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Characterization of the ferrioxamine uptake system of *Nitrosomonas europaea*

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The chemolithoautotroph *Nitrosomonas europaea* has two genes predicted to encode outer-membrane (OM) ferrioxamine transporters. Expression of the ferrioxamine uptake system required induction, as shown by the shorter lag phase in ferrioxamine-containing cultures when ferrioxamine-exposed cells were used as an inoculum. The two OM ferrioxamine siderophore transporters encoded by *foxA*$_1$ (NE1097) and *foxA*$_2$ (NE1088) were produced only in cells grown in Fe-limited ferrioxamine-containing medium. The inactivation of *foxA*$_1$, singly or in combination with *foxA*$_2$, prevented growth in Fe-limited medium containing excess desferrioxamine (DFX). The *foxA*$_2$-disrupted single mutant grew poorly in the regular Fe-limited (0.2 μM) medium with 10 μM DFX, but grew well when the Fe level was raised to 1.0 μM with 10 μM DFX. For efficient acquisition of Fe-loaded ferrioxamine, *N. europaea* needs both ferrioxamine transporters FoxA$_1$ and FoxA$_2$. FoxA$_1$ probably regulates its own production, and it controls the production of FoxA$_2$ as well.

INTRODUCTION

Nitrosomonas europaea derives all of its cellular energy from the oxidation of ammonia (NH_3) to nitrite (NO_2^-). Ammonia catabolism requires many Fe-containing proteins (Hooper *et al.*, 1972). Iron acquisition by *N. europaea* is supported by approximately 90 genes that have high similarity to Fe uptake genes in other organisms; these genes include approximately 29 genes with similarity to TonB-dependent outer-membrane (OM) siderophore transporter genes (Chain *et al.*, 2003; Wei *et al.*, 2006). Genomics analysis suggests that *N. europaea* has the potential to take up a broad spectrum of siderophores (Wei *et al.*, 2006). However, with the exception of genes for citrate synthesis, the *N. europaea* genome has no predicted genes for siderophore biosynthesis (Chain *et al.*, 2003). Although *N. europaea* has a greater Fe requirement than *Escherichia coli* and *Pseudomonas* species, under Fe-limiting conditions without added siderophores, *N. europaea* grows moderately well by assimilating most of the Fe present in the culture medium (Wei *et al.*, 2006). However, the cells grown under Fe-limited conditions have lower haem and cellular Fe contents and fewer membrane layers than cells grown under Fe-replete conditions, and they upregulate the production of several Fe siderophore receptors and diffusion proteins (Wei *et al.*, 2006).

There are several processes for Fe acquisition that are tightly regulated (reviewed by Braun & Killmann, 1999; Clarke *et al.*, 2001; Faraldo-Gomez & Sansom, 2003). Under Fe-replete conditions, the ferric uptake regulator (Fur, with Fe^{2+} as a co-factor) represses the expression of Fe uptake systems, while Fe limitation leads to derepression (Braun *et al.*, 2003; Escolar *et al.*, 1999; Visca *et al.*, 2002). There are additional levels of regulation, such as those through FecI–FecR–FecA (ECF σ-factor–anti-σ-factor–OM-transducer) (Mahren *et al.*, 2005). One main avenue for Fe acquisition is via siderophore uptake systems. Siderophores are low-molecular-mass, Fe-chelating compounds that micro-organisms produce and secrete in low-Fe environments to sequester Fe. The Fe-loaded siderophores are then recaptured by the micro-organisms via OM siderophore transporters (Braun & Killmann, 1999; Neilands, 1995). In some cases, siderophores produced by one micro-organism can be taken up by another. For instance, *Arthrobacter flavescens* is able to take up siderophores made by soil fungi and bacteria (Winkelmann, 1991), and the non-siderophore-producing *Pseudomonas fragi* uses exogenous siderophores, such as ferrioxamine, enterobactin and some pyoverdines, for its Fe nutrition (Champomier-Verges *et al.*, 1996). Exogenous siderophores, such as cepabactin and coprogen, promote Fe uptake by *Pseudomonas aeruginosa* PAO1, while others, such as ferrichrome A, have no effect on its Fe nutrition, indicating the requirement for specific siderophore transporters (Meyer, 1992). The hydroxamate siderophore ferrioxamine [also known as desferrioxamine (DFX) for the Fe-free form] is of particular interest because it is produced by many soil bacteria, such as *Erwinia*, *Nocardia*, *Streptomyces*, *Arthrobacter*, *Chromobacterium* and

Abbreviations: DFC, desferrichrome; DFX, desferrioxamine; ICP-MS, inductively coupled plasma mass spectrometry; OM, outer membrane; qRT-PCR, quantitative RT-PCR.

Pseudomonas species (Berner *et al.*, 1988; Gunter *et al.*, 1993; Meyer & Abdallah, 1980; Muller & Raymond, 1984; and references therein), and it is used by many other bacteria for Fe uptake (Deiss *et al.*, 1998; Kingsley *et al.*, 1999). In its predominant habitats (soils and wastewaters), with low available Fe levels, *N. europaea* is likely to encounter and use ferrioxamine for its Fe requirements.

