transmembrane sensor	+ 4.1
NE2486	Sigma factor, ECF subfamily	+ 2.9

[1] Potential novel iron transporters are boxed, gene orientation is indicated by left (clockwise) and right (counter clockwise) justification, and transcriptional organization is indicated by the vertical arrows.

[2] Fold increases (+) and decreases (-) in expression levels (with respect to wild type in Fe-replete medium) caused by Fe limitation are indicated by white and black backgrounds, respectively.

[3] Expression changes of atleast 2 fold and considered significant in two out of three biological replicates are shown with a star at their end.

[4] Expression changes of less than 2 fold and considered significant are shown in italics and were included because of their association with adjacent genes.

[5] TonB-dependent receptor pseudogenes shown in strikethrough font and are included for completion sake although there is no data for those genes.

3.4.1.1.2. Multi-copper oxidase dependent iron acquisition system genes

Transcripts of two multi-copper oxidase genes encoded by NE1543 and NE0315 were present at higher levels under iron limited conditions (Table 3.2). Multi-copper oxidases were shown to be essential for iron acquisition in *Saccharomyces cereviseae* [15, 16], and in *Chlamydomonas reinhardtii* [17]. Multi-copper oxidase associated high affinity iron uptake requires an Fe^{3+}-reductase and an Fe^{3+}-permease. Reductases first generate Fe^{2+} from Fe^{3+}; multi-copper oxidases then convert Fe^{2+} to Fe^{3+} which is immediately transported by Fe^{3+}-permease into the cytoplasm. Higher transcript levels of multi-copper oxidases in *N. europaea* under iron-limitation indicate that they might play a role in high affinity iron uptake. It has to be noted that homologs of neither Fe^{3+}-reductase nor Fe^{3+}-permease have been identified in *N. europaea* genome. However, 2 genes NE0314, NE0316 encoding hypothetical proteins also had higher transcript levels under iron-limitation conditions (Table 3.2). It is possible that these two hypothetical proteins along with NE0315 encoded multi-copper oxidase have a novel role in iron acquisition in *N. europaea.*

3.4.1.1.3. Potential novel iron acquisition genes

In addition to siderophore-dependent iron acquisition system homologs and multi-copper oxidase dependent iron acquisition system homologs, array analysis enabled us to identify several genes with potential novel roles in iron acquisition. Transcript levels of these genes were present at higher levels under iron limitation and can be organized into 8 gene clusters (Table 3.2).

Gene cluster NE1538-NE1539-NE1540 was particularly interesting with regards to iron acquisition. Gene NE1540 encodes a TonB-dependent receptor homolog and its

transcript showed highest change (195 fold high; Table 3.2) under iron limitation. BLAST searches indicated that this gene product is highly similar to HugA, the putative heme receptor of *Plesiomonas shigelloides* (e-value: 5e-148). The NE1540 coding sequence contains sequences found by Bracken et al. [18] to be present in most heme receptors but absent in outer membrane receptors not involved in heme uptake. ClustalW alignment of NE1540 with the HugA receptor of *P. shigelloides* or with the heme receptor HemR of *Yersinia enterocolitica* indicated that the two histidine residues implicated with their function are conserved in the amino acid sequence encoded by NE1540. FRAP and NPNL boxes and two conserved glutamic acids on the carboxy-terminal side of the NPNL box usually found in heme receptors are also present in the NE1540 sequence. Most HemR-expressing bacteria are able to use hemoglobin (Hb), heme, myoglobin, hemopexin, catalase, human and bovine serum albumin-heme, and haptoglobin-hemoglobin complexes as sources of iron. Interestingly HmbR expressing *Neisseria meningitidis* cells were able to use only hemoglobin (Hb) and heme [18] as sources of iron. Higher transcript level of a heme receptor in *N. europaea* iron-limited cells suggests that heme and related hemoproteins might be a potential source of iron for *N. europaea*.

In some pathogenic bacteria such as *N. meningitidis*, utilization of heme as iron source requires the oxidative cleavage of heme ring by a heme oxygenase (HemO) present in the cytoplasm [19]. *N. europaea* genome does not encode a *hemO* homolog; however, gene NE1539 whose transcript is also present at higher level in iron limited conditions, encodes a hypothetical protein with chelatase domain (8e-10 e-value; Table 3.2). Chelatases are a divergent group of enzymes that catalyze insertion of

metal ions into various tetrapyrroles. They are known for incorporation of metal ions into porphyrin molecules by distorting the porphyrin ring [20]. *N. europaea* genome encodes homologs of cobaltochelatase (*cobN;* NE0757; 0 e-value), ferrochelatase (*hemH;* NE1476; 7e-99 e-value) and siroheme synthase (*cysG;* NE0532; 9e-95) genes that are probably involved in cobalamin biosynthesis, heme biosynthesis and siroheme synthesis. There was no change in transcript levels of the above mentioned chelatase homologs in *N. europaea* due to iron limitation. Higher transcript levels of NE1539 in iron limited conditions indicates that NE1539 might play a potential role in iron metabolism. The possibility that NE1539 plays a role in acquiring iron from heme by distorting its ring structure needs to be investigated.

NE1538, encoding a hypothetical protein shows some similarity to a membrane bound metallopeptidase (0.004 e-value; Table 3.2). In *Plasmodium falciparum*, a malarial parasite that degrades hemoglobin, a novel metallopeptidase falcilysin was shown to degrade peptide fragments of hemoglobin although it was unable to cleave hemoglobin or denatured globin [21]. The NE1538 encoded protein might be playing a similar role in *N. europaea*.

It is not clear if genes NE1538, NE1539 and NE1540 are co-transcribed. Both NE1539 and NE1540 coding sequences have N-terminal signal peptides indicating that they are transported out of cytoplasm. No signal peptide was found in the predicted amino acid sequence of NE1538 coding sequence indicating that the protein is not transported out of cytoplasm. Homologs of this gene cluster are highly conserved in *N. eutropha* but completely absent in *N. multiformis*. In *N. oceani*, homologs of only NE1539 and

NE1540 are moderately conserved. These genes are likely to specify a new 3-component iron-uptake system.

Another cluster of genes NE0726-NE0727 was interesting with regards to iron acquisition. The transcript of NE0726, which encodes a hypothetical protein showed the second highest fold change under iron limitation (50 fold up; Table 3.2). Scanning the sequence against the Prosite patterns and motifs database identified a lipocalin signature domain (e-value: 0.012). Lipocalins are a functionally diverse family of proteins that generally bind small, hydrophobic ligands [22] and interact with cell-surface receptors [23]. Flo et al., [24] demonstrated that a protein called lipocalin 2 secreted by mammalian liver, spleen and macrophages can efficiently chelate Fe-enterobactin, thus keeping bacterial infections in check. Lipocalin 2 was shown to have high affinity for enterochelin (10^{-10} M), hence it has been renamed as siderocalin [25]. Lipocalin from human tears was also shown to bind bacterial and fungal siderophores [26].

Lipocalin members have been discovered in bacteria recently. The first bacterial lipocalin (Blc) identified in *E. coli* was shown to be an outer membrane lipoprotein expressed under conditions of environmental stress. Blc is thought to contribute to the adaptation of cells to both starvation and high growth medium osmolarity, which are conditions known to exert stress on the cell envelope [27]. Crystal structures of both human siderocalin and *E. coli* lipocalin Blc [28] have been determined and coding sequence of NE0726 is too distant from known lipocalin structures. ClustalW alignment of NE0726 with human lipocalin 2, tear lipocalin and bacterial lipocalins

indicated that NE0726 is more closely related to human lipocalin proteins than bacterial lipocalins.

NE0726 protein has an N-terminal signal peptide indicating that it is exported out of cytoplasm. A protein can be inserted into outer membrane by one of two ways [29], either through hydrophobic transmembrane sequences (which NE0726 does not seem to have) or through modification with a lipid (to become a lipoprotein) which NE0726 also does not seem to have. Hence, there are good chances that it is a periplasmic or secretory protein. We propose that NE0726 has a role in iron acquisition, perhaps involved in binding iron or an iron chelator, and then delivering it to a membrane protein for transport across the membrane.

The transcript level for gene NE0727, which is annotated as dihydroorotase (PyrC, 1e-148 e-value) and is downstream of gene NE0726 is also higher under iron limited conditions (Table 3.2). Dihydroorotase belongs to MEROPS peptidase family M38, where it is classified as a non-peptidase homolog. Dihydroorotase catalyses the third step in the *de novo* biosynthesis of pyrimidine, the conversion of ureidosuccinic acid (N-carbamoyl-L-aspartate) into dihydroorotate [30]. However, the *N. europaea* genome encodes another homolog of dihydroorotase (NE1664; 1e-103 e-value) that is clustered along with other pyrimidine biosynthesis enzymes like (CarA, CarB, PyrB, PyrX etc) and therefore likely to be involved in pyrimidine biosynthesis. It might be possible that NE0727 plays a role as a peptidase to release iron/iron-siderophores bound to NE0726 gene product.

Transcript levels for the gene cluster NE2038-NE2039 encoding a putative myeloperoxidase and a hypothetical protein were also higher under iron limited

conditions (Table 3.2). Both genes are co-polar with a 4 bp intergenic space. Myeloperoxidases are heme containing peroxidases that use hydrogen peroxide as the electron acceptor to catalyze a number of oxidative reactions. In *E. coli*, YcdB, a putative iron-dependent heme peroxidase was shown to be up regulated in iron starvation conditions and was thought to have a potential role in iron transport as a novel periplasmic oxidase or reductase [11]. However, in *E. coli*, *ycdB* was associated with two other genes: *ycdN*, a homolog of the high-affinity ferrous iron transporter (Ftr1p) of yeast and *ycdO*, a potential exported lipoprotein of unknown function. Homologs of the *ycdNOB* genes are found co-located in the chromosomes of at least 7 other bacteria indicating that these 3 genes form a functional unit [11]. In *N. europaea*, except for a hypothetical protein, there are no *ycdNO* homologs co-located with NE2038, the putative heme peroxidase. However homologs of ferrous iron transporter (NE0153, NE2323) and putative lipoproteins (NE1985, NE2466) that exist elsewhere in the genome had higher transcript levels under iron limited conditions (Table 3.2). Homologs of NE2038 & NE2039 are not present in the genome sequences of other ammonia oxidizers.

A gene cluster consisting of 3 co-polar genes NE2124-NE2125-NE2126, which encode homologs of a TonB-dependent receptor, a putative iron uptake protein, PiuC, a FMN-dependent alpha-hydroxy acid dehydrogenase also had higher transcript levels under iron limited conditions (Table 3.2). PiuC and FMN-dependent alpha-hydroxy acid dehydrogenase proteins encoded by genes NE2125 & NE2126 respectively may have an as-yet-unidentified role in iron metabolism.

Gene cluster NE0508-NE0509-NE0510 which encodes hypothetical proteins, also had higher transcript levels under iron limited conditions (Table 3.2). Hypothetical proteins encoded by NE0508, NE0510 appear to be conserved only in ammonia oxidizers such as *N. europaea, N. eutropha*, *N. multiformis* and *N. oceani* whereas the hypothetical protein encoded by NE0509 contains a Sco1 conserved domain (e-value: 7e-23) and its homologs are also present in other organisms. Sco1 proteins are copper chaperones that usually bind and insert copper ions into the active sites of cytochrome c oxidase subunits 1 and 2 [31]. Copper chaperones were also shown to be necessary for incorporation of copper into the multi-copper ferroxidase Fet3, which is required for high affinity iron uptake in *S. cerevisiae* [32, 33]. Iron limitation in *N. europaea* led to higher transcript levels of 2 multi-copper oxidase genes. It will be interesting to study the possibility that the gene products encoded by NE0508-NE0509-NE0510 acts play a copper chaperone role in biogenesis of multi-copper oxidases NE0315, NE1543 that are up regulated under iron limited conditions.

3.4.1.1.4. Putative membrane proteins with roles in iron acquisition

Transcripts of NE0230 and NE0231, which are oriented in opposite directions were present at higher levels under iron limited conditions (Table 3.2). These genes whose products are annotated as hypothetical proteins are unique to *N. europaea.* Blast searches indicated that part of the NE0230 protein sequence is similar to a multiple PDZ domain protein family member (mpz-1) of *Caenorhabditis elegans* with a 0.36 e-value. PDZ domains are found in many signaling proteins. One of their functions is to provide a scaffold for forming membrane-associated protein complexes by binding to the carboxyl termini of their partners. PDZ domains are also thought to play a signal

transduction role by propagating the information that binding has occurred to remote sites [34]. For example, in *E. coli*, a membrane bound protease DegS containing multiple PDZ domains were shown to sense accumulation of unfolded/misfolded outer membrane proteins (OMPs) in the periplasm, initiating a proteolytic cascade resulting in rescue or degradation of the misfolded OMPs in periplasm [35]. Iron limitation was shown to induce expression of OMPs in several organisms including *N. europaea* [4]. Higher transcript levels of NE0230 and NE0231 in iron limited conditions indicates that these genes have a potential role in iron metabolism.

Another cluster with higher transcript levels under iron limitation conditions consists of genes NE0543-NE0544 (Table 3.2). Blast searches indicated that the protein encoded by NE0544 gene is conserved across the bacterial kingdom and is annotated as putative transmembrane protein in some bacterial genomes. It has 7 predicted transmembrane helices. Gene NE0543, encoding another hypothetical protein is also conserved and in some organisms, it is annotated as a transmembrane anchor protein. It has 1 transmembrane helix. Both proteins have weak signal peptide predictions at their N-terminal end.

Transcript of NE2432, a hypothetical protein, was present at higher levels in iron limited conditions (Table 3.2). Homologs of this gene are found in *N. europaea* (not in other AOBs) and in other organisms. NE2432 belongs to COG3182 which is described as an uncharacterized iron-regulated membrane protein, PiuB. Genes NE1084, NE1984 also belong to this COG and their transcripts are also up regulated in iron limited conditions. All these 3 proteins have PepSY_TM domains which are associated with protease activity [36]. It should be noted that NE2432, NE1984,

NE1084 and two other genes NE0537, NE0550 (not up regulated in iron limited conditions) are adjacent to TonB-dependent receptors. Higher transcript levels of similar genes in *N. europaea* indicate that they might play a role in iron uptake.

3.4.1.1.5. Putative iron-responsive *fur* gene

Bacteria typically regulate their iron uptake in response to iron availability. In *E. coli* and many other bacteria this regulation is mediated by the ferric-uptake regulator protein (Fur) [37].). In *Helicobacter pylori*, the *fur* gene auto-regulates itself in response to iron [38]. In contrast, iron-responsive auto-regulation of the *fur* gene was not observed in *C. jejuni;* however, iron-limitation lead to up-regulation of *perR*, the peroxide stress regulator [13]. The *N. europaea* genome has 3 *fur* homologs [5]. Multiple *fur* homologs have been described for several bacteria including *B. subtilis* and are designated Fur, PerR [39, 40] and Zur [40] for their roles in regulating iron uptake, peroxide stress response and zinc uptake. While NE1722 encodes Zur (e-value: 1e-23), the remaining two *fur* homologs (NE0616; e-value: 1e-54 and NE0730; e-value: 8e-15), appear to encode Fe-sensing Fur proteins. *N. europaea* doesn't appear to have a *perR* homolog. The transcript of NE0616 whose deduced amino acid sequence has all conserved residues of Fe-sensing Fur proteins as other organisms, did not show any significant change in our microarrays. In contrast, the transcript level of NE0730, the second Fe-sensing *fur* homolog, was 15 fold higher in Fe limited conditions compared to Fe-replete conditions (Table 3.2).

3.4.1.2. Differential expression of genes related to energy production and conservation

Eighty genes encoding iron-containing proteins involved in energy metabolism had lower transcript levels under iron limited conditions (Table 3.3). These include genes encoding proteins involved in hydroxylamine metabolism (*hao-orf2-cycAB*). Hydroxylamine oxidoreductase (HAO), a key enzyme in the respiratory chain of *N. europaea* contains 24 c-type hemes per homotrimer [41]. The electrons extracted during oxidation of hydroxylamine may pass through two tetraheme cytochromes *c*554 (cycA) and *c*M552 (cycB) to the electron transport chain at the level of ubiquinone [42]. Down-regulating these iron-intensive proteins under iron limitation would provide a mechanism for *N. europaea* cells to conserve iron. There are 3 copies of *hao-orf2-cycA* and 2 copies of *cycB* (Table 3.3) in the genome and unique probes could not be made for these genes because they are identical or nearly identical. Hence, a single probe was used to interrogate these copies. Changes in expression for *cycAB* genes were less than 2.0 fold but were statistically significant. Although *hao-orf2-cycAB* genes are clustered together, separate transcripts for *hao* and *cycAB* were observed in *N. europaea* [43] which explains the differences in fold changes in *hao-orf2-cycAB* gene cluster. Lower transcript levels of *hao-orf2-cycAB* are consistent with lower respiration rates and lower NH_3- and NH_2OH-dependent O_2 uptake (AMO & HAO) activities observed in *N. europaea* iron limited cells [4].

Transcript levels of genes encoding several other cytochromes such as c553 and cytochrome P-460 were also lower under iron-limited conditions (Table 3.3). These results are consistent with lower heme C content of *N. europaea* iron-limited cells [4].

Table 3.3. Genes associated with metabolism (> 2.0 fold)

	Gene		Description[4]	Relative Fold Change[2,3]
	Energy production and conservation			
↑		NE0011	cytochrome P460 precursor	-2.3*
↓	NE0024		Cytochrome c, class I	-2.7
↑		NE0102	Cytochrome c-552 precursor	-3.5
		NE0620	Iron-containing alcohol dehydrogenase	-2.1*
↓	NE0622		cupin 2 like domain containing Fe^{2+} depedent dioxygenase??? E-value: 2e-04	-2.0*
↑		NE0675	glcD; glycolate oxidase subunit GlcD	-2.2
		NE0676	probable oxidoreductase	-2.1*
↓	*NE0735*		*Cytochrome c553*	*-1.4*
↓	NE0736		Cytochrome c553	-3.2
↑		NE0820	Zinc containing alcohol dehydrogenase superfamily	-2.1*
↓	NE0824		cytochrome C or P-460 domain??? E-value: 0.006	-2.2
↑		NE0852	Nitrite and sulfite reductase	-2.0*
↓	NE0870		Manganese and iron superoxide dismutase (SODM)	-2.6
↓	NE0887		ATP/NADPH-dependent carboxylic acid reductase?? E-value: 5.7	-2.4

Table 3.3. continued

NE0959		
NE2336	*Cytochrome C_M552 - cycB*	*-1.6*
NE0960;		
NE2042;		
NE2337	*Cytochrome c-554 - cycA*	*-1.7*
NE0961;		
NE2043;		
NE2338	hypothetical protein - orf2	-2.6
NE0962;		
NE2044;		
NE2339	Hydroxylamine oxidoreductase - hao	-3.4
NE1002	aconitate hydratase	-2.2*
NE1010	protoheme IX farnesyltransferase - cytochrome oxidase assembly factor	-2.3*
NE1011	Sco1/SenC/PrrC involved in biogenesis of respiratory and photosynthetic systems	-2.1*
NE1012	Putative transmembrane cytochrome oxidase c SURF1 family	-2.1
NE1232	catalase??? E-value: 6e-32	-6.9
NE1233	putative iron dependent peroxidase?? E-value: 4e-06	-6.6
NE1234	Cytochrome P-450 domain??? E-value: 2e-28	-8.9
NE1235	impB/mucB/samB family protein??? E-value: 2.2	-11.5
NE1236	Cytochrome C monohaem domain??? E-value: 2.0	-13.6
NE1237	Glucose-methanol-choline (GMC) oxidoreductase e-value: 6e-64	-3.4
NE1239	Lipoxygeanse	-2.3*
NE1240	cycloygenase-2	-2.2*
NE1241	Tyrosinase	-2.2*
NE1243	dehydrogenase domain??? E-value: 1.2	-2.8*
NE1244	hypothetical protein - kazal type proteinase inhibitor domain??	-4.6*
NE1245	Kazal-type serine protease inhibitor domain	-3.5*
NE1315	cytochrome c551 peroxidase	-4.9
NE1319	Thioredoxin	-2.0*
NE1320	2-isopropylmalate synthase	-2.0*
NE1529	Thioredoxin related	-2.7

Table 3.3. continued

NE1701	peptide methionine sulfoxide reductase - msrA;	-2.1*
NE1886	Catalase	-2.0*
NE2000	Aldehyde dehydrogenase family	-2.4*
NE2001	hypothetical protein	-2.7*
NE2003	nitric oxide reductase, cytochrome c-containing subunit	-3.2
NE2004	Cytochrome c oxidase, subunit I	-2.5
NE2005	norQ protein	-2.1*
NE2151	Rieske (2Fe-2S) domain-containing membrane protein??? E-value: 1e-13	-6.7*
NE2152	short chain dehydrogenase/reductase??? E-value: 0.69	-5.2*
NE2303	Uncharacterized NAD(FAD)-dependent dehydrogenase	-3.4
NE2304	Isochorismatase hydrolase family	-2.3
NE2305	DUF1130 superfamily -[Fe_4S_4 cluster]??? E-value: 4e-34	-2
NE2510	NADH:flavin oxidoreductase/NADH oxidase	-2.4*

[2, 3, 4] – Details as in Table 3.2.

Transcript levels of certain Fe-rich proteins such as bacterioferritin, superoxide dismutase and catalase that protect cells from oxidative damage were also lower under iron limited conditions (Table 3.3). Lower transcript levels of genes encoding such proteins under iron-starved conditions have been observed in *E. coli* [11], *P. aeruginosa* [12], *V. cholerae* [44] etc. In addition, transcript levels of genes encoding some hypothetical proteins that appeared to contain iron were also lower under iron limitation. Such hypothetical proteins and the e-values of the iron-containing domains are included in Table 3.3.

3.4.1.3. Differential expression of miscellaneous genes:

Iron limitation in *N. europaea* also affected a diverse number of cellular processes as indicated by changes in transcript levels of 104 genes belonging to various functional categories. Of these, 43 genes encode hypothetical proteins of unknown function (Appendix A). These genes did not have clear homology with any known or characterized proteins hence it is difficult to say whether they have an as-yet-unidentified role in iron metabolism.

Transcript levels of 16 genes associated with information storage & processing (Appendix B) were higher under iron limiting conditions. Transcript levels of some heme biosynthetic enzymes and cytochrome c biogenesis proteins were also lower under iron limitation (Appendix B). This is probably the reason for lower heme C-content of *N. europaea* cells under iron limitation conditions [4].

Transcripts of 45 genes associated with cellular processes (Appendix C) are differentially expressed under iron limited conditions. Higher transcript levels of genes encoding GroEL, GroES chaperones upon iron limitation is consistent with their role

in assisting folding and assembly or degradation of damaged proteins, respectively [45]. Of particular interest is the higher transcript level of iron-dependent Q biosynthesis genes (Appendix C) upon iron limitation especially when the cell is down regulating all other genes encoding non-essential iron containing proteins.

Genes NE0169, NE0170, NE0171 are co-polar and their transcript levels were higher under iron limited conditions (Appendix C). They are annotated as AcrA (acroflavin resistance protein A) belonging to HlyD secretion protein family, outer membrane efflux protein resembling TolC efflux pump OprM and a salt induced outer membrane protein. Multicomponent efflux pumps like AcrAB-TolC and MexAB-OprM which are found exclusively in Gram-negative bacteria including *P. aeruginosa* allow extrusion of substrates such as pyoverdine directly into the external medium [46, 47]. *N. europaea* genome does not carry genes required for siderophore biosynthesis and it is unknown what substrates it is pumping out under iron limited conditions.

Transcripts of genes NE2296, NE2297, NE2298, NE2299, NE2300 (bioDCHFB), homologs of biotin biosynthesis genes were all present at higher levels under iron limited conditions (Appendix C). Biotin biosynthesis genes are induced in response to low iron in a number of organisms; these include *bioA* in *C. jejuni* [13] and *bioB* in *H. pylori* [48], *bioB* in *V. cholerae* [44].

Transcript levels of genes NE0728-NE0729, NE0937-NE0938 and NE1923-NE1924, homologs of two component sensor systems (Appendix C) were differentially expressed under iron limited conditions. Bacteria use two-component signal transduction pathways to sense both the extra cellular and intracellular environment and to coordinate cellular events according to changing conditions [49]. *N. europaea*

probably uses above two component sensor system genes to adapt to a low iron environment.

3.5. Discussion:

This study revealed that *N. europaea* may have multiple routes for iron uptake in addition to siderophore-dependent iron acquisition. Although *N. europaea* genome encodes 42 TonB-dependent outer membrane receptors, only 9 are up-regulated under iron limited conditions in the absence of siderophores. It is interesting to note that of the upregulated 9 TonB dependent receptors, 7 were not directly contigous with anti-σ/ECF-σ homologs (Table 3.2). All these receptors were previously identified by mass spec analysis under low iron conditions [4].

NE1540, a TonB-dependent receptor on the other hand is probably involved in utilizing iron from heme or heme containing proteins. Up regulation of the transcript for the heme transporting receptor under iron limitation in *N. europaea* (Table 3.2) is surprising because the ability to take up heme or heme containing proteins is considered as a pathogenic bacterial strategy [50]. *N. europaea* probably utilizes heme and hemoproteins that enter the extracellular space following cell turnover and breakdown so it can recycle the limited iron efficiently.

Another surprising observation was a 50 fold higher transcript levelof NE0726 that encodes a hypothetical protein with a putative lipocalin domain (Table 3.2). Perhaps *N. europaea*, rather than producing and secreting siderophores, has acquired this mechanism to capture iron-siderophores. In such a case, this strategy gives *N. europaea* an edge while competing with other organisms. These findings raise many issues. For instance, does lipocalin chelate siderophore bound iron or just iron?

What happens to lipocalin once it has chelated the iron or siderophore and its bound iron?

In our previous study, we showed that *N. europaea* was able to grow in a medium with relatively low iron by adjusting its physiology. This study has revealed all those genes encoding Fe-containing proteins that *N. europaea* has down regulated in order to adapt to growth under iron limited conditions. Down regulation of genes encoding Fe-rich proteins under iron limitation represents a newly recognized iron homeostatic mechanism, which is under the control of a small regulatory RNA molecule, RyhB in *E. coli* [51], *V. cholerae* [52] and two functionally homologous sRNAs PrrF1 & PrrF2 in *P. aeruginosa* [53]. This mechanism allows the cellular demand for iron to be reduced, enabling available iron to be utilized more economically and ensures that production of Fe-requiring proteins does not exceed iron availability. RyhB like small RNA has not yet been identified in *N. europaea* but down regulation of transcripts coding for Fe-storage and several Fe-containing proteins indicates that such mechanism might exist in *N. europaea* too.

It is noteworthy that not all Fe-protein-encoding genes are down-regulated under iron limitation. This may reflect the high importance of some Fe-proteins, such as the DNA polymerase (Appendix B), the aerobic ribonucleotide reductase required for DNA biosysnthesis, Queuosine biosynthesis enzymes (Appendix C) whose transcripts were up regulated under iron limited conditions.

In conclusion, the evaluation of differential gene expression in *N. europaea* in response to iron limitation enabled us to gain a better understanding of the metabolic changes occurring in response to this stress. The study also enabled us in identifying

several new potential iron acquisition systems that might be unique to *N. europaea* or to ammonia oxidizing bacteria or present in other environmentally important bacteria. Clearly, iron acquisition in *N. europaea* might require more systems than what was previously thought, and further studies will be necessary to establish their role during iron limitation.

3.6. Aknowldgements

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CHARACTERIZATION OF *Nitrosomonas europaea* *nitABC* IRON TRANSPORTER

CHAPTER 4

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4.1. Abstract

The *Nitrosomonas europaea* genome has a single three-gene operon (*nitABC*) annotated as encoding an iron ABC-transporter system. Further phylogenetic analysis of *nitB* clustered the encoded product with Fe^{3+}-ABC transporter permease components from other organisms. An *N. europaea* strain deficient in *nitB* (*ΔnitB*) was constructed to study the role of *nitABC* encoded ABC transporter in iron acquisition. The *N. europaea ΔnitB* mutant strain grew well in either Fe-replete or Fe-limited media and also in Fe-limited medium containing the catecholate-type siderophore enterobactin or the mixed-chelating type siderophore aerobactin. However, in contrast to the wild type, the *ΔnitB* strain was unable to grow in Fe-limited media containing either hydroxamate-type siderophores ferrioxamine, ferrichrome or the mixed-chelating type siderophore pyoverdine. These results indicate that the *nitABC* operon encodes a unique ABC type iron transporter capable of transporting Fe^{3+}-bound ferrioxamine, ferrichrome or pyoverdine siderophores and that there are as yet undetermined transporters for transport of iron either in Fe^{3+} or Fe^{2+} forms and also Fe^{3+}-bound enterobactin and aerobactin siderophores across the cytoplasmic membrane.

4.2. Introduction

Iron is vital for most organisms since it is a component of several key molecules that include cytochromes, ribonucleotide reductase and other metabolically linked compounds [1]. Iron is the fourth most abundant metal on earth; however in oxidative environments at physiological pH, iron is not always easily bio-available since it exists in an insoluble ferric (Fe^{3+}) state. Hence, microorganisms have evolved diverse strategies to acquire this essential nutrient.

The majority of microorganisms employ siderophore-dependent iron acquisition systems to acquire iron when it is limiting [2]. These systems involve use of small non-proteinaceous Fe^{3+}-chelating compounds called siderophores that are synthesized and secreted into the environment. Based on structure, siderophores can be categorized into hydroxamate, catecholate and mixed-chelating types [3]. In Gram-negative bacteria including *Escherichia coli* [1] and *Yersinia pestis* [4], Fe^{3+}-siderophore complexes are taken up by siderophore specific outer membrane receptors and specific ABC transporters [1]. Siderophore-dependent iron acquisition systems are highly efficient but can be metabolically demanding since they involve expression of numerous gene products to synthesize and take up siderophores.

Alternatively, some pathogenic bacteria including *Haemophilus influenzae* [5] and *Neisseria gonorrhoeae* [6], utilize a siderophore-independent iron transport system. Such siderophore-independent high affinity iron acquisition systems involve specific outer membrane receptors that directly bind to host iron binding proteins transferrin or lactoferrin, extract Fe^{3+} and transport it into the periplasm [7]. The free Fe^{3+} is then transported from periplasm to the cytosol by the Fe^{3+}-ABC transporter [5]. The

fundamental difference between siderophore-dependent and siderophore-independent iron transport involves the chemical nature of the substrate. In the former, iron is bound and transported into the cytosol as an intact Fe^{3+}-siderophore complex and in the later, free Fe^{3+} is bound and transported into the cytosol [5, 7].

ABC transporters couple ATP hydrolysis with substrate translocation across biological membranes. A typical prokaryotic binding protein-dependent ABC transporter system involves (i) one or several extracellular binding proteins, (ii) one or two different (homodimer or heterodimer) integral membrane permease proteins and (iii) one or two different ATP-hydrolases that face the cytoplasm and supply energy by hydrolysis of ATP. Binding proteins bind to the substrate with high affinity and mediate the first steps in the passage of substrate across the cytoplasmic membrane. Binding proteins usually have a cleavable signal sequence, processed by the leader peptidase and subsequently released as mature protein into the cytoplasm. The second step of the transport process is binding of the binding protein to the integral membrane permease protein and substrate transport across the cytoplasmic membrane [8].

Nitrosomonas europaea is a Gram-negative chemolithoautotrophic bacterium that derives its energy for metabolism by oxidization of ammonia (NH_3) and whose genome has been sequenced and annotated [9]. One of the interesting revelations of the annotation efforts was the presence of ~95 genes dedicated to iron transport and none for siderophore synthesis [9] supporting the physiological relevance for *N. europaea's* high iron requirement [10]. Of these 95 genes, 42 are homologs of TonB-dependent outer membrane siderophore receptors that are specific to various hydroxamate, catelcholate and mixed-chelating type siderophores. This is a relatively

large number compared to 11 receptor homologs in the chemolithoautotroph *Acidithiobacillus ferroxidans* [11] or six in the heterotroph *Escherichia coli* and 35 genes in the heterotroph *Pseudomonas aeruginosa* [1]. Despite the plethora of Fe^{3+}-siderophore receptor homologs in *N. europaea*, the genome annotation revealed only three genes that were homologs of binding protein dependent Fe-ABC transporter system [9]. This was surprising since other bacteria such as *E. coli* and *Y. pestis* have at least 3 complete sets of Fe-ABC transporters [1]. We hypothesized that *N. europaea* utilizes this single ABC transporter system as a common iron carrier responsible for iron uptake into the cell interior. In other words, in *N. europaea,* a single ABC transporter system was thought to serve as convergence point for transport of iron across the cytoplasmic membrane.

In this study, we tested the role of the iron ABC transporter encoded by NE1029-NE1030-NE1031 genes by constructing an *N. europaea* strain deficient in the permease component (NE1030). We demonstrated that *nitABC* transporter is involved in transport of iron associated with few siderophores but not iron from other sources.

4.3. Materials and Methods

4.3.1. Bacterial cultures and siderophore feeding experiments

N. europaea (ATCC 19178) was cultured as described with minor modifications [12, 13]. The standard (Fe-replete) medium contained 10 μM Fe^{3+} ($FeCl_3$) complexed with EDTA to prevent iron precipitation. Fe-limited medium was made from reagent-grade chemicals, without addition of any iron salt, and contained 0.2 μM Fe [14]. All media, buffers and other reagents were made in deionized water. All glassware was soaked in 1% HNO_3 overnight, and then rinsed thoroughly with deionized water. Fe-free

ferrioxamine (desferal), ferrichrome and pyoverdine were purchased from Sigma (St. Louis, MO). Fe-free enterobactin and aerobactin were purchased from Biophore Research Products (Tübingen, Germany). No siderophores pre-loaded with Fe were used in this study. Siderophores were dissolved in deionized water, filter-sterilized, and added to Fe-limited medium in the siderophore feeding experiments. In this study 10 μM siderophore was used to ensure the complete chelation of Fe (0.2 μM) in the Fe-limited medium. Notable among the siderophores is enterobactin which has low solubility in water. Addition of 2% MeOH increased its solubility but inhibited *N. europaea* growth significantly. Hence, we suspended enterobactin in water and stirred it well before addition to *N. europaea* Fe-limited media. Strains and plasmids used in this study are listed in Table 4.1.