A major challenge to the characterization of the function and regulation of the OM siderophore transporters in *N. europaea* is the large number of such genes, and their deduced functional redundancy (Chain *et al.*, 2003; Wei *et al.*, 2006). For example, multiple genes are predicted to encode OM ferrichrome (hydroxamate-type) transporters, catechol-type siderophore transporters, and OM transporters for other siderophores. However, of the hydroxamate-type siderophore transporters in *N. europaea*, only NE1097 and NE1088 putatively code for OM ferrioxamine transporters. These two genes are located in one of the two islands on the *N. europaea* chromosome where Fe-transport-related genes are concentrated (Chain *et al.*, 2003; Wei *et al.*, 2006). NE1097 is more similar than NE1088 to the well-characterized *foxA* genes of other bacteria. Thus, in this study, NE1097 is designated *foxA*$_1$, and NE1088 as *foxA*$_2$ (and the corresponding proteins are designated FoxA$_1$ and FoxA$_2$). In other bacteria, ferrioxamine transporters and ferrichrome transporters cannot substitute for each other (Gaspar *et al.*, 1999; Killmann & Braun, 1998), thereby allowing biochemical and genetic characterization of one type of hydroxamate-type siderophore transporter, without interference from another.

This study characterized the role of OM Fe-ferrioxamine transporters in Fe acquisition in *N. europaea* through both physiological and genetic approaches. Mutants of *N. europaea* lacking functional ferrioxamine transporter genes lost the ability to grow in media containing excess DFX (all Fe being chelated in ferrioxamine). This study provides evidence for the functionality of two of the many predicted Fe-acquisition genes in *N. europaea*, and support for the view that this bacterium can acquire Fe siderophores secreted by other microbes in natural environments.

METHODS

Bacterial cultures and siderophore feeding experiments. *N. europaea* (ATCC 19178) was cultured as described, with minor modifications (Ensign *et al.*, 1993; Stein & Arp, 1998). The standard (Fe-replete) medium contained 10 μM Fe^{3+} ($FeCl_3$) complexed with EDTA to prevent Fe from precipitation. Fe-limited medium was made from reagent-grade chemicals, without addition of any Fe salt, and contained 0.2 μM Fe (Wei *et al.*, 2006). All media, buffers and other reagents were made in double-deionized water. All glassware was soaked in 1 % HNO_3 overnight, and then rinsed thoroughly with double-deionized water. *N. europaea* cultures were grown at 30 °C on a rotary shaker, and late-exponential-phase cells were collected by centrifugation and thorough washes for the analyses. NO_2^- concentrations in *N. europaea* cultures were determined colorimetrically by the Griess reagent (Hageman & Hucklesby, 1971), and were used as a second indicator for the growth rates and growth phases, since the accumulation of NO_2^- is consistently proportional to the increase in cell mass during growth. Citric acid and Fe-free ferrichrome (desferrichrome, DFC) and Desferal (deferoxamine/DFX mesylate) were purchased from Sigma. No siderophores pre-loaded with Fe were used in this study. Siderophores were dissolved in double-deionized water, filter-sterilized, and added to Fe-limited medium, or to media with specified Fe concentrations, in the siderophore feeding experiments. The generic siderophore name ferrioxamine is also used to describe a medium or a culture containing both Fe and desferrioxamine (often in excess), and Fe-ferrioxamine (Fe-DFX) is used to refer to the Fe-loaded form, when necessary. In this study 10 μM DFX was used to ensure the complete chelation of Fe (0.2 μM) in the Fe-limited medium. In the media that contained other metals, and whose pH changed at different culture phases as a result of NO_2^- accumulation, complete chelation of Fe appeared to require at least fivefold DFX (1.0 μM) relative to the Fe level (0.2 μM) in the regular Fe-limited medium, as indicated by the complete inhibition of the growth of the double ferrioxamine transporter mutant (see below).

Determination of Fe and haem contents. Total Fe contents in thoroughly washed *N. europaea* cells were determined by the ferrozine assay following HNO_3 (5 %) digestion of cells at 100 °C (Carter, 1971). Fe concentrations below 1 μM were determined by inductively coupled plasma mass spectrometry (ICP-MS), as described (Houk, 1994). Preparation of a cell-soluble fraction, and determination of haem contents following extraction with pyridine, were done as described (Berry & Trumpower, 1987; Wei *et al.*, 2006).

^{59}Fe siderophore complex uptake experiments. Isotopic iron (^{59}Fe) in ferric chloride form [specific activity: >5 Ci g^{-1} (185 GBq g^{-1})] was purchased from Perkins-Elmer Life and Analytical Sciences. ^{59}Fe was chelated to DFX by mixing it with DFX in double-deionized water (adjusted to pH 6.0). Cultures (OD_{600} approx. 0.02) of the wild-type (in Fe-limited medium, with and without DFX) and the double mutant (in Fe-limited medium) were divided into treatment and control, and tested for the uptake of ^{59}Fe-DFX. To the control culture (for non-specific binding of ^{59}Fe), 200 μM allylthiourea (an inhibitor of ammonia monooxygenase activity) and 3 mM NaN_3 were added before the addition of ^{59}Fe-DFX (concentrations specified in Results). Fractions of the cultures were taken over a time course, and filtered through a 0.45 μm type HA filter (Millipore) to separate cells from unincorporated ^{59}Fe-DFX. The cells retained on the filters were washed with 5 ml 10 mM sodium citrate solution (pH 7), followed by 20 ml double-deionized water. The radioactivity in the cells attached to the filters was measured by liquid scintillation counting.