4.3.2. RNA extraction and RT-PCR analysis

RNA was isolated as described [15]. Briefly, 250 ml acid phenol, 250 ml chloroform, SDS to 1% and sodium acetate to 0.3 M were added to 500 ml cell suspension in buffer (2 mM $MgCl_2$ and 50 mM NaH_2PO_4, pH 7.5). The cell suspension was mixed thoroughly and centrifuged for 5 min at 16000 *g*. The total RNA was precipitated with ethanol and resuspended in diethylpyrocarbonate (DEPC)-treated water. When necessary, cells were resuspended in a commercial RNA stabilizer solution (RNAlater; Ambion, Austin, TX), to prevent mRNA changes and degradation during sample preparation. Reverse Transcription (RT) was done with M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions, with a 50 ^{0}C extension temperature. RNA templates for RT-PCR were treated with RQ1 DNase (Promega) or 'DNA-free' DNase (Ambion) multiple times until no DNA product was

detected by Taq DNA polymerase in a PCR with any of the primers used. The primers used in the PCR and RT-PCR experiments are listed in Table 4.2.

4.3.3. DNA manipulations, mutagenesis and mutant isolation

DNA preparation, restriction digestions and agarose gel electrophoresis were done as described [16]. The iron ABC transporter permease component *nitB* (Fig. 4.1a) was amplified by PCR using Taq DNA polymerase (Promega) on an iCycler Thermal Cycler (Bio-Rad, Hercules, CA) as described by the manufacturers (see Table 4.2 for primers). The resulting DNA fragments were cloned into the pGEM-T Easy vector (Promega). A kanamycin-resistance cassette (from pUC4KSAC [17]) was inserted into the BglII site of the *nitB* ORF (Fig. 4.2a). The plasmid construct with the insertion was introduced into the *N. europaea* wild-type cells by electroporation on the ElectroPorator (Invitrogen) at 1300 V, with a capacitance at 50 μF, and a load resistance at 500 Ω. Successful transformants were selected in liquid medium using kanamcyin sulfate (20 mg ml-1). Aliquots from these cultures were streaked onto Nylon disk membranes, which were placed on semisolid plates, to isolate clonal mutant strains, as described ([18]. The *N. europaea ΔnitB* mutant was verified by Southern analysis (Fig. 4.2b, and Results).

For Southern hybridization analysis, DNA was resolved in 1% agarose gels [16]. Prior to electrophoresis, DNA was stained with ~5 mg ethidium bromide ml^{-1} in the loading buffer. The DNA was blotted onto Nytran membranes (Schleicher & Schuell BioScience, Keene, New Hampshire, USA). Probes for hybridization were generated

Table 4.1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
E. coli		
DH5α	F′ ø80d*lacZ*ΔM15 *endA1 recA1 gyrA96 thi-1 hsdR17(r_K^- m_K^+) supE44 relA1 deoR Δ*(*lacZYA-argF*)U169	[19]
N. europaea		
ATCC 19178	wild-type	American Type Culture Collection
ΔnitB	Insertion of *Kan cassette* into BglII site of NE1030 gene	This study
Plasmids		
pUC4 pKSAC	Plasmid containing the *kan* cassette from Tn903	Pharmacia
pGEM-T Easy	Vector for cloning PCR products; Amp^r	Promega
pNE1030	1984-bp PCR fragment containing NE1030 gene cloned into pGEM-T Easy vector	This study
pNE1030::*kan*	*kan* cassette from pUC4 pKSAC inserted into BglII site of pNE1030	This study

Table 4.2. Primers used in this study.

For RT-PCR analysis		
NE1029-1	5'-AATTTTCTGGCCAAATCAGG-3'	This study
NE1029-2	5'-AATATGGAATATCCCGCCAA-3'	This study
NE1030-1	5'-CCACCGCGATTTATAAAACCT-3'	This study
NE1030-2	5'-GATGCGTTTTTCTGAAAGCA-3'	This study
NE1031-1	5'-TGATACGCCCTGATGATGTT-3'	This study
NE1031-2	5'-ATCATGCCATTGGTGAGAAA-3'	This study
For cloning, mutagenesis and mutant confirmation		
NE1030ud-1	5'-CGAACAGGCACAAAAACTGT-3'	This study
NE1030ud-2	5'-ACATTGGCGGTTACAGTCAG-3'	This study

by PCR with primers specific for *nitB* gene and labeled by random priming (Prime-a-Gene Labelling System, Promega) with [α–^{32}P]dCTP (3000 Ci mmol^{-1}, 110 TBq mmol^{-1}; ICN). Hybridization was carried out as described by [16, 20]. Images were obtained by phosphorimaging as described by the manufacturer (Molecular Dynamics, Sevenoaks,

Kent, United Kingdom).

4.3.4. Phylogenetic tree construction

ClustalW was used for sequence alignment applying default parameters (altered gap penalties were not applied). Gaps in the alignment were not omitted. The phylogenetic tree was built by Parsimony method with the distance matrix generated by ClustalW and was represented with the program DrawTree 3.6 available at (http://mobyle.pasteur.fr/). The reliability of each node was established by bootstrap methods.

4.3.5. Hidden Markov Model-based Fur binding site prediction

A group of experimentally validated Fur boxes from *E. coli*, *S. typhimurium*, *P. aeruginosa* and *S. aureus* used by Quatrini et al., [21] along with 3 experimentally confirmed *N. europaea* fur boxes were used to build HMM profiles and to search for fur binding sites in the promoter regions of (600 nt from the proposed initiation of translation of the potential target gene).

N. europaea sequence data was obtained from DOE Joint Genome Institute (JGI) website (http://genome.ornl.gov/microbial/neur/). Sequence similarity searches of the available nucleotide and protein databases were performed with the BLAST program,

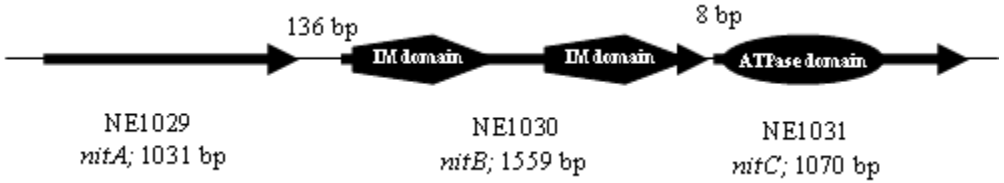
available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast/).

4.4. Results

4.4.1. Sequence analysis of the *nitABC* genes

Three genes encoding a complete set of putative iron uptake ABC transporter were annotated in the *N. europaea* genome [9]. In this study, these genes were named *nitABC* for *Nitrosomonas* iron transporter ABC components. The *nitA* (NE1029, 343 aa residues), *nitB* (NE1030, 519 aa residues) and *nitC* (NE1031, 356 aa residues) genes are co-polar (Fig. 4.1a). Sequence similarity searches of the *nitABC* genes using BLASTX predicted that *nitA* encodes an iron transport system periplasmic binding protein, *nitB* encodes an iron transport system permease protein and *nitC* encodes an iron transport system ATPase protein. The *nitA* encoding sequence contains a signal peptidase I cleavage site signal sequence (probability 0.998; between residues 33 and 34) as predicted by SignalP 3.0 HMM program [22]. The *nitB* encoding sequence contains 2 ABC transporter integral membrane type-1 domains (residues 33-235 and 307-513) characteristic of ABC transporter permease components [23]. The *nitC* encoding sequence contains an ATP-binding cassette, ABC transporter-type domain (residues 5-237) and also contains Walker A (residues 37-43) and B (residues 157-162) motifs, ATP-binding domain (residues 37-44) and ABC transporter signature domain (residues 137-151) characteristic of ABC transporter ATPase components [23]. Homologs of *nitABC* are absent in sequenced genomes of other ammonia oxidizing bacteria except in *Nitrosospira multiformis* [24]. Comparison of *nitB* coding sequence from *N. europaea* and *N. multiformis* with iron ABC transporters from

a



b

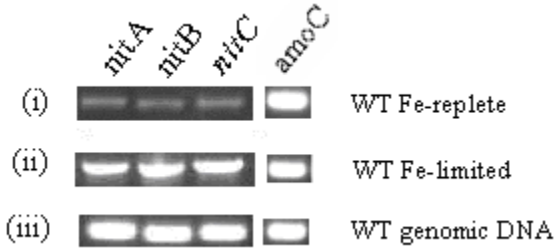


Fig. 4.1. Genetic map of *nitABC* and mRNA levels under Fe-replete and Fe-limited growth conditions. (a) Gene organization and domains. (b) RT-PCR analysis of transcript levels of *nitABC* genes from *N. europaea* wild-type (WT) grown under (i) Fe-replete and (ii) Fe-limited conditions and (iii) PCR analysis of *nitABC* genes using *N. europaea* wild-type (WT) genomic DNA as positive control. *amoC* gene from *N. europaea* was used as a control for RT-PCR.

other bacteria placed *nitB* in the subgroup of Fe^{3+}-ABC transporters (Fig. 4.2). *nitA* and *nitC* genes also grouped with Fe^{3+}-ABC transporters (data not shown).

Generally, transcription of genes encoding iron uptake systems is repressed in iron-replete conditions. In most gram-negative bacteria, a Fur-Fe^{2+} dimer binds to a conserved DNA sequence, called a Fur box, which usually overlaps the −10 and/or −35 promoter motifs, blocking access of RNA polymerase to the promoter [25]. We have used an *in silico* approach, fed with experimentally confirmed *N. europaea* Fur boxes (unpublished data), to identify candidate Fur-binding sites in promoter regions of *nitABC* genes. A potential Fur box (5'-GATTGATAACTATTCTT-3') in the promoter region of *nitA*, -142 bp upstream of the proposed initiation of translation of the *nitA* gene was found. No putative Fur boxes were identified upstream of *nitB* or *nitC* genes.

4.4.2. Transcriptional analysis of *nitABC* genes

The *nitB* ORF starts 136 bp after the stop codon of *nitA* and *nitC* starts 8 bp after the stop codon of *nitB*. In order to determine whether *nitABC* genes are co-transcribed in a single operon, we performed reverse transcriptase PCR (RT-PCR) amplification analysis using combinations of primers designed to amplify the intergenic regions of the *nitABC* ORFs. RT-PCRs across the *nitA*/*nitB* and *nitB*/*nitC* junctions gave products identical in size to the positive control reactions with genomic DNA as template (data not shown) suggesting that *nitABC* genes are linked in a transcript. To determine whether the *nitABC* genes were expressed differentially in *N. europaea* under Fe-replete (10 μM) and Fe-limited (0.2 μM) conditions, we once again performed RT-PCR analysis. The *nitABC* transcripts were detected at higher levels in

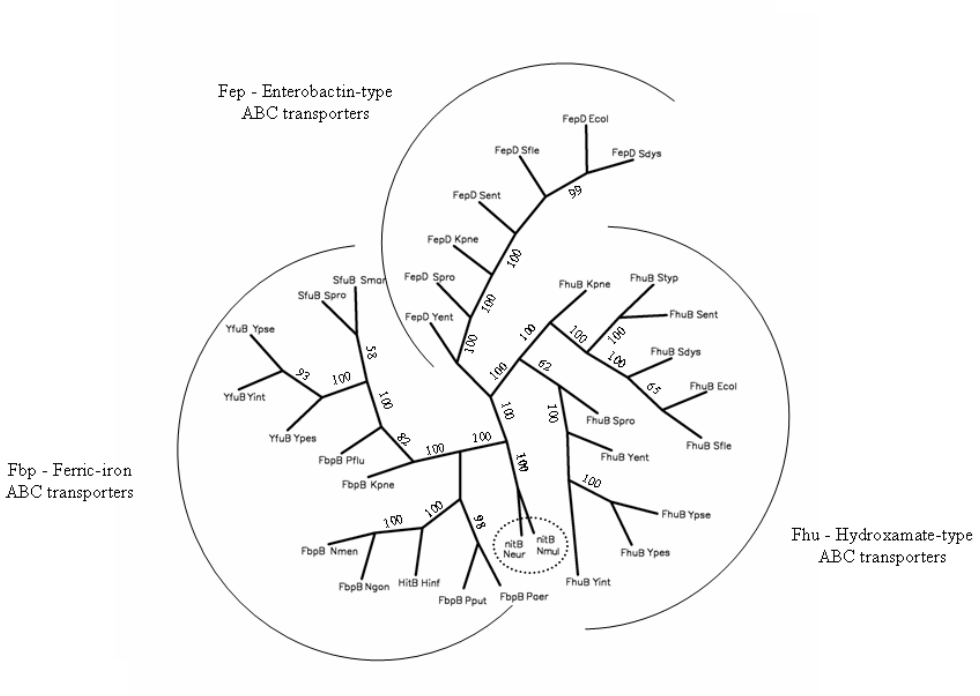


Fig. 4.2. Unrooted phylogenetic tree showing the relationship of *N. europaea* NitB-CDS to iron-ABC transporters from other organisms using Parsimony method.
Ecol, *Escherichia coli*; Hinf, *Heamophilus influenzae*; Kpne, *Klebsiella pneumoniae*; Ngon, *Neisseria gonorrhoaea*; Nmen, *Neisseria meningitidis*; Neur, *Nitrosomonas europaea*; Nmul, *Nitrosospira multiformis*; Sent, *Salmonella enterica*; Styp, *Salmonella typhimurium*; Spro, *Serratia proteamaculans***;** Smar, *Serratia marcescens*; Sdys, *Shigella dysenteriae*; Sfle, *Shigella flexneri*; Yent, *Yersinia enterocolitica*; Yint, Yersinia intermedia; Ypes, *Yersinia pestis*; Ypse, *Yersinia pseudotuberculosis*; Paer, *Pseudomonas aeruginosa*; Pflu, *Pseudomonas fluorescens*; Pput, *Pseudomonas putida*.

cells grown in Fe-limited medium than in Fe-replete medium. Transcripts of ammonia monooxygenase C *(amoC)* component used as positive control both for the efficiency of the RT-PCR procedure and for RNA and cDNA recovery showed no significant difference in expression in Fe-replete and Fe-limited medium (Fig. 4.1b).

4.4.3. Construction of the *N. europaea ΔnitB* strain

The *N. europaea ΔnitB* strain containing disrupted *nitB* was constructed by insertional mutagenesis in wild-type background and used to analyze the function of ABC transporter encoded by the *nit* operon. The *nitB* construct with a kanamycin-resistance cassette (Km^r) insert (Fig. 4.3a) was electroporated into *N. europaea* wild-type cells. The *ΔnitB* mutant was obtained through homologous recombination and confirmed by PCR (data not shown) and Southern hybridization (Fig. 4.3b). The *nitB* probe detected a 2.2 kb *EcoR1-Kpn1* fragment in the wild type and a 3.1 kb fragment (calculated size based on the DNA sequences) in the *ΔnitB* mutant. The Km^r probe detected the same 3.1 kb fragment in *ΔnitB* mutant but not in the wild type. These results confirm that a single copy of Km^r was correctly inserted in *nitB* gene of the *N. europaea* genome. We performed RT-PCR analysis to determine whether kanamycin insertion in *nitB* gene led to disruption of *nitB*. Transcripts for either *nitB* or *nitC* were not detected in *ΔnitB* mutant (Fig. 4.3c). The absence of the *nitC* transcript might be due to a polar effect since it is downstream to *nitB*. Transcripts of ammonia monooxygenase C *(amoC)* component used as positive control both for the efficiency of the RT-PCR procedure and for RNA and cDNA recovery showed no significant difference in expression in Fe-replete and Fe-limited medium (data not shown).

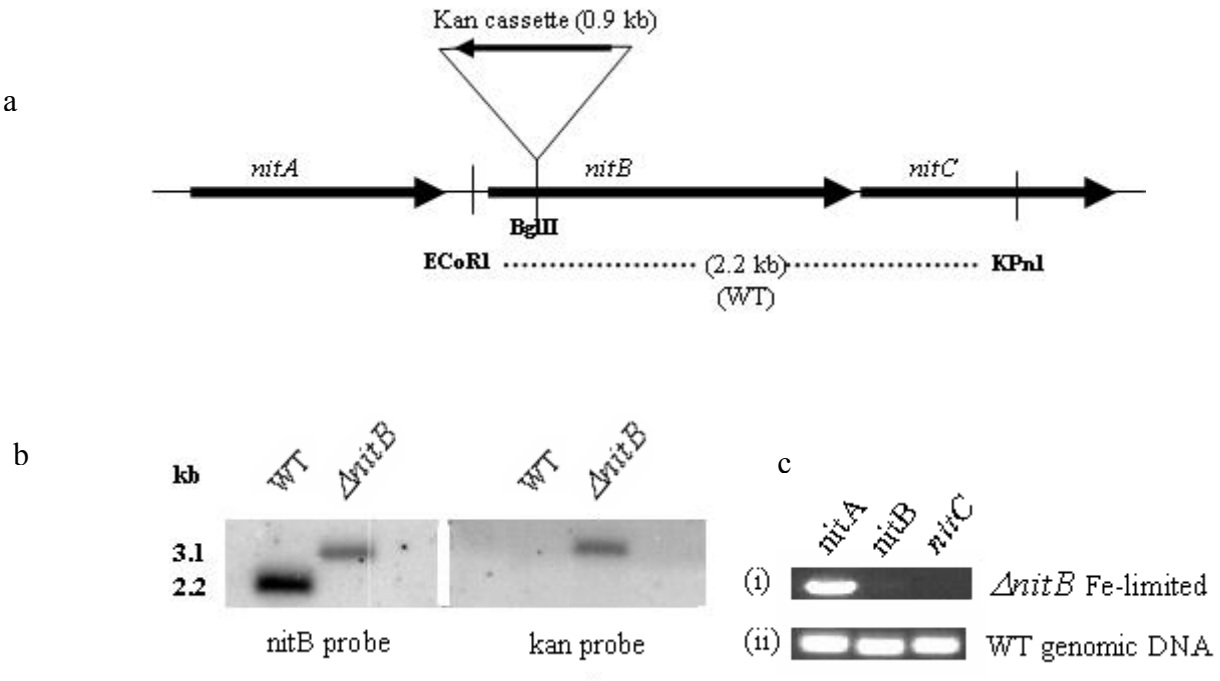


Fig. 4.3. Insertional mutagenesis scheme and mutant confirmation by Southern Analysis. (a) Restriction map of *nitABC* genes and mutagenesis strategy. (b) Verification of mutagenesis of *nitB* gene in *N. europaea* by Southern hybridization. Genomic DNA from the wild-type (WT), *ΔnitB* mutant were digested with EcoR1 and Kpn1, and probed with (left) *nitB* ORF sequence and (right) kan sequence. (c) (i) RT-PCR analysis of transcript levels of *nitABC* genes from *ΔnitB* mutant grown under Fe-limited conditions and (ii) PCR analysis of *nitABC* genes using *N. europaea* wild-type (WT) genomic DNA as positive control. Fe-replete medium contained 10 μM Fe and Fe-limited medium contained 0.2 μM Fe.

4.4.4 Effect of *nitB* mutation on growth of *N. europaea*

Growth of the *N. europaea ΔnitB* strain was compared to that of wild-type strain in both Fe-replete and Fe-limited medium. Surprisingly, there was no significant difference in growth of *ΔnitB* in both Fe-replete and Fe-limited medium compared to wild type (Fig. 4.4a). The possibility that the *nitABC* transporter plays a role in transport of iron from all sources across the cytoplasmic membrane as hypothesized could not be demonstrated. Apparently in the absence of siderophores, *N. europaea* has alternative means for transport of Fe^{3+} or ferrous (Fe^{2+}) ions across the cytoplasm. To further investigate the role of *nitB*, we examined growth of both the wild-type and the *ΔnitB* strain in Fe-limited medium containing Fe^{3+}-chelating siderophores such as ferrioxamine, ferrichrome (hydroxamate-type siderophores), enterobactin (catecholate-type siderophore), aerobactin or pyoverdine (mixed-chelating type siderophores). The *ΔnitB* mutant strain could not grow in presence of 10 μM ferrioxamine, ferrichrome or pyoverdine while the wild-type strain grew well in their presence (Fig. 4.4b). Interestingly, both the *ΔnitB* mutant and the wild-type strain grew in presence of 10 μM enterobactin and 10 μM aerobactin (Fig. 4.4c) after a lag period of 4 to 9 days. Perhaps *N. europaea* induces the systems responsible for uptake of Fe^{3+}-associated with enterobactin and aerobactin during this lag period, though it may also be that changes ot the medium or to the siderophores during the lag lead release of Fe^{3+} and growth of *N. europaea*.

These results indicate that the *nitABC* operon encodes an ABC transporter capable of taking up Fe^{3+}-bound ferrioxamine, ferrichrome or pyoverdine into the cytoplasm, and is not involved in uptake of Fe^{3+} bound to enterobactin or aerobactin siderophores.

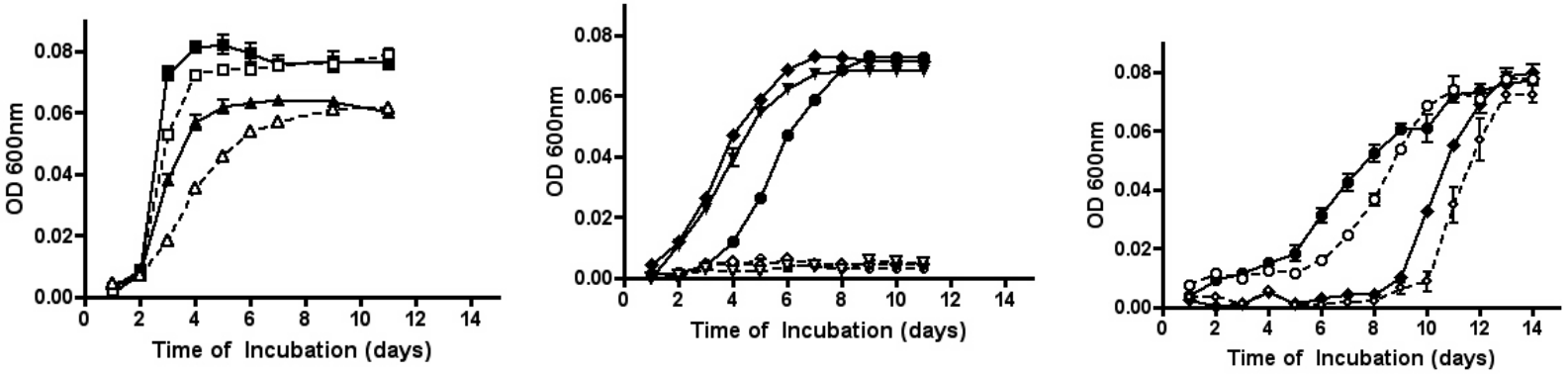


Fig. 4.4. Growth of the *N. europaea* wild-type (WT) (straight lines, filled symbols) and *ΔnitB* mutants (dotted lines, open symbols)**.** (a) Fe-replete (squares) and Fe-limited (triangles) media. (b) Fe-limited medium with ferrioxamine (circles), ferrichrome (diamonds) and pyoverdine (triangles) (c) Fe-limitied medium with enterobactin (circles) and aerobactin (diamonds). Fe-replete medium contained 10 μM Fe and Fe-limited medium contained 0.2 μM Fe.Data shown are means of triplicates, with variation less than 10%. The experiment was repeated and produced similar results.

4.5. Discussion

Many Gram-negative bacteria encode multiple ABC transporters with different substrates for iron uptake. For instance, *E. coli* utilizes FepBCDG ABC transporter for transport of Fe^{3+}-enterobactin complexes [26, 27], FecBCDE for Fe^{3+}-citrate transport [28] and FhuBCD ABC transporter for transport of several Fe^{3+}-hydroxamate complexes [29]. A common feature of the characterized ABC transporters appears to be that that they can only transport structurally similar Fe^{3+}-loaded siderophores [8]. Other bacteria, such as *S. marcescens*, *N. meningitidis*, *Y. enterocolitica* and *H. influenzae*, utilize Fe^{3+}-ABC transporters (e.g. SfuABC, FbpABC, YfuABC, HitABC) to sequester the unchelated Fe^{3+} in the periplasm and transport it into the cytoplasm [8]. Such Fe^{3+}-ABC transporters function in the absence of siderophores and clearly differ from Fe^{3+}-siderophore ABC transporters. Except for few signatures such as the conserved region (CR) in the integral membrane permease components or the Walker A and B motifs in the ATPase components, Fe^{3+}-siderophore ABC transporters and Fe^{3+}-ABC transporters do not share obvious sequence similarity [8].

In the *N. europaea* genome sequence, only 1 complete set of ABC transporter (*nitABC*) with similarity to known iron uptake transporters was annotated [9]. The *nitABC* operon encodes a classical ABC transporter, with a periplasmic binding protein, a cytoplasmic membrane protein which potentially acts as a membrane permease and an ATP-binding protein. Transcription assays using RT-PCR analysis confirmed that the *nitABC* region is expressed and is organized as a single expression unit in *N. europaea*. The *nitABC* operon is preceded by a Fur box consensus, suggesting regulation by iron and our RT-PCR analysis confirmed that transcript

levels of *nitABC* genes were higher in Fe-limited medium compared to Fe-replete medium. Transcript levels of *nitABC* operon were ~ 2.5 fold higher in Fe-limited medium compared to Fe-replete medium in our microarray experiments (data not yet published).

On the basis of phylogenetic analysis, the *nitABC* operon appeared to encode a Fe^{3+}-ABC transporter. Hence, we hypothesized that *N. europaea* transports iron from all sources across the cytoplasmic membrane through a single ABC transporter. However, in this study, we showed that the *ΔnitB* mutant strain had no growth defect in Fe-replete or Fe-limited medium indicating that there must be other mechanisms for transporting iron into the cytoplasm. Such redundancy of iron transporters within a genome has often hampered investigation of a particular iron transporter's function, with single mutations affecting an iron transporter frequently resulting in no discernible phenotype [30, 31].

However, *ΔnitB* mutant strain could not grow in Fe-limited medium containing hydroxamate-type siderophores ferrioxamine, ferrichrome and mixed-chelating type siderophore pyoverdine indicating that *nitABC* operon encodes an Fe^{3+}-siderophore transporter. Further evidence that nitABC transporter is involved in transport of Fe^{3+} associated with ferrioxamine and ferrichrome came from our experiments with another ammonia oxidizing bacterium *Nitrosomonas eutropha. N. eutropha* genome has been sequenced and does not contain nitABC homologs [42] and is unable to grown in presence of Fe^{3+}-bound ferrioxamine or ferrichrome (unpublished data).Therefore, the transporter encoded by *N. europaea nitABC* appears to be an atypical Fe^{3+}-siderophore transporter that is capable of transporting structurally dissimilar siderophores. In

contrast, other organisms such as *E. coli*, *Y. pestis* [4] have specific ABC transporters for structurally similar siderophores [8]. Of course we cannot discard the possibility that *nitABC* transporter is involved in transport of Fe^{3+} released from above mentioned Fe^{3+}-siderophore complexes. Release of iron from Fe^{3+}-siderophore complexes is considered as the last step in siderophore-mediated iron acquisition. The extreme difference in the affinity of siderophores for Fe^{3+} and Fe^{2+} forms favors the hypothesis of a reductive mechanism [32]. Fe^{3+}-siderophore reductase activities in the cytoplasm, as well as the periplasm and membrane cell fractions, have been reported from several microorganisms [33-35]. Release of Fe^{2+} due to Fe^{3+}-siderophore reductase activity would lead to accumulation of toxic Fe^{2+} which would generate damaging free hydroxyl radicals through Fenton chemistry hence an Fe^{2+}-trap must be present to trap free Fe^{2+} [36, 37]. In *Saccharomyces cerevisiae* [38, 39], *Chlamydomonas reinhardtii* [40], *Pseudomonas aeruginosa* [41], ferroxidases (also called multicopper oxidases) were shown to trap Fe^{2+} released by membrane-surface localized Fe^{3+}-reductase with high affinity and oxidize it to Fe^{3+}. The Fe^{3+} was then shown to be transported into cytoplasm via a high affinity ferric permease [38, 39]. The *N. europaea* genome encodes at least seven multi-copper oxidases. Out of these, one multicopper oxidase was shown to be highly expressed in Fe-limited conditions [14] and transcript levels of two multicopper oxidases were shown to be higher in Fe-limited conditions compared to Fe-replete conditions in our microarray studies (data not yet published). Perhaps the *N. europaea nitABC* encoded iron transporter is involved in transport of Fe^{3+}-associated with siderophores whose reductases are present in periplasm (Fig 4.5). Reductases that release Fe^{3+} from Fe^{3+}-

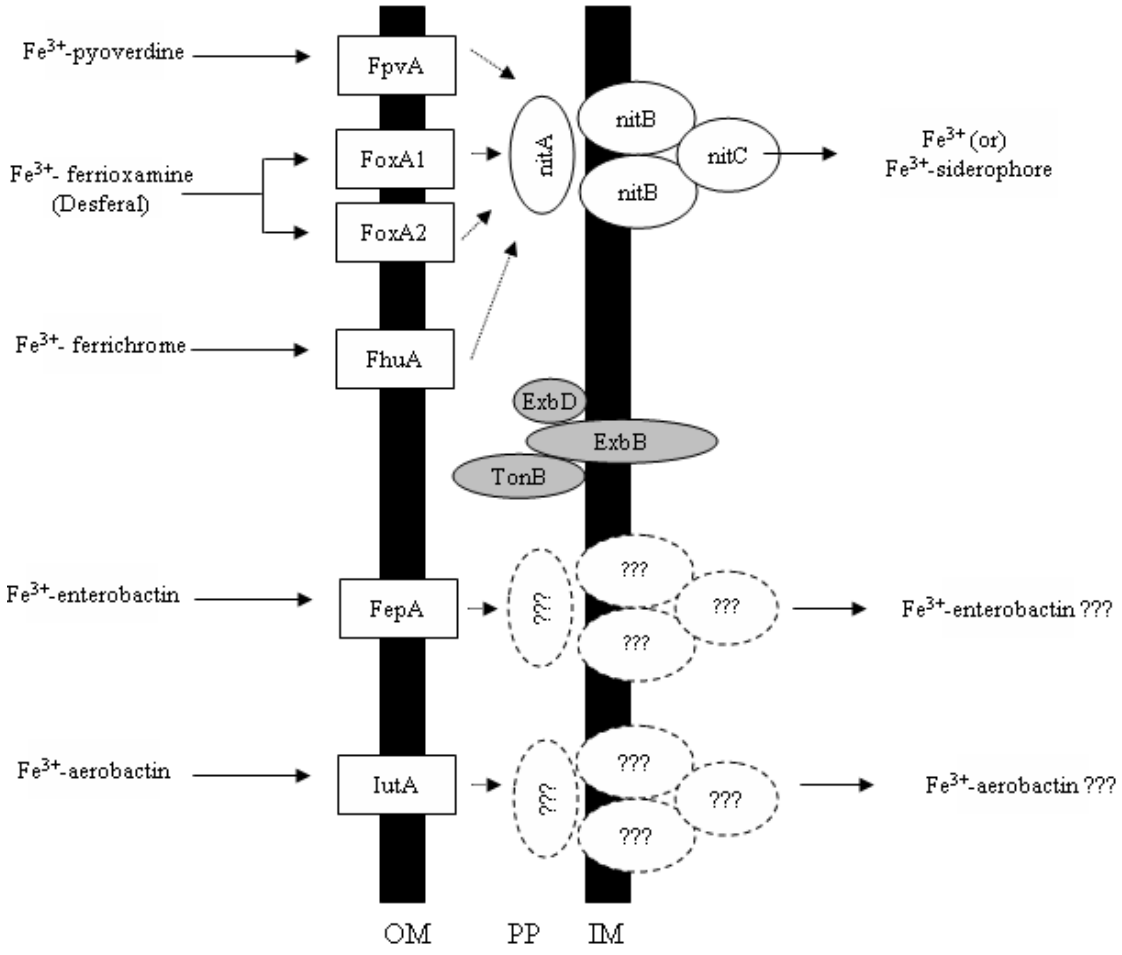


Fig. 4.5. Schematic of siderophore associated Fe uptake through *nitABC* transporter in *N. europaea*.

enterobactin and Fe^{3+}-aerobactin might be located in the cytoplasm; hence iron bound to them does not enter the cytoplasm via *nitABC* operon. Upon careful scanning of the *N. europaea* genome, gene cluster NE1209-NE1210-NE1211 appeared to have some, albiet low, similarity to ATPase and permease components of Fe^{3+}-enterobactin type ABC transporter. Gene NE1207, found in close proximity, was annotated as a bacterial transferase hexapeptide repeat and has low similarity to a truncated periplasmic binding protein. However, transcripts of gene products of NE1207, NE1209 and NE1211 did not show any differential expression in Fe-limited medium compared to Fe-replete medium. This observation does not rule out the possibility that they might be involved in Fe^{3+}-enterobactin transport. Further investigation is required to explain which ABC transporter systems are used by *N. europaea* to acquire Fe^{3+}-enterobactin and Fe^{3+}-aerobactin.

In summary, our results confirm that the *N. europaea nitABC* operon is involved in iron acquisition and provide further evidence that the *nitABC* transporter is involved in transport of iron associated with ferrioxamine, ferrichrome and pyoverdine siderophores. These studies form the basis of continuing investigations aimed at understanding iron transport systems in *N. europaea*.

4.6. Acknowledgments:

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ROLE OF A *fur* HOMOLOG IN IRON METABOLISM AND GENE REGULATION IN *Nitrosomonas europaea*

CHAPTER 5

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5.1. Abstract

Environmental iron concentrations coordinately regulate transcription of genes involved in iron acquisition via the ferric uptake regulation (*fur*) system. The genome of *Nitrosomonas europaea*, an ammonia oxidizing bacterium, carries three genes (NE0616, NE0730 and NE1722) encoding proteins belonging to Fur family. *In vivo* experiments with the three cloned *fur* homologs indicated that the *fur* homolog encoded by NE0616 complemented the *Escherichia coli* H1780 *fur* mutant. A *N. europaea fur-kanP* mutant strain was created by insertion of kanamycin-resistance cassette in the promoter region of *fur* homolog encoded by NE0616. The total cellular iron contents of the *fur-kanP* mutant strain increased by 1.5-fold compared to wild-type when grown in Fe-replete media. The *fur-kanP* mutant exhibited increased sensitivity to iron at or above 500 μM concentrations. The *fur-kanP* mutant was capable of utilizing iron-bound ferrioxamine without any lag phase unlike wild-type. Transcript profiling indicated that *fur* gene is involved in regulation of at least three Fe-regulated genes in *N. europaea.*

5.2. Introduction

The molecular basis for the coordinated regulation of iron acquisition systems by iron was first described for *Escherichia coli* [1]. Since then it has been shown that a number of bacteria regulate their iron acquisition systems via Fur (ferric uptake regulator) [2-5]. Fur is a sequence-specific DNA-binding protein and acts as a negative regulator of transcription in vivo by complexing with ferrous (Fe^{2+}) ion to repress expression of iron-regulated genes [6]. Mutations in the *fur* gene result in constitutive expression of siderophores and outer membrane Fe^{3+}-siderophore receptors potentially useful for iron uptake [7].