RNA extraction and real-time RT-PCR. RNA extraction was conducted as described (Wei *et al.*, 2004). RNA samples were treated with DNase (Ambion), and then quantified by A_{260} measurements using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The levels of gene expression (mRNA) in late-exponential-phase cells were determined by real-time (quantitative) RT-PCR (qRT-PCR) in a MyiQ real-time PCR system, with a SYBR-Green-I-based detection kit (Bio-Rad), as described by the manufacturer; the relative expression levels for the genes tested were calculated according to the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001; Pfaffl, 2001), using the *N. europaea* 16S rRNA as a reference. All primers (Table 1) for qRT-PCR were designed to produce PCR products of approximately 150 bp, with an annealing temperature at 59 °C.

DNA preparation, PCR, cloning, mutagenesis and mutant isolation. General DNA manipulations were done as described (Sambrook *et al.*, 1989). The ferrioxamine transporter genes *foxA*$_1$ and *foxA*$_2$ (Fig. 1a) were amplified by PCR using *Taq* DNA polymerase (Promega) on an iCycler Thermal Cycler (Bio-Rad), as described by the manufacturers (see Table 1 for primers). The

Table 1. Primers used for determination of the expression levels by qRT-PCR, and for cloning and mutagenesis of the *N. europaea* OM ferrioxamine transporter genes

Gene	Primer sequence (5′→3′)	
	Forward	Reverse
For mRNA quantification by real-time RT-PCR		
NE1097* (*foxA*$_1$)	TACGATCCGGTTTATGGTGA	TGGAAAAATCGTAACGGACA
NE1088* (*foxA*$_2$)	CGTTACTGGGTGTTGACTGG	TGATCGATCTCTTGCTTTGC
16S rRNA gene	AAGCGGTGGATTATGTGGAT	ATCTCACGACACGAGCTGAC
For cloning, mutagenesis and mutant confirmation		
NE1097Cln	AAGCGAATCAGAGATTTTTATCC	GCACCTGACTGATTTTACCG
NE1088Cln	CGTTACTGGGTGTTGACTGG	TGATCGATCTCTTGCTTTGC
NE1097Mut	CAGGAGTGACGACATTCTTCTG	GCAATGATGATGGCAGCTATAC
NE1088Mut1	TGGCAAGTGAAAAGAAGATGC	ACCCGACAGCTGATACGACT
NE1088Mut2	GAACCGATATGCCATAGATCG	TGAAAACTCTCTATAACCGGAAA

*The primers were designed to be specific for each gene (i.e. regions where the two genes have high sequence similarity were avoided), and at the similar distance from the start codon. The two primer sets have similar PCR amplification efficiency (experimentally determined), allowing quantitative comparison of the RNA levels of the two genes.

resulting DNA fragments were cloned into the pGEM-T Easy vector (Promega) to form two clones. A gentamicin-resistance cassette (from pUCGM; Schweizer, 1993) was inserted into the *Hin*dIII site of the ORF of *foxA*$_1$ (Fig. 1b). For *foxA*$_2$, a 540 bp *Eco*RV segment was deleted from the ORF. A kanamycin-resistance cassette (Kovach *et al.*, 1995) was end-filled, and then ligated into the *foxA*$_2$ ORF (Fig. 1b). The plasmid constructs with the insertions were 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 gentamicin sulfate (15 μg ml^{-1}) or kanamycin sulfate (20 μg ml^{-1}). Aliquots from these cultures were streaked onto Nylon disk membranes, which were placed on semi-solid plates, to isolate clonal mutant strains, as described (Hommes *et al.*, 1996). The double-knockout mutant was obtained similarly by using the single mutant strains as recipient cells for electroporation, selected in the presence of both kanamycin and gentamicin. All mutants were verified by Southern analysis (Fig. 1c, and Results). Southern blotting, labelling of DNA probes, hybridization and imaging were done as described previously (Wei *et al.*, 2004).

Cell fractionation, protein quantification, electrophoresis and MS analysis. Total cell membranes were prepared as previously described (Wei *et al.*, 2006). 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 (Hyman & Arp, 1993). HPLC tandem MS (HPLC/MS/MS) analyses and identification of proteins were performed as previously described (Wei *et al.*, 2006).

RESULTS

Growth of *N. europaea*, and Fe incorporation in the presence of DFX

N. europaea could use the siderophore ferrioxamine for its Fe uptake; it grew well in Fe-limited (0.2 μM) medium with 20 μM added DFX. But the addition of DFX to the Fe-limited medium led to a longer lag phase than in the medium without added DFX (Wei *et al.*, 2006), suggesting that the ferrioxamine uptake system in *N. europaea* requires the presence of the siderophore for its induction. We tested whether the lag phase would be shortened by using cells grown in the presence of ferrioxamine as the inoculum (0.2 μM Fe, 10 μM DFX). The lag phase was shorter when the inoculum used was grown in medium containing ferrioxamine than when the inoculum was grown in Fe-limited medium in the absence of ferrioxamine (Fig. 2). The lag phase of the culture inoculated with ferrioxamine-exposed cells was comparable to that of the ferrioxamine-free Fe-limited culture (Wei *et al.*, 2006).

We also tested whether genetic changes (e.g. phase variation) were the cause for the loss of the long lag phase of *N. europaea* after growth in the presence of DFX. Inocula of cells grown in DXF-containing medium were used to start cultures in regular Fe-replete and Fe-limited media without ferrioxamine. These cells, when used to start cultures in DXF-containing Fe-limited medium, exhibited again the long lag phase similar to that observed initially. This result indicates that no genetic changes or enrichment of variants occurred during growth of *N. europaea* in medium with ferrioxamine-sequestered Fe.

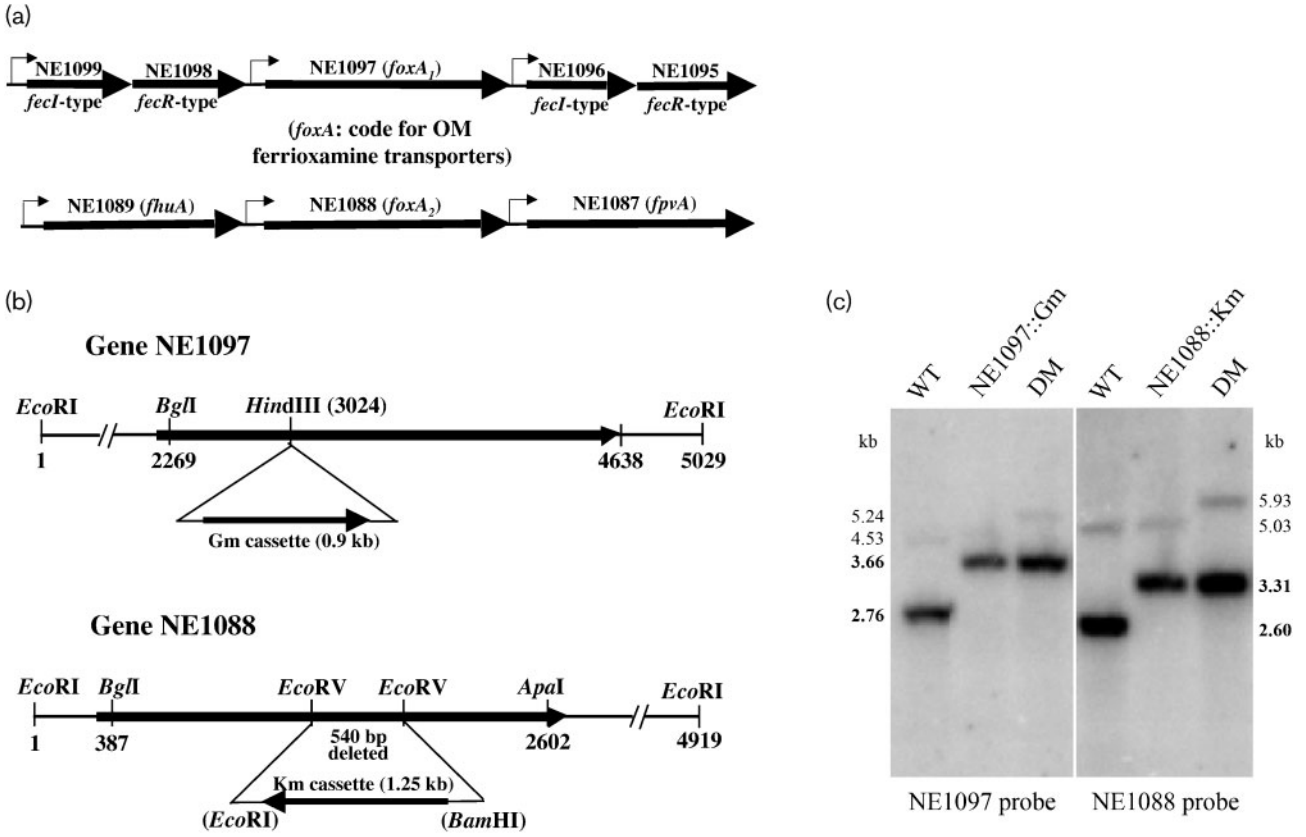

Fig. 1. Gene organization, and insertional mutagenesis scheme and confirmation. (a) Organization of $foxA_1$ and $foxA_2$ (NE1097 and NE1088), and neighbouring genes. *fhuA*, ferrichrome receptor gene; *fpvA*, pyoverdine transporter gene. Thin arrows above the genes indicate putative promoters. (b) Restriction maps of $foxA_1$ (2475 bp) and $foxA_2$ (2454 bp), with flanking regions and the mutagenesis strategy. Maps are approximately proportional to the fragment sizes, except breaks (//). (c) Verification of mutagenesis of ferrioxamine transporter genes in *N. europaea* mutants by Southern hybridization. Left, genomic DNAs from the wild-type (WT), the single mutant $foxA_1$::Gm, and the double mutant (DM), were digested with *Bgl*I and *Eco*RI, and probed with the $foxA_1$ (NE1097) ORF sequence. The major wild-type band (2.76 kb) corresponds to the fragment of *Bgl*I–*Eco*RI on the right (downstream) of NE1097, and the upper low-intensity wild-type band (4.53 kb) corresponds to the *Bgl*I-downstream *Eco*RI fragment of NE1088. Right, genomic DNAs from the wild-type, the single mutant $foxA_2$::Km, and the double mutant, were digested with *Eco*RI and *Apa*I, and probed with the $foxA_2$ (NE1088) ORF sequence. The major wild-type band (2.60 kb) corresponds to the fragment of *Eco*RI (left) to *Apa*I of NE1088, and the low-intensity wild-type band (5.03 kb) to the *Eco*RI–*Eco*RI fragment of NE1097. Shown are the calculated sizes of the DNA fragments, and all the hybridized fragments appeared at the expected positions.