Nitrosomonas europaea is an aerobic chemolithoautotroph that uses NH_3 and CO_2 for growth [8]. Mechanisms for iron transport are essential for maintaining the many cytochromes and putative heme-binding proteins involved in ammonia-oxidizing metabolism [9, 10]. The genome of *N. europaea* has 4% of its genes encoding iron acquisition proteins and no genes for siderophore production [8]. *N. europaea* responds to iron limitation by elevating production of Fe^{3+}-siderophore receptors normally repressed under iron-replete conditions [11, 12]. Several *N. europaea* iron-repressible genes contain sequences similar to the *Escherichia coli* Fur box (unpublished data) in their promoter regions; hence it is likely that a Fur-like repressor regulates iron uptake genes in *N. europaea* as well. Indeed, the *N. europaea* genome contains three genes encoding *fur* homologs (NE0616, NE0730, NE1722) containing characteristic Fur domain [8].

Multiple *fur* homologs have been described for several bacteria. Different species have a variable number of genes bearing the Fur domain, for example, *Escherichia coli*

[13], *Bacillus subtilis* [14], *Mycobacterium smegmatis* have three, *Staphylococcus aureus* and some species of *Brucella* have four and *Thermoanaerobacter tengcongensis* has five *fur* homologs [15]. The apparent redundancy in *fur* homologs has been clarified by a considerable amount of experimental data obtained from genetic and biochemical analysis in bacteria such as *E. coli* and *B. subtilis* [13, 14, 16-18] . The experimental data suggests that the Fur protein family has several subclasses with different functions [17]. The major Fe-sensing Fur subclass is mainly involved in the control of iron homeostasis [19]. A second class controls the expression of genes involved in the response of bacteria to oxidative stress (i.e. PerR), but it does not appear to be involved in the cellular response to iron [14]. A third class of Fur homologs called Zur (zinc uptake regulator) controls the uptake of zinc in *E. c*oli [13, 18] and *B. subtilis* [16].

The Fe-sensing Fur protein has been extensively studied and is shown to act as a global regulator in response to environmental iron concentration due to its involvement in the regulation of activities as varied as the acid tolerance response, the oxidative stress response, metabolic pathways, and virulence factors and has been extensively studied [6]. In this study, we aimed to characterize the regulatory role of a *fur* homolog from *N. europaea*. Using genetic complementation studies, we demonstrated that only one *fur* homolog (NE0616) out of three in *N. europaea* encoded a functional Fur protein. Here we report the construction of an *N. europaea fur* promoter knock-out mutant (*fur-kanP*) strain, its effect on the expression of Fe-regulated genes and the physiology of *N. europaea.*

5.3. Materials and Methods

5.3.1. Bacterial cultures and siderophore feeding experiments

N. europaea (ATCC 19178) was cultured as described, with minor modifications [20, 21]. The standard (Fe-replete) medium contained 10 μM Fe^{3+} ($FeCl_3$) complexed with EDTA to prevent Fe precipitation. Fe-limited medium was made from reagent-grade chemicals, without addition of any Fe salt, and contained 0.2 μM Fe [12]. All media, buffers and other reagents were made in double-deionized water. All glassware was soaked in 1% HNO3 overnight, and then rinsed thoroughly with double-deionized water. Fe-free Desferal (deferoxamine/DFX mesylate) was purchased from Sigma (St. Louis, MO). Desferal was dissolved in double deionized water, filter-sterilized, and added to Fe-limited medium in the siderophore feeding experiments. In this study 10 μM Desferal was used to ensure the complete chelation of Fe (0.2 μM) in the Fe-limited medium. *N. europaea* cultures were grown at 30 ^{0}C on a rotary shaker, and mid-exponential-phase cells were collected by centrifugation and thorough washes for the analyses. *Escherichia coli* DH5α, *E. coli* H1780 (*fiu*::λp*lac*Mu53 and inactivated *fur*), and *E. coli* H1717 (*aroB fhuF::λplac*Mu were cultured on Luria-Bertani (LB) agar plates or in liquid LB medium in the presence of the appropriate antibiotic (ampicillin [100 μg ml^{-1}] and/or kanamycin [20 μg ml^{-1}]) under the conditions described above.

5.3.2. DNA preparation, PCR, cloning, mutagenesis and mutant isolation

General DNA preparation, restriction digestions and agarose gel electrophoresis were done as described by [22]. The three *N. europaea fur* homologs (Fig. 5.4a) were amplified by PCR using Taq DNA polymerase (Promega, Madison, WI) on an iCycler

Thermal Cycler (Bio-Rad, Hercules, CA), as described by the manufacturers (see Table 5.1 for primers). The resulting DNA fragments were cloned into the pGEM-T Easy vector (Promega) and named pFur616, pFur730 and pFur1722 respectively. For insertion of Kanamycin resistance cassette (Km^r) into plasmid pFur616, the EZ::TN <KAN2> kit from Epicentre (Madison, Wis.) was used to insert a transposon conferring kanamycin resistance (Km^r) into the promoter region (pFur-kanP) and C-terminal region (pFur-kanC) of *fur* following the directions of the manufacturer. The insertion of the Km^r gene was localized by nucleotide sequence determination at 117 nt upstream of the ATG start codon of *fur* (pFur-kanP) and 312 nt downstream of the ATG start codon of *fur (*pFur-kanC*)* in plasmid pFur616. The pFur616-kanP plasmid construct with the Km^r insertion was introduced back into the *N. europaea* wild-type cells by electroporation on the ElectroPorator (Invitrogen) at 1300 V, with a capacitance at 50 μF, and a load resistance at 500 Ω. Successful transformants were selected in liquid medium using kanamcyin sulfate (20 mg ml^{-1}). Aliquots from these cultures were streaked onto Nylon disk membranes, which were placed on semisolid plates, to isolate clonal mutant strains, as described [23]. The mutant was verified by Southern analysis (Fig.5.4B, and Results). Southern blotting, labelling of DNA probes, hybridization and imaging were done as described previously [24]. Attempts to generate *fur* null mutant by using pFur-kanC construct were unsuccessful.

5.3.3. Fur Titration Assays (FURTA)

Plasmids (listed in Table 5.1) were introduced into *E. coli* H1717 and H1780 (*fur* inactivated) strains and *lacZ* expression was assessed by visualization of a change in colony color from white to red on MacConkey lactose plates (Difco) supplemented

Table 5.1. Bacterial strains, plasmids and primers used in this study

Strain, plasmid or primer	Description	Reference
Strains		
E. coli		
DH5α	F′ ø80d*lacZ*ΔM15 *endA1 recA1 gyrA96 thi-1 hsdR17(r_K^- m_K^+) supE44 relA1 deoR Δ*(*lacZYA-argF*)U169	[25]
H1717	*aroB fhuF*::λp*lac*Mu	[26]
H1717 (pFur616)	*E. coli* H1717 carrying pFur616	This study
H1717 (pFur616-kanP)	*E. coli* H1717 carrying pFur616-kanP	This study
H1717 (pFur616-kanC)	*E. coli* H1717 carrying pFur616-kanC	This study
H1780	araD139Δ[a]argF-lacU169rpsL150 relA1 flbB5301deoC1 ptsF25 rbsR fiu::lacZ fusion lacking Fur	[26]
H1780 (pFur616)	*E. coli* H1780 carrying pFur616	This study
H1780 (pFur616-kanP)	*E.coli* H1780 carrying pFur616-kanP	This study
H1780 (pFur616-kanC)	*E.coli* H1780 carrying pFur616-kanC	This study
H1780 (pFur730)	*E. coli* H1780 carrying pFur730	This study
H1780 (pFur1722)	*E. coli* H1780 carrying pFur1722	This study
N. europaea		
ATCC 19178	wild-type	American Type Culture Collection
fur-kanP	Insertion of *kan* cassette in the fur box upstream of NE0616 gene	This study
Plasmids		
pGEM-T Easy	Vector for cloning PCR products; Amp[r]	Promega

Table 5.1. continued.

pFur616	pGEM-T Easy vector containing NE0616 u&d region	This study
pFur616-kanP	In vitro transposon mutagenesis of pFur616 with EZ-Tn5 <KAN-2> with k*an* cassette insertion in *fur box* located in promoter region of NE0616	This study
pFur616-kanC	In vitro transposon mutagenesis of pFur616 with EZ-Tn5 <KAN-2> with k*an* cassette insertion in C-terminal region of NE0616	This study
pFur730	pGEM-T Easy vector containing NE0730 u&d region	This study
pFur1722	pGEM-T Easy vector containing NE1722 u&d region	This study
Primers		
For cloning, mutagenesis and mutant confirmation		
NE0616ud-1	5'-ATCCTGGAAGAAAACGGTCA-3'	This study
NE0616ud-2	5'-TGCAGGTTTCAAACGAAAAA-3'	This study
NE0730ud-1	5'-TTTCAGACGTTGCTGACAAAA-3'	This study
NE0730ud-2	5'-TCATTTTGGCTGTTCATTTCA-3'	This study
NE1722ud-1	5'-TATGGCTTACGGAAAACGGTA-3'	This study
NE1722ud-2	5'-ACAAAAACAGACACGGAGGAA-3'	This study

with 30 μM Ferrous ammonium sulfate. Plates were examined after 24 h of growth at 37°C. The assays were performed in triplicate for each sample.

5.3.4. Determination of Fe and heme contents and enzyme activities

Total Fe contents in thoroughly washed *N. europaea* cells were determined by the ferrozine assay following HNO_3 (5 %) digestion of cells at 100 ^{0}C [27]. Measurements of Fe concentrations below 10 μM were made using a Teledyne Leeman Prodigy ICP-OES (Hudson, NH) at the W.M. Keck Collaboratory for Plasma Spectrometry, Oregon State University. Preparations of a cell-soluble fraction, and determination of heme contents following extraction with pyridine, were done as described [12, 28]. Whole cell NH_3-dependent and hydroxylamine dependent O_2 uptake activities were measured as described [12, 29].

5.3.5. Cell fractionation, protein quantification and SDS-PAGE analysis

Total cell membranes were prepared as previously described [12]. Briefly, cells were broken by ultrasonication, the sonicated material was centrifuged at 1500 g for 1 min to pellet unlysed cells, and the top phase (cell lysate) was transferred to ultracentrifuge tubes. Crude total membranes were collected by ultracentrifugation of the cell lysates, and washed thoroughly by homogenization in Tris buffer (0.1 M, pH 7.8) containing 1 M KCl. Total membranes were collected again by ultracentrifugation, and resuspended in Tris buffer (50 mM, pH 7.8). Protein contents in whole cells and cell fractions were estimated by using the Micro BCA Protein Assay kit (Pierce), and BSA was used as a protein standard. The peptide composition of cell membranes was analysed using SDS-PAGE [with 12% (w/v) acrylamide in resolving gels], as described previously [12, 30].

5.3.6. Microarray experiment and DNA microarray construction

Cells from three independent experiments (three iron-replete [300ml culture at 0.04 OD] and three iron-limited cultures [500ml culture at 0.025 OD]) of *fur-kanP* mutant were harvested at mid exponential phase by centrifugation. Total cellular RNA was extracted using 1ml of Trizol (Ambion Inc., Austin, TX) following the manufacturer's instructions. The extracted RNA was purified with an RNeasy Mini kit (Qiagen Inc., Valencia, CA.) and treated with RNase-free DNase I (Qiagen) to digest residual chromosomal DNA. The concentration of purified RNA was determined by using a Nanodrop spectrophotometer (Nanodrop Technologies, Rockland, DE) and RNA quality was checked by the A260/A280 ratio and RNA 6000 Nano LabChip kit on the Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Palo Alto, CA.). Intact, good quality RNA (>9.0 RIN score) was used for the experiments.

The NimbleChip 4-plex Made-to-Order array for *N. europaea* was manufactured by Roche NimbleGen Systems, Inc. based on the published genome sequence (AL954747) (14). Identified genes in *N. europaea* are represented on the array by the probe sets with 14 pairs of 60-mer perfect match/mismatch oligo probes. Microarray analyses were performed in triplicate (three controls and treatments), using RNA samples extracted from independent experiments. cDNA synthesis, labeling, hybridization, scanning and data normalization were performed at Roche Nimblegen Core Facility, Iceland. DNASTAR ArrayStar v2.1 software was used to analyze normalized gene expression data obtained from Roche Nimblegen's NimbleScan software. NimbleScan normalizes expression data using quantile normalization. Gene calls are generated using the Robust Multichip Average (RMA) algorithm.

Differentially regulated genes were detected using a 2.0 fold change as a minimum for up- or down regulation. Student's t-Test with a cutoff p-value of 0.05 was used to compare the means of gene expression values for two individual replicates or for two groups of replicates for a given gene. Biological functions of up regulated or down regulated hypothetical genes were searched by BLAST.

5.3.7. Phylogenetic tree construction

ClustalW was used for sequence alignment applying default parameters (altered gap penalties were not applied) [31]. Gaps in the alignment were not omitted. The phylogenetic tree was built by Phyml 3.0 with the distance matrix generated by ClustalW and was represented with the program TreeDyn 198.3 available at (http://www.phylogeny.fr/) [32]. The reliability of each node was established by bootstrap methods.

5.3.8. Hidden Markov Model-based Fur binding site prediction

A group of experimentally validated Fur boxes from *E. coli*, *S. typhimurium*, *P. aeruginosa* and *S. aureus* used by Quatrini et al., [33] along with 3 experimentally confirmed *N. europaea* fur boxes were used to build HMM profiles and to search for fur binding sites in the promoter regions of (600 nt from the proposed initiation of translation of the potential target gene).

N. europaea sequence data was obtained from DOE Joint Genome Institute (JGI) website (http://genome.ornl.gov/microbial/neur/). Sequence similarity searches of the available nucleotide and protein databases were performed with the BLAST program, available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast/).

5.4. Results

5.4.1. Sequence analysis of *N. europaea fur* homologs

N. europaea genome contains three genes that encode putative Fur-like repressors, NE0616, NE0730, NE1722 respectively [8]. The *N. europaea* Fur-like repressors are only distantly related to each other with 25% to 35% amino acid identity. The Fur homolog encoded by NE0616 is most similar in sequence to various Gram-negative Fe-sensing Fur proteins. The recent elucidation of the crystal structure of the *P. aeruginosa* Fur protein provided considerable insight into its 2 metal binding sites. Binding Site 1 represents the putative iron binding regulatory site and is coordinated by amino acids H86, D88, E107, and H124 and Site 2 is coordinated by H32, E80, H89 and E100 [17]. All these residues are conserved only in the *N. europaea* NE0616 Fur homolog but not in Fur homologs encoded by NE0730 and NE1722 (Fig. 5.1). Phylogenetic analysis of Fur homolog coding sequences from *N. europaea* with Fur proteins from other bacteria placed NE0616 in the group B comprised of Fe-sensing Fur proteins, NE1722 in the group A comprised of Zn- sensing Zur proteins. Surprisingly, NE0730 *fur* homolog was also placed in group B. No Fur homologs of *N. europaea* grouped with peroxide sensing PerR proteins i.e., group C (Fig. 5.2). The *fur* gene itself is classically iron regulated and there is strong evidence that this is through a mechanism of autoregulation [34, 35]. Fur recognizes and binds specifically to a DNA sequence, known as the Fur box, that is typically located in proximity to the −10 and/or −35 promoter elements of target genes [6]. Analysis of several Fur-binding