The total cellular Fe content [14.7 ± 0.68 nmol (mg protein)$^{-1}$] in cells grown in DFX-containing Fe-limited medium was similar to that in cells grown in DFX-free Fe-limited medium [13.6 ± 2.2 nmol (mg protein)$^{-1}$; Wei *et al.*, 2006]. The haem content in the soluble fraction of the *N. europaea* cells cultured in Fe-limited DFX-containing medium was also similar to that in DFX-free Fe-limited medium [3.04 ± 0.03 versus 2.87 ± 0.71 nmol (mg protein)$^{-1}$; Wei *et al.*, 2006]. These results indicate that *N. europaea* can take up and process ferrioxamine-chelated Fe. Furthermore, in the presence of DFX, cells took up about 90 % of the Fe in the Fe-limited medium, compared with 70 % in the absence of DFX (Wei *et al.*, 2006).

Ferrioxamine induction of $foxA_1$ and $foxA_2$ expression, and of FoxA protein production

Induction of the ferrioxamine uptake system was further confirmed by the elevated mRNA levels of $foxA_1$ and $foxA_2$ in cells grown in ferrioxamine-containing medium (Table 2). The mRNA levels of $foxA_1$ and $foxA_2$ increased about twofold by Fe limitation alone, but the inclusion of ferrioxamine resulted in additional eight- and sixfold increases, respectively, in mRNA level. The expression levels of $foxA_1$ were higher than those of $foxA_2$ under all three growth conditions (Table 2). Clearly, the expression of $foxA_1$ and $foxA_2$ was highly induced by the presence of ferrioxamine.

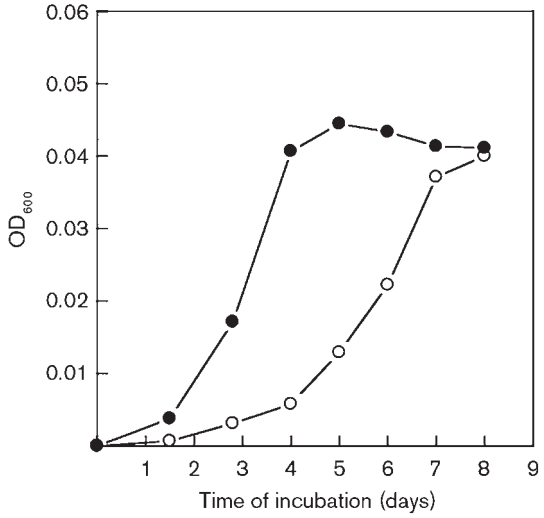

Fig. 2. Effects of inoculum source on the early growth rates of *N. europaea* in medium containing 0.2 μM Fe and 10 μM DFX; ○, inoculated with cells not exposed to ferrioxamine; ●, inoculated with cells previously exposed to ferrioxamine. The two inoculum cultures were at a similar growth stage, and equal inoculum amounts were used (normalized by OD_{600}). Data shown are means of triplicates, with variation less than 10 % among the triplicates. The experiment was repeated several times, and produced similar results or the same trend.

We also examined induction of the ferrioxamine uptake system at the protein level. Cells of *N. europaea* were grown in Fe-replete medium, Fe-limited medium, and Fe-limited medium supplemented with siderophores. Total membranes isolated from these cells were analysed for protein compositions by SDS-PAGE. Several protein bands (e.g. bands 2, 3 and 4 in lane 3, Fig. 3) were uniquely observed in the cells cultured in ferrioxamine-containing medium, compared with cells grown in Fe-limited medium (lane 2, Fig. 3) and those grown in ferrichrome-containing medium (lane 4, Fig. 3). The identities of the membrane proteins differentially expressed under Fe-limited ferrioxamine-supplemented conditions were determined by HPLC/MS/MS analyses. Given that the predicted molecular masses of the two mature proteins (minus signal peptides) $FoxA_1$ and $FoxA_2$ are 87.4 and 86.3 kDa, band 4 in lane 3 (Fig. 3) was a good candidate to contain these proteins. Indeed, band 4 contained two proteins identified as the products of *foxA*$_1$ (NE1097) and *foxA*$_2$ (NE1088) that are homologous to the *foxA* of other bacteria (Bell *et al.*, 2004; Killmann & Braun, 1998). The Mascot (www.matrixscience.com) scores for $FoxA_1$ and $FoxA_2$ obtained from this protein sample were 3310 and 670 respectively, both being greatly above the peptide Mascot cutoff value of 30. $FoxA_1$ and $FoxA_2$ were highly produced in cells grown in the ferrioxamine-containing Fe-limited medium, and not in the cells grown in Fe-limited medium containing ferrichrome (Fig. 3, lane 4) or citrate (not shown). These results indicate that production of OM ferrioxamine transducers requires ferrioxamine, consistent with the elevated *foxA*$_1$ and *foxA*$_2$ mRNA levels detected in cells grown in ferrioxamine-containing medium (Table 2), and suggest a role for $FoxA_1$ and $FoxA_2$ in ferrioxamine uptake.

Table 2. *foxA*$_1$ and *foxA*$_2$ mRNA levels in *N. europaea* grown in Fe-replete (10 μM Fe) and Fe-limited (0.2 μM) media, and in Fe-limited medium supplemented with 10 μM DFX

Treatment	*foxA*$_1$ mRNA level*	*foxA*$_2$ mRNA level*	Ratio of *foxA*$_1$/*foxA*$_2$
Fe-replete	3.53 ± 0.04	1.0*	3.53
Fe-limited	7.94 ± 0.04	1.66 ± 0.01	4.78
Fe-limited, + DFX	67.62 ± 0.20	10.41 ± 0.02	6.50

*All mRNA levels in each treatment were normalized using the 16S rRNA level as a standard; the lowest level was set to be 1.0, and all other values are relative mRNA levels calculated with the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001; Pfaffl, 2001), allowing reliable comparisons among different treatments.

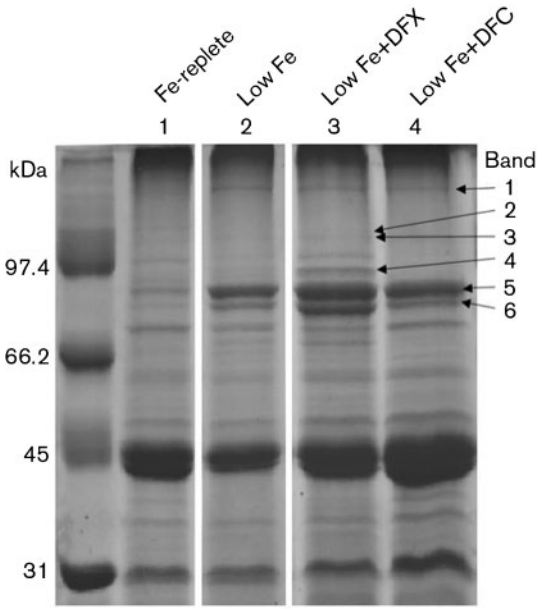

Fig. 3. SDS-PAGE analysis of total membrane proteins of *N. europaea* wild-type grown in Fe-replete medium (lane 1), Fe-limited (low Fe, 0.2 μM) medium without siderophores (lane 2), Fe-limited medium with 10 μM DFX (lane 3), and Fe-limited medium with 10 μM DFC (lane 4). All lanes shown were from the same gel; for clarity, some lanes next to lane 2 are not shown. The amount of total membrane protein loaded was 4.5–5.5 μg per lane. Membrane proteins that were differentially expressed under different conditions are indicated by arrows and numbers. Bands 1, 5 and 6 were common to lanes 2, 3 and 4, and each band from each lane was separately subjected to MS/MS analyses.

Ferrioxamine-exposed cells also shared two major protein bands with cells grown in siderophore-free and ferrichrome-containing media (bands 5 and 6, Fig. 3). These two bands contained the same siderophore transporters (Wei *et al.*, 2006), except that an additional OM siderophore transducer encoded by NE2433 was detected in the ferrioxamine-exposed cells (data not shown).

Generation and characterization of ferrioxamine transporter mutants

To further characterize the ferrioxamine uptake system, we focused on the *foxA* genes, including construction of insertional knockouts of each gene. $FoxA_1$ has a 34 % aa identity (54 % similarity) to the characterized ferrioxamine receptor (FoxA) of *Salmonella enterica* (Chiu *et al.*, 2005; Kingsley *et al.*, 1999), while $FoxA_2$ has 32 % aa identity (51 % similarity) to the *S. enterica* FoxA. $FoxA_1$ and $FoxA_2$ have, respectively, 33 and 31 % aa identity to the *Yersinia enterocolitica* FoxA (Baumler & Hantke, 1992), which is highly similar to another characterized FoxA in *Erwinia herbicola* (Berner & Winkelmann, 1990; Deiss *et al.*, 1998). $FoxA_1$ and $FoxA_2$ share a 46 % aa identity (61 % similarity) with each other. Interestingly, the primary sequences of $FoxA_1$ and $FoxA_2$ are typical of OM transducers (with an extended N terminus). Thus, $FoxA_1$ and $FoxA_2$ are about 100 aa longer than the OM ferrioxamine receptors in the enteric species mentioned above. Both $FoxA_1$ and $FoxA_2$ have a 36 % aa identity to a transducer-type FoxA recently reported in *P. aeruginosa* (Llamas *et al.*, 2006).