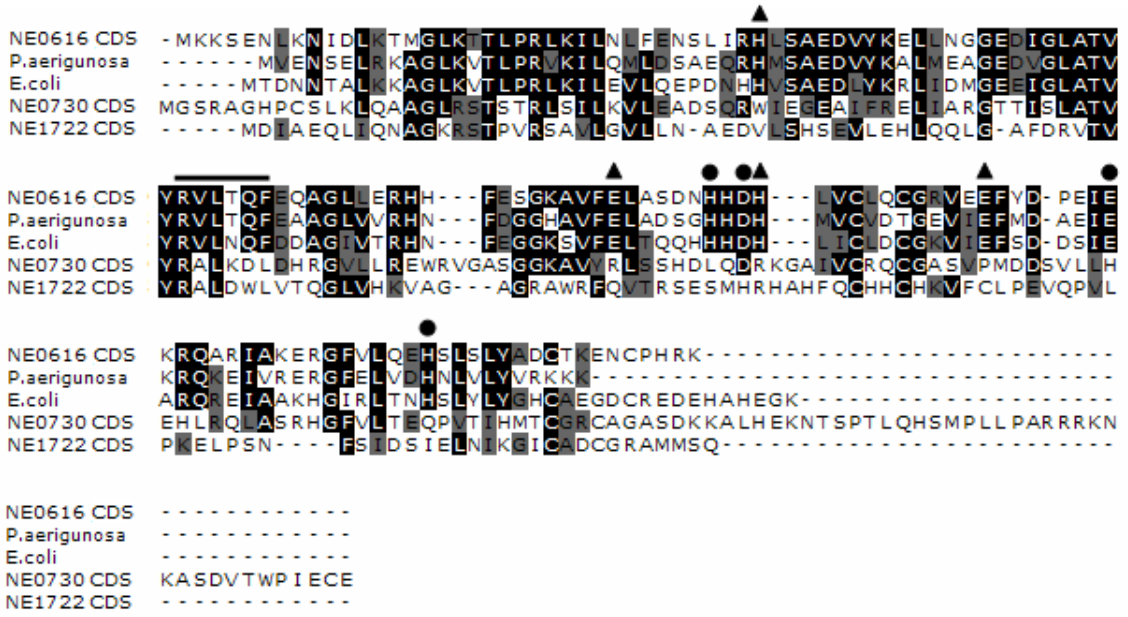
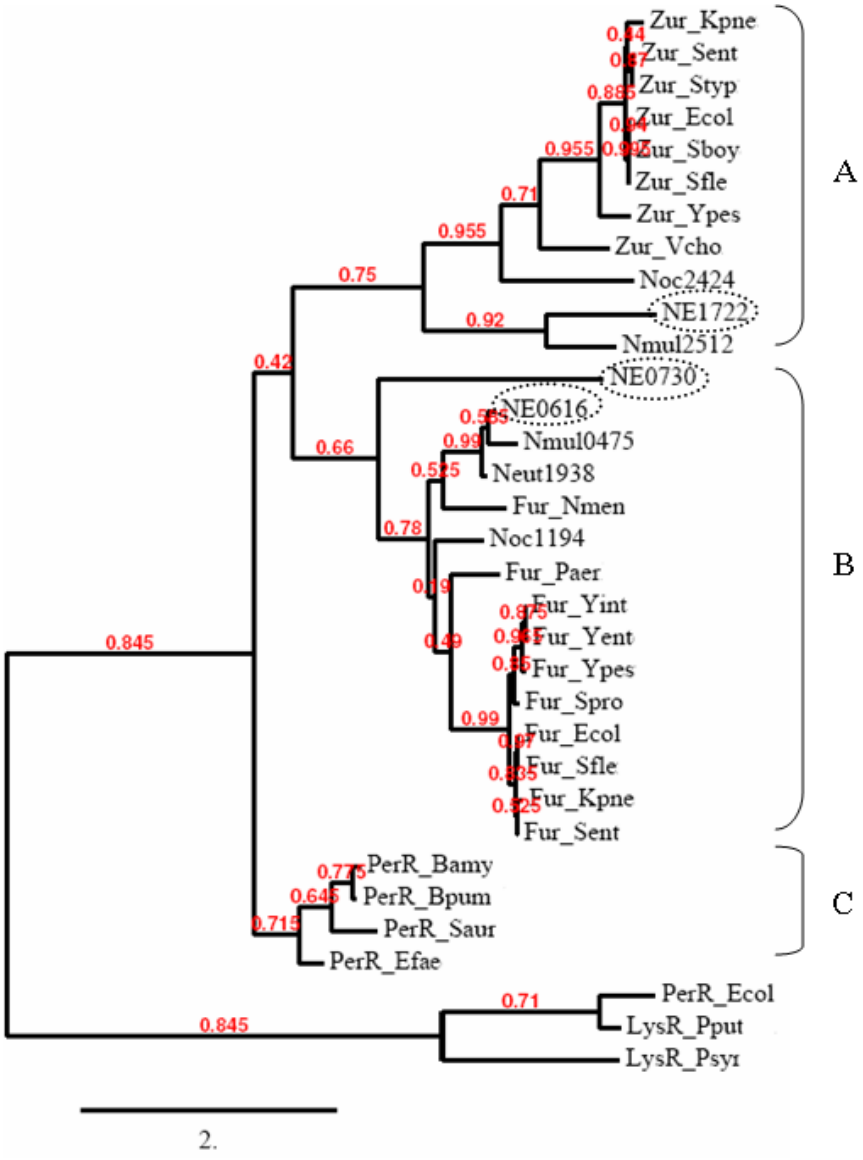


Figure 5.1: Alignment of *N. europaea* Fur homolog coding sequences with *E. coli* and *P. aeruginosa* Fur proteins using ClustalW [31]. Identical residues are shaded black, with similar residues shaded grey. Metal binding site 1 residues are indicated with circles, and site 2 residues are indicated with triangles, as identified from the crystal structure of *P. aeruginosa* Fur. Residues indicated by straight line highlight a motif thought to be involved in DNA binding.

Figure 5.2. Maximum-Likelihood tree of the Fur homologs. Phylogenetic tree of Fur coding sequences generated by Phyml analysis. The numbers beside nodes are the percentages of bootstrap values calculated for 200 replicates: The three groups – A, B and C – mentioned in the text are indicated on the right side of the tree. Bamy, *Bacillus amyloliquefaciens*; Bpum, *Bacillus pumilus*; Ecol, *Escherichia coli*; Efae, *Enterococcus faecalis*; Kpne, *Klebsiella pneumoniae*; Nmen, *Neisseria meningitidis*; Paer, *Pseudomonas aeruginosa*; Pput, *Pseudomonas putida*; Psyr, *Pseudomonas syringae*; Saur, *Staphylococcus aureus*; Sboy, *Shigella boydii*; Sent, *Salmonella enterica*; Sfle, *Shigella flexneri*; Spro, *Serratia proteamaculans*;Styp, *Salmonella typhimurium*; Vcho, *Vibrio cholerae*; Yent, *Yersinia enterocolitica*; Yint, Yersinia intermedia; Ypes, *Yersinia pestis*; Ypse, *Yersinia pseudotuberculosis*; NE, *Nitrosomonas europaea*; Neut, *Nitrosomonas eutropha*; Nmul, *Nitrosospira multiformis*; Noc, *Nitrosococcus oceanii*;



sites allowed the early definition of a 19-bp consensus Fur box in *E. coli* [6]. Since then, canonical Fur boxes have been described in several bacteria such as *P. aeruginosa* [36], *N. gonorrhoeae* [37] and *V. cholerae* [38]. We have used an *in silico* approach, fed with experimentally confirmed *N. europaea* Fur boxes (unpublished data), to identify candidate Fur-binding sites in promoter regions of all 3 *N. europaea fur* homologs. A potential Fur box (5'-TAATAATACGTATCTTTAT-3') in the promoter region of NE0616 gene, -121 bp upstream of the proposed initiation of translation of the *fur* gene was found. We were unable to find potential Fur boxes in the promoter region of the other *N. europaea fur* homologs, NE0730 and NE1722.

5.4.2. Complementation of an *E. coli fur* mutant by *N. europaea fur* homologs

In order to determine which *fur* homolog of *N. europaea* encodes the Fe-sensing Fur protein, pFur616, pFur730 and pFur1722 plasmids (Table 5.1) were used to transform the *E. coli fur* mutant H1780 [26]. *E.coli* H1780 strain was engineered to be *fur* deficient and to include the Fur-regulated gene *fiu* fused to a promoterless *lacZ* gene. This reporter gene, *fiu-lacZ*, cannot be repressed in this strain due to the *fur* mutation, and therefore the gene encoding the enzyme β-galactosidase is constitutively expressed and the strain always shows Lac^+ phenotype. [26]. The pFur616-kanC (Table 5.1) plasmid carrying kanamcyin resistance cassette (Km^r) insertion in the C-terminal region of NE0616 gene was used to transform H1780 as a negative control. All strains evaluated for Lac phenotype were grown on McConkey Lactose plates with 30 μM iron supplement, since iron is required to ensure that Fur is functional [6]. In these studies, *E. coli* H1780, H1780 (pFur616), H1780 (pFur616-kanC), H1780 (pFur730) and H1780 (pFur1722) strains were compared. Lac^+ phenotype was

observed for *E. coli* H1780 whether grown in the presence or absence of added Fe supplement as predicted since it is deficient in Fur protein (data not shown). Complementation of *E. coli* H1780 with pFur616 rescued the Fur defect of this strain and resulted in the repression of transcription of the *fiu-lacZ* reporter gene, as shown by the Lac^- phenotype (Fig. 5.3A; upper left quadrant). When pFur616-kanC plasmid containing the disrupted NE0616 gene, was transformed into the *E. coli* H1780 mutant, Lac^+ phenotype was restored (Fig. 5.3A; upper right quadrant).When pFur730 and pFur1722 plasmids containing the
N. europaea fur homologs NE0730 and NE1722 were transformed separately into *E. coli* H1780 strain, Lac^+ phenotype was observed (Fig. 5.3A; lower left and right quadrants). These results clearly demonstrate that the *N. europaea* NE0616 *fur* homolog is expressed in *E. coli* in a functional form and is capable of regulating the Fur-dependent *fiu* promoter in H1780. The other *N. europaea fur* homologs (NE0730 and NE1722) were not capable of regulating the *fiu* promoter in H1780. NE0616 is here after referred to as *N. europaea fur*.

5.4.3. The *N. europaea fur* promoter is repressed by Fur

Several studies have employed *E. coli* H1717 strain to allow the detection of iron-regulated promoters in bacteria such as *E. coli* and *Salmonella typhimurium* [39, 40]. *E. coli* H1717 strain has a chromosomal iron-regulated *fhuF* promoter fused to *lacZ*. This fusion is exceptionally sensitive to small changes in iron concentration because of the weak affinity of the *fhuF* promoter for the Fur-Fe^{2+} repression complex. Introduction of a multi-copy plasmid carrying Fur-binding sites into the test strain depletes the intracellular Fur pool. This gives rise to the dissociation of the repressor

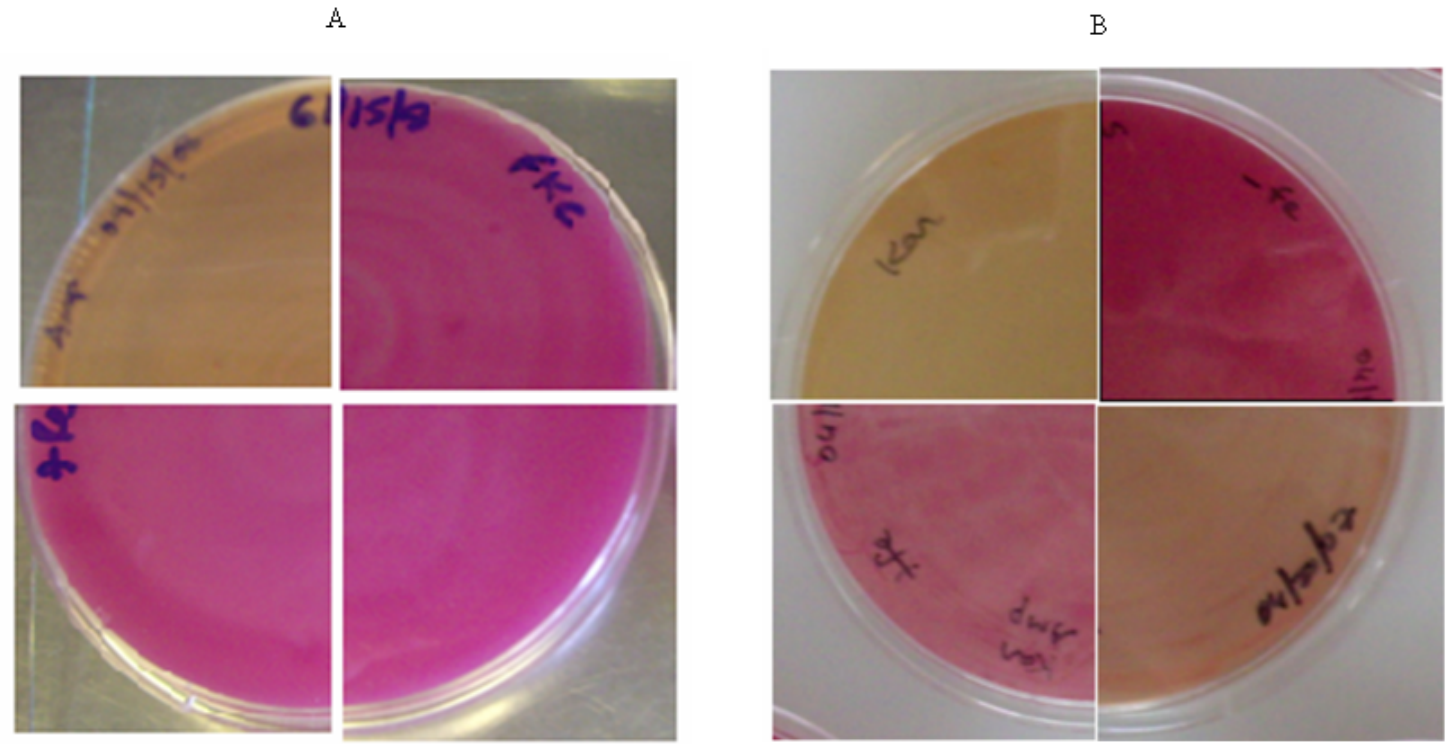


Figure 5.3. Fur Titration Assays (FURTA)
A. Complementation of an *E. coli fur* mutant H1780 by *N. europaea* Fur homologs. A. *E. coli* H1780 (pFur616) - upper left quadrant; H1780 (pFur616-kanC) - upper right quadrant; H1780 (pFur730) - lower left quadrant; H1780 (pFur1722) - lower right quadrant plated on McConkey medium with 30 μM Fe supplement and grown at 37^0C for 24 hrs.
B. *E. coli* H1717 plated on McConkey medium with 30 μM Fe supplement - upper left quadrant, no Fe supplement - upper right quadrant; H1717 (pFur616) - lower left quadrant; H1717 (pFur616-kanP) - lower right quadrant plated on McConkey medium with 30 μM Fe supplement and grown at 37^0C for 24 hrs.

from the fusion promoter, thereby allowing expression of enzyme β-galactosidase. We have screened plasmids pFur616 carrying intact Fur box and pFur616-kanP carrying disrupted Fur box using

E. coli H1717 strain to determine NE0616 Fur box functionality. The pFur616-kanC plasmid (Table 5.1) carrying Km^r insertion in the C-terminal region of NE0616 gene was also used to transform *E. coli* H1717 as a positive control.

In these studies, *E. coli* H1717 in the presence and absence of Fe supplement, H1717 (pFur616), H1717 (pFur616-kanP) and H1717 (pFur616-kanC) strains were compared. Lac^- phenotype was observed for *E. coli* H1717 when grown in the presence of 30 μM Fe supplement, since it does not carry any multi-copy plasmid with a functional Fur box on it (Fig. 5.3B upper left quadrant). Lac^+ phenotype was observed when H1717 was grown with no added Fe supplement, since there is not enough Fe to suppress *fhuF-lacZ* fusion (Fig. 5.3B; upper right quadrant). When pFur616 carrying putative Fur box was transformed into *E. coli* H1717 and the resulting strain was grown in presence of 30 μM Fe supplement, it resulted in derepression of the *fhuF-lacZ* reporter gene, as shown by the Lac^+ phenotype (Fig. 5.3B; lower left quadrant). This indicates that the predicted Fur box is functional and must has titrated intracellular Fe pool. When apFur616-kanP plasmid containing the disrupted NE0616 Fur box, was transformed into the *E. coli* H1717 strain, Lac^- phenotype was restored (Fig. 5.3B; lower right quadrant) indicating that the Km^r insertion led to disruption of Fur box functionality. When a pFur616-kanC plasmids containing Km^r insertion in the C-terminal region of NE0616 gene was transformed into *E. coli* H1717 strain, Lac^+ phenotype was observed (data not shown) indicating

that Km^r in C-terminal region of NE0616 did not affect its Fur box functionality. These results demonstrate that the promoter of *N. europaea* NE0616 *fur* homolog carries a Fur box and it is functional as recognized by *E. coli* Fur protein.

5.4.4. Isolation of the *N. europaea fur-kanP* mutant strain

To address the physiological role *fur* plays in *N. europaea*, we attempted to generate an *N. europaea fur* null mutant but were unsuccessful. However, we were successful in isolating an *N. europaea fur-kanP* mutant strain with Km^r inserted in the Fur box located in the promoter region of NE0616 gene (Fig. 5.4A). The pFur616-kanP plasmid was electroporated into *N. europaea* wild-type cells. The *fur-kanP* mutant was obtained through homologous recombination and confirmed by PCR (data not shown) and Southern hybridization (Fig. 5.4B). The *fur* probe detected a ~ 4 Kb EcoR1 fragment and a ~ 4.9 Kb Pst1 fragment in wild-type and a ~ 5 Kb EcoR1 fragment and a ~ 4.3 Kb Pst1 fragment (calculated size based on the DNA sequences) in *fur-kanP* mutant strain. The kanamycin-cassette probe detected the same ~ 5 Kb EcoR1 fragment and the ~ 4.3 Kb Pst1 fragment in *fur-kanP* muant but not in the wild type. These results confirm that a single copy of Km^r was correctly inserted in the Furbox located in the promoter region of NE0616 gene of the *N. europaea* genome (Fig. 5.4A). Preliminary studies by RT-PCR analysis indicated that there was no *fur* transcript in the *fur-kanP* mutant strain as opposed to wild-type where *fur* transcript was abundant. Transcripts of ammonia monooxoygenase C (*amoC*) component used as positive control both for the efficiency of the RT-PCR procedure and for RNA and cDNA recovery showed no significant difference in expression in wild-type and the *fur-kanP* mutant (data not shown).

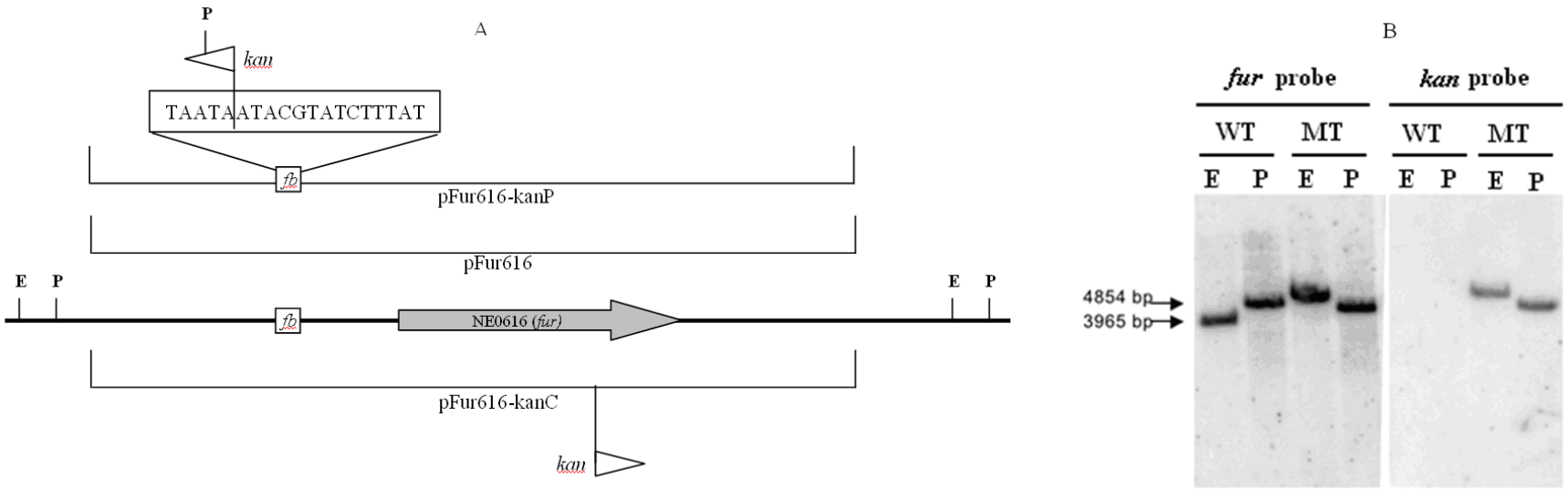


Figure 5.4: In-vitro transposon mutagenesis scheme and mutant confirmation.
A.The physical structure of a 5,810-bp fragment of the *N. europaea* chromosome is shown in the center (heavy black line), with positions of NE0616 (*fur)* gene shown as gray arrow, the fur box (*fb*) located in NE0616 promoter region shown as white rectangle. The regions covered by the plasmids pFur616, pFur616-kanP, pFur616-kanC whose DNA sequences were determined are shown as thin black lines with the names of the respective plasmids shown below each line. The position and relative orientation of each in vitro-constructed *Tn5-Kan2* cassette insertion mutation are indicated by a flag on the lines. The restriction endonuclease sites P (Pst1) and E (EcoR1) used for Southern Blot confirmation are indicated. B. Verification of mutagenesis of *fur-kanP* in *N. europaea* by Southern hybridization. Genomic DNA from the wild-type (WT), *fur-kanP* mutant (MT) were digested with E (EcoRI) and P (Pst1), and probed with (left) *fur* ORF sequence and (right) *kan* sequence.

5.4.5. Effect of *fur-kanP* mutation on growth of *N. europaea*

Growth of the *N. europaea fur-kanP* strain was compared to that of the wild-type strain in both Fe-replete (10 μM Fe) and Fe-limited (0.2 μM Fe) media. Surprisingly, there was no significant difference in growth of *fur-kanP* in both Fe-replete and Fe-limited media compared to wild type (Fig. 5.5A). The *fur-kanP* mutant did not exhibit a growth advantage over the wild type when iron was limiting or show increased sensitivity to iron-induced redox stress when grown in the presence of Fe (up to 250 μM Fe; data not shown). However growth of *fur-kanP* mutant was affected when grown in medium containing 500 μM Fe (Fig. 5.5B). The mutant was unable to grow in media containing more than 500 μM Fe (data not shown). Growth of wild-type was inhibited only when concentrations of Fe exceeded 1 mM [12].

N. europaea can use the siderophore ferrioxamine for its iron uptake after a 3 – 4 day lag period suggesting that the ferrioxamine uptake system in *N. europaea* requires induction [11, 12]. When *N. europaea fur-kanP* mutant was grown in Fe-limiting media containing ferrioxamine, there was no lag phase (Fig. 5.5B) indicating that the ferrioxamine uptake system was already induced in the *fur-kanP* mutant.

5.4.6. Effect of *fur-kanP* mutation on induction of Fe-regulated outer membrane proteins in *N. europaea*

Previous studies have shown that *N. europaea* grown in Fe-limited medium stimulated expression of several Fe-regulated outer membrane proteins (TonB-dependent receptors) with molecular masses of ~ 80 kDa [11, 12]. Growth of *N. europaea* in Fe-replete medium resulted in a coordinate loss of expression of these TonB-dependent receptors. To determine whether the expression of these proteins was regulated by *fur*

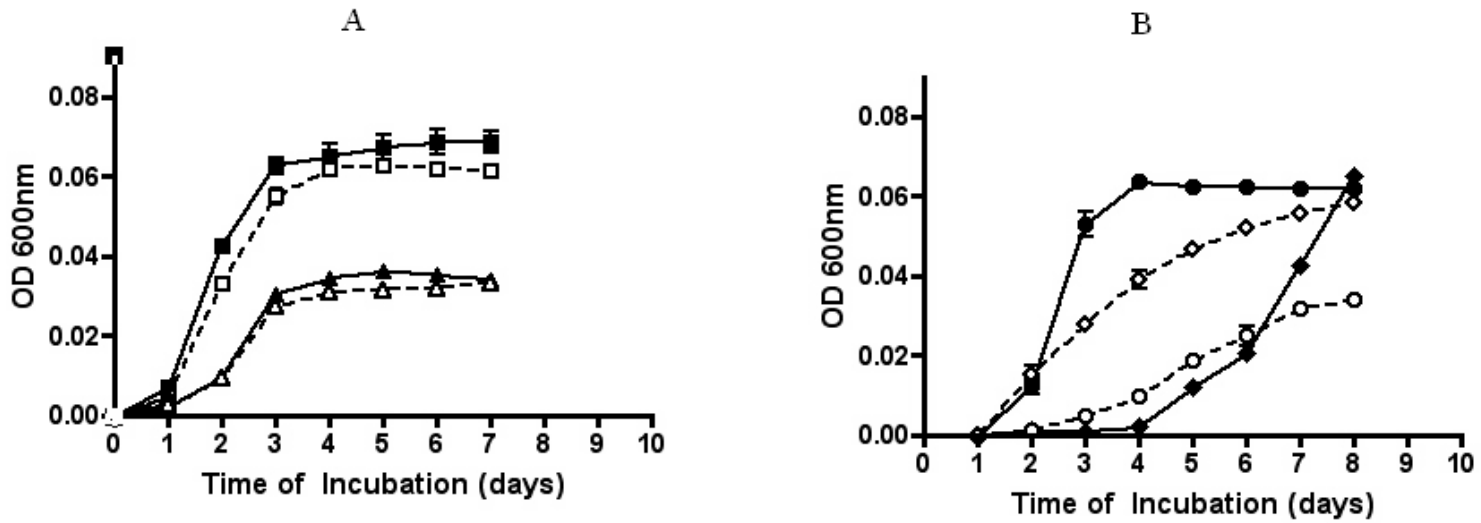


Figure 5.5. Growth curves of the *N. europaea* wild-type (solid lines, filled symbols) and *fur-kanP* mutants (dotted lines, open symbols) as measured by OD**.** (A) in Fe-replete (squares) and Fe-limited (triangles) media. (B) in 500 μM Fe medium (circles) and in Fe-limited media with 10 μM ferrioxamine (diamonds). Data shown are means of triplicates, with variation less than 10%. The experiment was repeated and produced similar results.

in *N. europaea*, wild-type and *fur-kanP* mutant were cultured in Fe-replete and Fe-limited media and their total outer membrane proteins were isolated. SDS-PAGE analysis of the outer membrane protein profiles demonstrated that *fur-kanP* mutant shared a major protein band (Fig. 5.6) with wild-type cells grown in Fe-limited media irrespective of the concentration of iron in the medium. This band contained several TonB-dependent OM Fe^{3+}-siderophore receptors [11, 12]. This result is consistent with the model in which the TonB-dependent receptors with putative roles in iron uptake are regulated by *fur* [6].

5.4.7. Effect of *fur-kanP* mutation on Fe and heme contents of *N. europaea*

Fur mutants generally express iron transport systems constitutively (with respect to iron), and have increased free cellular iron levels (although total cellular iron levels are actually reduced) [41, 42]. To determine the effect of *fur-kanP* mutation on iron contents of *N. europaea,* wild-type and *fur-kanP* mutant cells were cultured in Fe-replete and Fe-limited media and their total cellular iron contents were measured by ICP-OES analysis. *N. europaea* Fe-limited cells showed lower iron contents compared to Fe-replete cells irrespective of the *fur* mutation as observed previously [12]. The *fur-kanP* mutant had nearly 1.5 fold more iron than the wild type cells when grown in Fe-replete media (Table 5.2). The iron contents of wild-type and the *fur-kanP* mutant did not show variation when grown in Fe-limited medium (Table 5.2). The *fur-kanP* mutation also influenced both the amount of soluble cytochromes produced and the proportion of iron distributed to cytochromes (Table 5.2). These data suggest that in *N. europaea*, Fur regulates the concentration of intracellular free iron through modulation

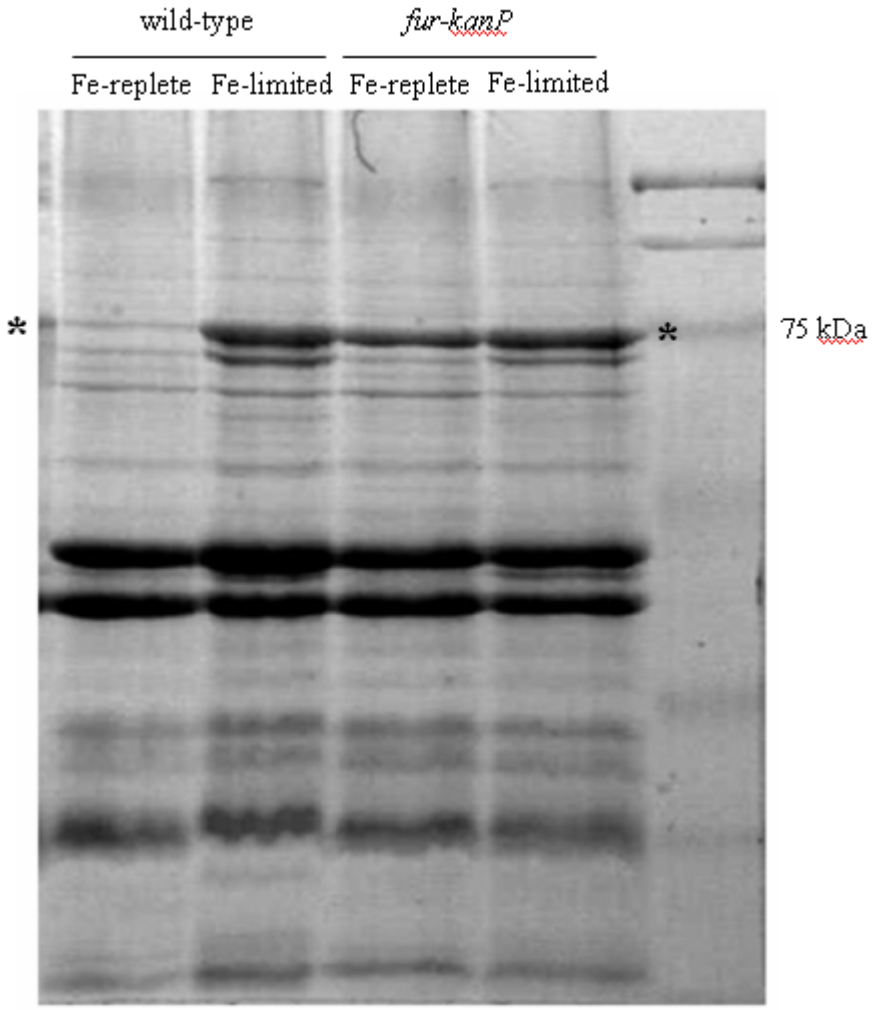


Figure 5.6. SDS-PAGE Analysis of total membrane proteins of *N. europaea* wildtype and *fur-kanP* mutant in Fe-replete (10µM) (lanes 1, 3) and Fe-limited (0.2µM) media (lanes 2, 4). Over-expression of proteins with molecular weights similar to outer membrane Fe-siderophore receptors indicated by * was observed in *fur-kanP* mutant in both Fe-replete and Fe-limited media.

Heme and Fe content in	wild-type		*fur-kanP* mutant	
	Fe-replete	**Fe-limited**	**Fe-replete**	**Fe-limited**
Soluble Fraction				
Heme C (nmol/ml culture)	0.85 ± 0.02	0.38 ± 0.05	0.48 ± 0.02	0.21 ± 0.04
Heme C (nmol/mg protein)	7.77 ± 0.23	4.04 ± 0.53	5.67 ± 0.31	5.04 ± 0.91
Whole Cell				
Fe (nmol/ml culture)	1.36 ± 0.15	0.15 ± 0.01	2.04 ± 0.09	0.11 ± 0.01
Fe (nmol/mg protein)	90.43 ± 6.01	26.41 ± 2	136.23 ± 14	24.91 ± 3
Cellular Fe concentration (mM)	8.27 ± 0.94	1.99 ± 0.13	12.48 ± 0.57	1.98 ± 0.18

Table 5.2. Heme C content in the cells soluble (membrane-free) fraction and the total cell Fe content in *N. europaea* wild-type and *fur-kan-P* mutant grown in Fe-replete (10 μM) and Fe-limited (0.2 μM) media

of iron acquisition and iron consumption, and that, in the absence of Fur, *N. europaea* is unable to regulate its iron acquision.

5.4.8. Effect of *fur-kanP* mutation on AMO and HAO activities of *N. europaea*

As indicators of the overall cell activity, NH_3- and NH_2OH-dependent O_2 uptake (AMO and HAO) rates in wild-type and *fur-kanP* mutant cells grown in Fe-replete and Fe-limited media were measured. *N. europaea* Fe-limited cells showed lower AMO and HAO activities compared to Fe-replete cells irrespective of the *fur* mutation as observed previously [12]. The AMO and HAO activities of wild-type and *fur-kanP* mutant did not show much variation when grown in Fe-replete media (Table 5.3). The AMO activities when measured at per mg basis were not affected; however the HAO activity was about two-fold lower in *fur-kanP* Fe-limited cells compared to wild-type Fe-limited cells (Table 5.3). This is consistent with our previous observation of lower heme contents in *fur-kanP* mutant. HAO requires 24 hemes per enzyme for function [43] and the lower specific activity of HAO in Fe-limited cells of *fur-kanP* mutant might be due to the lowered available heme under Fe-limited conditions. This data also suggests that the *fur-kanP* mutation lead to an improper balance of iron allocation in *N. europaea.*

5.4.9. Transcriptional profiling of *N. europaea* Fur-regulated gene expression

To identify *N. europaea* genes regulated by Fur, we used DNA array-based transcriptional profiling with RNA isolated from *N. europaea fur-kanP* mutant grown in Fe-replete and Fe-limited medium and compared them with transcript profiles of *N. europaea* wild-type cells grown in Fe-replete and Fe-limited media (data not yet published). Surprisingly only three genes encoding proteins with putative roles in iron

Activity	wild-type		*fur-kanP* mutant	
	Fe-replete	**Fe-limited**	**Fe-replete**	**Fe-limited**
Whole Cell				
AMO (nmol/min/OD)	94.55 ± 4.10	38.12 ± 6	88.21 ± 2.5	21.77 ± 0.6
AMO (nmol/min/mg protein)	1500 ± 63	779 ± 17	1446 ± 40	680 ± 18
HAO (nmol/min/OD)	25.98 ± 0.2	10.87 ± 2.4	25.7 ± 4.8	4.66 ± 0.2
HAO (nmol/min/mg protein)	412 ± 2.8	222 ± 5	421 ± 2.5	146 ± 6.4

Table 5.3. Enzyme-catalyzed activities of *N. europaea* wild-type and *fur-kanP* mutant grown under Fe-replete (10 μM) and Fe-limited (0.2 μM) conditions

uptake displayed iron-dependent Fur-repressed expression (Table 5.4). One of them is an ABC transporter homolog, second one is a *fur* homolog and third one is a TonB-dependent receptor homolog. Several genes encoding proteins with putative roles in iron uptake showed only partial de-repression in *fur-kanP* mutant under Fe-replete conditions (data not shown). Interestingly, the transcript for NE0616 *fur* gene with Km^r inserted in its promoter region was detected in *fur-kanP* mutant and was 2.9 fold lower when compared to wild-type. It is unknown for now whether the *fur* transcript level detected in *fur-kanP* mutant is below background level or the *fur-kanP* mutant infact retained the ability to express *fur* gene at low level. The *fur-kanP* mutations effect on intracellular levels of Fur (i.e., effects on *fur* expression level) will need to be studied before we define the Fur regulon in *N. europaea*.

5.5. Discussion:

The *N. europaea* genome sequence has revealed three distinct *fur* homologs (encoded by genes NE0616, NE0730 and NE1722) [8]. In this study, we provide several lines of evidence showing that the *fur* homolog encoded by *N. europaea* gene NE0616 is the Fe-sensing Fur protein. First, we have shown that NE0616 displays 8 out 8 amino acid identity with the metal binding sites of *P. aeruginosa* Fur (Fig. 5.1) [17] and that the *fur* homolog encoded by NE0616 is clustered with Fe-sensing Fur proteins from other bacteria (Fig. 5.2). An *E. coli* Fur titration assay (FURTA) system for Fur analysis was utilized as a second method to confirm that the cloned NE0616 *fur* encodes a functional protein. The H1780 (pFur616) strain carrying *fur* homolog encoded by NE0616 on a plasmid was evaluated for its ability to utilize lactose as described by Hantke et al., [26]. Utilization of lactose by H1780 (pFur616) strain was detected by

Table 5.4. List of genes encoding proteins with putative roles in iron uptake that displayed iron-dependent Fur-repressed expression

		Relative Fold Change[2,3]	
Gene[1]	**Description**	***fur-kanP* +Fe vs wild-type +Fe**	***fur-kanP* -Fe vs wild-type -Fe**
NE0054	ABC-type multidrug transport system ATPase and permease components	+3.7	+1.6*
NE0616	Fe-sensing Ferric uptake regulator protein	-2.9	-2.9
NE0730	Ferric uptake regulator family protein	+21.8	+1.3*
NE0731	TonB-dependent receptor protein - catecholate type??	+12.1	--

[1] Gene orientation is indicated by left (clockwise) and right (counter clockwise) justification, and transcriptional organization is indicated by the vertical arrows.