Constructs of *foxA*$_1$ and *foxA*$_2$ with antibiotic-resistance cassette inserts (Fig. 1b) were electroporated into the *N. europaea* wild-type cells. Both single and double insertional mutants were obtained through homologous recombination, and were verified by Southern hybridizations (Fig. 1c). The gentamicin-cassette probe detected the band corresponding to the 3.66 kb (the calculated size based on the DNA sequences) *Bgl*II–*Eco*RI fragment in the single mutant *foxA*$_1$::Gm (NE1097::Gm) and in the double mutant (*foxA*$_1$::Gm/*foxA*$_2$::Km), as detected with the *foxA*$_1$ probe (not shown). The kanamycin-cassette probe detected the same 3.31 kb (the calculated size based on the DNA sequences) *Eco*RI–*Apa*I fragment in single mutant *foxA*$_2$::Km (NE1088::Km) and in the double mutant, as detected with the *foxA*$_2$ probe (not shown). These results confirm that a single copy of antibiotic-resistance cassette was correctly inserted in each targeted gene in the whole genome. In addition, weak hybridization bands were observed because *foxA*$_1$ and *foxA*$_2$ have regions of high nucleotide sequence homology (approx. 550 bp at the 5′ end having approx. 88 % nucleotide identity to each other). These bands (Fig. 1c, upper low-intensity bands) with correct predicted fragment sizes (refer to Fig. 1b, c, and legend) further confirmed that only the targeted gene had an insert in the two single mutants, while both genes had an insert in the double mutant, and no undesired recombination occurred between the two genes.

Characterization of these ferrioxamine transporter mutants showed that, as predicted, the double mutant could not grow in Fe-limited (0.2 μM) medium containing DFX at 10 μM, nor could it grow in DFX-containing medium with increased Fe (1.2 μM) (Fe-sequestered). Surprisingly, the single mutant with a disrupted *foxA*$_1$ had the same phenotype as the double mutant (Fig. 4), suggesting that this transporter ($FoxA_1$) has an essential role in the Fe-ferrioxamine-acquisition system. Growth of the single mutant *foxA*$_2$::Km was tested in 10 μM DFX-containing medium with 0.2, 0.4 and 1.2 μM Fe. Little growth was observed after 8 days' incubation with Fe at 0.2 μM (Fig. 4). When Fe was at approximately 0.4 μM, the growth rate and cell mass after 10 days' incubation (data not shown) were less than half of those of the wild-type grown in Fe-limited (0.2 μM) medium with 10 μM DFX. Interestingly, when the Fe (Fe-ferrioxamine) concentration was raised to 1.2 μM, the growth rate and final cell mass (Fig. 4) were comparable to those for the wild-type grown in medium with 0.2 μM Fe and 10 μM DFX (Fig. 2).

All three mutants grew to levels that were similar to those of the wild-type in the regular Fe-replete medium, in siderophore-free Fe-limited medium, and in Fe-limited medium with 10 μM added DFC (data not shown). These results indicate that *foxA*$_1$ and *foxA*$_2$ are not involved in siderophore-independent Fe uptake or the uptake of ferrichrome (another hydroxamate-type siderophore), and that the mutations have no apparent pleiotropic or adverse effect on growth under any of the conditions used

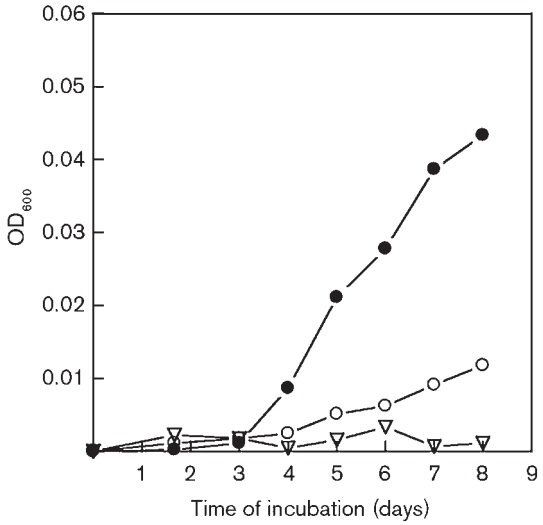

Fig. 4. Growth phenotypes of the single ferrioxamine transducer mutants of *N. europaea* in DFX-containing media; ▽, single mutant NE1097::Gm in medium with 1.2 μM Fe and 10 μM DFX; ○, single mutant NE1088::Km growing in medium with 0.2 μM Fe and 10 μM DFX; ●, single mutant NE1088::Km growing in medium with 1.2 μM Fe and 10 μM DFX. Data shown are means of duplicates, with variation less than 10 %. The experiment was repeated and produced similar results.

in this study, except in the presence of ferrioxamine. The efficient acquisition of ferrioxamine-bound Fe needs both functional $FoxA_1$ and $FoxA_2$, providing direct genetic evidence for the functionality of the *N. europaea* $foxA_1$ and $foxA_2$.