[2] Fold increases (+) and decreases (-) in expression levels indicated by white and black backgrounds, respectively.

[3] Expression changes of lower than 2 fold and considered significant are indicated with a * at end.

color change of colonies from white to red on McConkey lactose plates indicating the formation of lactic acid. Lactose utilization was not detected when H1780 strain carrying plasmids pFur616-kanC, pFur730, pFur1722 were plated on McConkey lactose plates (Fig. 5.3A).

One of the major limitations in the research on the role of Fur has been the inability to make a *fur* null mutant. Null mutations have been successfully isolated for *E. coli* [44, 45], *Vibrio cholerae* [46], *Shigella flexneri* [47], *Neisseria meningitidis* [34]. Unsuccessful attempts to isolate insertional null mutants have been reported for *Pseudomonas aeruginosa* [48], *Pseudomonas putida* [49], and *Neisseria gonorrhoeae* [50]. To date, multiple attempts to generate a *N. europaea fur* mutant have been unsuccessful. Loss of the *fur* gene may be a lethal mutation in *N. europaea*, as occurs in some other gram-negative bacteria [48]. However, we were successful in generating an *N. europaea fur* promoter knock-out mutant (*fur-kanP*). Southern analysis with probes internal to *fur* or the Km^r corroborated insertion of Km^r in the promoter region of the *fur* gene and hence *fur-kanP* mutant strain was selected for further analysis.

The effects of *fur-kanP* mutation on *N. europaea* were broad. Inactivation of the *fur* gene (resulting in deregulation of iron metabolism) increases sensitivity to redox stress when grown under iron-rich conditions in some bacteria such as *E. coli* [51]. In *N. europaea,* the wild-type and *fur-kanP* mutant strain showed similar growth patterns when grown in Fe-replete (10 μM Fe) and Fe-limited (0.2 μM Fe) media (Fig. 5.5A). However, the *fur-kanP* mutant exhibited a growth defect when cultured in media containing 500 μM iron (Fig. 5.5B). The *fur-kanP* mutant strain was unable to grow beyond 500 μM Fe concentrations while the wild-type strain was able to withstand

iron concentrations up to 1 mM (data not shown). These results indicate that *N. europaea* Fur plays a role in regulating uptake of iron during iron sufficient growth. Increased intracellular free iron is likely to result from deregulated iron uptake by the *fur* mutant [41]. The *N. europaea fur-kanP* mutant strain grown to mid exponential phase in Fe-replete media (10 μM Fe) contained 1.5 fold higher iron than that of the wild-type strain as measured by ICP-OES (Table 5.2). Our measurements of total acid-soluble non-heme iron cannot distinguish between free iron and iron bound to proteins. Hence we measured the heme contents of wild-type and *fur-kanP* mutant and observed that the *fur-kanP* mutant had 1.4 fold lower heme contents compared to wild-type (Table 5.2). In addition, the activity of iron-rich HAO enzyme was lower in *fur-kanP* mutant strain (Table 5.3). These results indicate that the balance between acquiring enough iron and allocating it to various Fe-dependent proteins is lost in *N. europaea fur-kanP* mutant.

N. europaea protein profiles showed over expression of several outer membrane proteins upon Fe-limitation [11, 12]. We have observed similar over expression of outer membrane proteins in *N. europaea fur-kanP* mutant (Fig 5.6, band indicated by *) irrespective of iron availability. These data are consistent with previous studies describing *fur* mutations in other bacterial species [52, 53].

In an attempt to study the global effects of *fur-kanP* mutation on gene expression in *N. europaea*, we have used DNA microarrays. Rather surprisingly, while *fur-kanP* mutation affected many cellular processes, it did not affect the gene expression much. Only three genes with putative roles in iron uptake showed Fur-dependent gene expression in *fur-kanP* mutant strain (Table 5.4). Interestingly, the microarray data

revealed that the NE0616 *fur* transcript was 2.9 fold lower when compared to wild-type (Table 5.4). Since our *fur-kanP* mutant is a promoter knock-out, it is possible that there is some leaky transcription of *fur* which we were unable to detect using traditional RT-PCR. That might explain the partial de-repression of several iron uptake genes in *fur-kanP* mutant. Or it could also be possible that the second *fur* homolog encoded by NE0730 whose transcript was highly up-regulated in our *fur-kanP* mutant might be complementing for the loss of NE0616 encoded *fur* in *fur-kanP* mutant. Further studies are required to quantify the expression level of *fur* in our *fur-kanP* mutant before any of these mechanisms can be further explored.

5.6. Acknowledgements:

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CONCLUSIONS

CHAPTER 6

The ability to use multiple sources of iron for survival, the redundancy of iron transport systems and their regulation by iron attests the importance of iron for *N. europaea*. The essential requirement for iron and its role in regulation of various iron transport systems, however, are not unique for *N. europaea*; many other bacteria have demonstrated an essential requirement for iron [1]. There are some common themes of iron acquisition that *N. europaea* shares with other bacteria and there are some aspects that are different from most other bacterial systems.

Some of the common features between iron transport in *N. europaea* and other bacterial iron transport systems include membership in the TonB-dependent family of siderophore receptors, transport through the periplasm by ABC-like transporters and regulation by iron via the Fur repressor protein. (i) Several *N. europaea* TonB-dependent receptors have homology to known ferric siderophore receptors [2] and role of two such receptors in transport of iron-loaded ferrioxamine (*foxA1* and *foxA2*) has been demonstrated [3]. (ii) Transport of iron-loaded hydroxamate type siderophores and pyoverdine requires an ABC transport system, consisting of a periplasmic binding protein, transmembrane permease, and inner-membrane-associated ATPase (*nitABC*) (Chapter 4). Transport of iron-loaded enterobactin and aerobactin might be through similar ABC transport proteins or through an as yet unknown mechanism. (iii) Thus far, atleast 3 genes with putative roles in iron transport appear to be iron regulated through the Fur repressor protein in *N. europaea* (Chapter 5). The Fur regulon in *N. europaea* may in fact consist of as yet un-identified broad array of genes. Important

questions that remain unanswered for all TonB-dependent iron transport systems in *N. europaea* are:

(a) Do they show dependence on the energy-transducing Ton system like in other organisms?

(b) Does the induction of these systems involves a signaling cascade that starts at the siderophore receptor on cell surface and proceeds to the cytoplasm via regulatory proteins FecR and FecI that are so abundant in *N. europaea* genome?

(c) How does *N. europaea* strip iron from the iron-loaded siderophores?

Some of the uncommon features of *N. europaea* iron transport systems include inability to synthesize siderophores, existence of genes that encode hypothetical proteins with lipocalin, ferrochelatase like domains. (i) Unusual, but not unique, is the lack of siderophore production by *N. europaea* accompanied by the potential use of siderophores secreted by other microorganisms [2-4]. There is a possibility that *N. europaea* makes a siderophore but has not yet been identified. (ii) Although the effect of iron on *N. europaea* gene expression has been investigated; the functions of relatively few of the proteins are understood. Most of the iron-repressed proteins are thought to be involved in iron acquisition. Among them, particularly interesting are few highly repressed ones that carry lipocalin and ferrochelatase like domains (Chapter 3). Further work will be necessary to determine the role of such proteins in *N. europaea* iron acquisition.

In any scientific field, increased understanding generates more questions. This study has revealed that, despite much work, there is still a great deal to be discovered concerning iron metabolism in *N. europaea.* So many questions remain unanswered. Some of these are as follows

(a) How does *N. europaea* acquire iron in the absence of siderophores?

(b) Can *N. europaea* acquire iron from heme and other hemoproteins?

(c) If so, how can *N.europaea* utilize iron from heme without a heme oxygenase?

(d) Does *N. europaea* have ferric-reductase like activities?

(e) What role do multicopper oxidases play in *N. europaea* iron acquisition?

Answers to these questions will enhance our understanding of iron transport and utilization by *N. europaea*.

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6783-90.

APPENDICES

Appendix A: Genes poorly characterized (> 2.0 fold)

	Gene	Description[4]	Relative Fold Change[2,3]
	General function prediction only		
↑	NE0129	putative lipoprotein	-2.0*
↑	NE1090	conserved plasmid protein	+2.2
↑	NE1287	putative host factor-i protein - Hfq	-2.1
↓	NE1897	Peptidase family M48	-2.0
↑	NE2570	probable transmembrane protein	-2.5*
↓	NE2571	Metallo-beta-lactamase superfamily	-2.1*
	Hypothetical proteins		
↑	NE0064	putative signal peptide protein	-2.0*
	NE0065	hypothetical protein	-2.3
↑	NE0096	hypothetical protein	+2.3*
↑	NE0099	hypothetical protein	+2.4*
↑	NE0109	hypothetical protein	-5.7
↑	NE0183	possible (U92432) ORF4 [Nitrosospira sp. NpAV]	-2.1
↑	NE0256	hypothetical protein	+2.1*
↑	NE0300	Hypothetical protein - Transcirptional regulator TetR??	-2.0
↑	NE0493	hypothetical protein	-2.2

Appendix A continued:

↑	NE0523	hypothetical protein - predicted membrane protein??	+2.2*
↑	NE0524	CBS domain - txnl regulator	+2.1*
↓	NE0823	TPR repeat	-2.3*
↓	NE0824	Hypothetical protein	-2.2
↑	NE0825	ABC-type antimicrobial peptide transport system permease component	-2.2*
↓	NE0895	hypothetical protein	+2.2*
↓	NE0910	hypothetical protein	-2.1
↑	NE0912	hypothetical protein	+2.0
↓	NE0914	hypothetical protein	-2.0*
↓	NE0928	Hypothetical protein - Transciptional regulator Rrf2??	-2.9
↑	NE0941	hypothetical protein	-2.3
↑	NE1042	hypothetical protein	+2.5*
↓	NE1066	hypothetical protein	-2.8
↓	NE1128	Hypothetical protein - involved in quorum sensing for biofilm formation	-2.3
↓	NE1129	hypothetical protein	-2.1*
↓	NE1162	hypothetical protein	+2.0*
↓	NE1164	hypothetical protein	+2.2*
↑	NE1179	putative transmembrane protein	-2.0*

Appendix A continued:

↓	NE1225	Hypothetical protein - PilT domain?? - involved in cell motility	-2.1*
↑	NE1268	hypothetical protein	-2.2*
↓	NE1279	hypothetical protein	+2.7
↑	NE1337	hypothetical protein	+2.2
	NE1338	hypothetical protein	+2.1
↓	NE1366	hypothetical protein	+2.1*
↓	NE1377	Protein of unknown function DUF132	+2.1*
↑	NE1799	hypothetical protein	+2.2
↑	NE2145	hypothetical protein	-2.1
↑	NE2218	hypothetical protein	-23.0
↓	NE2230	hypothetical protein	+2.1*
↓	NE2439	hypothetical protein	+5.1*
	NE2440	hypothetical protein	+2.1*
	NE2441	hypothetical protein	+2.0
↓	NE2466	putative lipoprotein	+2.0*
↑	NE2573	putative periplasmic protein	+2.0*

[2, 3, 4] – Details as in Table 3.2.

Appendix B: Genes associated with Information Storage & Processing (> 2.0 fold)

Gene	Description[4]	Relative Fold Change[2,3]
Translation, ribosomal structure and biogenesis		
NE0400	30S ribosomal protein S10	+2.1*
NE0416	50S ribosomal protein L6	+2.2*
NE0417	Ribosomal protein L18P/L5E:Ribosomal protein L18	+2.0*
NE0419	Ribosomal protein L30	+2.4*
NE0615	possible outer membrane lipoprotein OmlA	+2.1*
NE0954	pheS; phenylalanyl-tRNA synthetase, alpha-subunit	+3.0
NE1563	Protein of unknown function DUF79	+2.3*
NE2072	Amidase:Glutamyl-tRNA(Gln) amidotransferase A subunit	+2.8*
NE2143	30S ribosomal protein S4	+3.3
transcription		
NE0838	Bacterial regulatory proteins, MerR family	+2.1*
NE1035	transcription termination factor Rho	+2.7
DNA replication, recombination repair		
NE0001	dnaA; chromosomal replication initiator protein	+2.1
NE0002	*DNA polymerase III, beta chain*	*+1.9*
NE0197	Primosomal replication protein PriB domain??	+2.2*
NE0341	Transposase IS4 family	+3.7
NE0716	Transposase IS4 family	+3.7

Appendix C: Genes associated with cellular processes (>2.0 fold)

Gene	Description[4]	Relative Fold Change[2,3]
Inorganic Ion transport and Metabolism		
NE0448	Ammonium transporter family	-2.3*
NE0576	cysA; sulfate transport ATP-binding ABC transporter	-2.1
NE0577	cysW; sulfate transport ABC transporter protein	-2.6*
NE0578	cysU; sulfate transport ABC transporter protein	-3.2
NE0581	possible predicted diverged CheY-domain	-3.7
NE0582	Prokaryotic sulfate-/thiosulfate-binding protein	-3.7
NE0583	hypothetical protein	-4
Post translational modification, turonover, chaperones		
NE0027	co-chaperonin GroES	+2.7*
NE0028	chaperonin GroEL	+3.0
NE0029	Short-chain dehydrogenase/reductase (SDR) superfamily	+2.6
NE0035	putative ATP-dependent protease LA, putative	+2.3*
NE0221	Radical activating enzyme - fe-s oxidoredutase domain??	+2.7
NE0222	ExsB protein - Queuosine biosynthesis ykvJ???	+2.9*
NE0592	possible uroporphyrin-III C-methyltransferase - hemX	-2.0*
NE0593	putative protein porphyrin biosynthesis - hemY	-2.1*
NE0767	cytochrome c-type biogenesis protein CcmE	-2.6
NE0768	cytochrome c-type biogenesis protein CcmF	-2.0*
NE0769	Periplasmic protein thiol:disulfide oxidoreductase DsbE	-2.0*
NE0771	cytochrome c-type biogenesis protein	-2.5*

Appendix C continued:

↑	NE0906	ftsH; cell division protein	-2.2
↑	NE1183	Serine protease, subtilase family	-2.2*
↓	NE1574	Serine protease, subtilase family	+4.1*
↑	NE1876	coproporphyrinogen III oxidase - hemF	-2
↑	NE2074	Heat shock hsp20 (alpha crystallin) proteins family	-2.4
	Cell envelope biogenesis, outer membrane		
↓	NE1054	putative alginate O-acetylation protein	+2.0*
↑	NE0169	HlyD family secretion protein	+2.5*
↑	NE0170	Outer membrane TolC efflux protein??	+2.4*
↑	NE0171	hypothetical protein - salt induced OMP??	+2.3
	Signal transduction mechanisms		
↑	NE0728	Sensory transduction histidine kinase	+3.7
↑	NE0729	Response regulators consisting of a CheY-like receiver domain	-2.1*
↓	NE0937	possible Response regulators - colR??	+2.8*
↓	NE0938	hypothetical protein - sensory histidine kinase colS??	+2.0*
↑	NE1201	Universal stress protein (Usp)	-2.0*
↓	NE1923	Response regulator receiver domain	+7.9
↓	NE1924	putative chemotaxis protein CheZ	+2.0

Appendix C continued:

Intracellular trafficing and Secretion

NE0902	Tol biopolymer transport system - Periplasmic component	+2.4*
NE2290	Bacterial type II secretion system protein E:GAF domain	+2.2
NE2326	Bacterial leader peptidase 1 (S26A) family:Signal peptidase	+2.5*

Carbohydrate transport and metabolism
Genes involved in Carbon fixation

NE1918	von Willebrand factor type A domain - cbbO	-2.1
NE1919	nitric oxide reductase NorQ protein - cbbQ	-2.1*
NE1920	Ribulose bisphosphate carboxylase, small chain - cbbS	-2.1
NE1921	*Ribulose bisphosphate carboxylase, large chain - cbbL*	*+1.2*
NE1922	Bacterial regulatory protein, LysR family - cbbR	+2.2

Amino acid transport and metabolism

NE0034	Aminotransferase class-V	+2.6
NE0643	hisF Imidazoleglycerol-phosphate synthase	+2.6
NE0871	ATP phosphoribosyltransferase catalytic subunit	-2.4
NE2229	possible long-chain N-acyl amino acid synthase	+9.4

Lipid Metabolism

NE1467	Fatty acid desaturase, type 2:Fatty acid desaturase, type 1	-2.6

Coenzyme metabolism

NE2298	possible BioH, catalyzes early step in biotin biosynthesis	+2.4*
NE2299	Aminotransferase class-I	+3.2*
NE2300	Biotin synthase	+2.7*

Nucleotide Transport and metabolism

NE2422	ribonucleotide-diphosphate reductase subunit beta	+2.3*
NE2423	ribonucleotide-diphosphate reductase subunit alpha	+2.2*