Uptake of ^{59}Fe-ferrioxamine by *N. europaea* wild-type and mutants

Late-exponential-phase cultures of *N. europaea* wild-type grown in Fe-limited DFX-containing medium were fed with ^{59}Fe (0.1 μM) and DFX (10 μM). During a 4 h incubation, DFX-induced *N. europaea* wild-type cells could take up ^{59}Fe-DFX, and they accumulated 28 pmol ^{59}Fe (ml cells)$^{-1}$ or 28 % of the total added ^{59}Fe. Cells cultured in DFX-free Fe-limited medium (i.e. not induced with DFX) did not take up ^{59}Fe-DFX during a 21 h incubation, but did take up approximately 50 % of the free ^{59}Fe (0.1 μM, without DFX). These results are consistent with the growth patterns and the requirement of a 3-day induction for non-DFX-induced cells to grow on DFX-chelated Fe (Fig. 2). As expected, the double ferrioxamine transporter mutant failed to accumulate ^{59}Fe in a 3-day incubation after cells were transferred from Fe-limited medium to the same medium amended with 2 μM ^{59}Fe and 10 μM DFX.

DISCUSSION

This study provides evidence for the role of $foxA_1$ and $foxA_2$ in ferrioxamine uptake in *N. europaea*. First, correlation was observed between the presence of ferrioxamine and the elevated transcription of the two genes (Table 2). Second, the proteins $FoxA_1$ and $FoxA_2$ were produced only in the cells grown in ferrioxamine-containing medium (Fig. 3). Third, inactivation of $foxA_1$ *and* $foxA_2$ abolished the uptake of Fe-DFX for cell growth (^{59}Fe feeding, and Fig. 4). This study is the first to confirm the functionality of two of the many siderophore transporters in *N. europaea*.

Addition of the siderophore ferrioxamine or ferrichrome to Fe-limited medium led to increased *N. europaea* growth (total cell mass), indicating that both siderophores can be used for efficient Fe uptake (Wei *et al.*, 2006). However, these two siderophores reveal two different regulatory responses used by *N. europaea* cultured under low Fe and in the presence of these siderophores. *N. europaea* cultured in ferrioxamine-containing medium showed a prolonged lag phase, indicating that cells initially could not take up Fe-ferrioxamine. The significant reduction of the lag phase by exposure of the inoculum to ferrioxamine (Fig. 2) indicated an induction mechanism. In contrast to the ferrioxamine, addition of excess ferrichrome to Fe-limited medium did not prolong the lag phase (Wei *et al.*, 2006), which is consistent with the observation that one ferrichrome transporter (a receptor, encoded by NE1089) is already highly expressed in cells grown in ferrichrome-free Fe-limited medium (Wei *et al.*, 2006).

Our results also reveal the specificity of the siderophores towards their transporters. Both ferrioxamine and ferrichrome are hydroxamate-type siderophores, and ferrioxamine transporter genes and ferrichrome transporter genes of *N. europaea* are phylogenetically close (data not shown). Nonetheless ferrichrome did not induce the expression of OM ferrioxamine transporters in *N. europaea* (Fig. 3), and this indicates a high specificity of each transporter to its corresponding siderophore, and hence the specificity for the regulation pathways involving particular σ and anti-σ factors. The failure of the double mutant to grow in media where all Fe was chelated by DFX also confirmed that none of the other predicted OM siderophore transporter genes, including transducer gene NE2433 that was uniquely expressed in ferrioxamine-grown cells, could compensate for the inactivated $foxA_1$ and $foxA_2$.

Induction (or autoregulation) of the expression of OM siderophore transporters by their binding of specific siderophores has been observed in other bacteria (e.g. Braun, 1997; Kim *et al.*, 1997; Llamas *et al.*, 2006), and typically involves a regulatory pathway with σ and anti-σ factors. OM transducers are those transporters that have N-terminal extensions that interact with anti-σ factor (FecR-type) that activates the σ factor (FecI-type), which turns on the transcription of the OM transducer genes (Braun *et al.*, 2003; Schalk *et al.*, 2004). Both *N. europaea* $FoxA_1$ and $FoxA_2$ have an N-terminal extension typical of OM transducers. Therefore, the *N. europaea* ferrioxamine uptake system is likely to be subjected to dual regulations: global regulation by available Fe levels through Fur, and specific regulation by available ferrioxamine through σ/anti-σ factors; our results (Fig. 3, Table 2) indicate that the latter plays a major role. However, only $foxA_1$ has cognate σ and anti-σ factor genes (Fig. 1a), similar to the *fecI–fecR–fecA* gene cluster in *E. coli* (Mahren & Braun, 2003; Visca *et al.*, 2002).

The phenotypes of the two single mutants described above (Fig. 4) point to possible different roles of $FoxA_1$ and $FoxA_2$ in the transport of ferrioxamine across the OM, and possibly in signal transduction. First, one may conclude that $FoxA_2$ is simply nonfunctional, since the single mutant $foxA_1$::Gm (with a functional $foxA_2$) failed to grow when all the Fe was chelated in ferrioxamine (Fig. 4). However, the other single mutant $foxA_2$::Km (functional $foxA_1$) could not grow at the same rate as the wild-type in ferrioxamine-containing Fe-limited medium, indicating a role of $FoxA_2$ in Fe-ferrioxamine uptake. An alternative explanation could be that $FoxA_2$ is functional for transporting Fe-ferrioxamine, but that it cannot activate the signal transduction pathway for its own expression when $foxA_1$ is nonfunctional (see below). Second, qRT-PCR results (Table 2) suggest that $foxA_1$ is expressed constitutively at low levels under Fe limitation in the absence of ferrioxamine, probably for the initial sensing of the siderophore. Therefore, both $FoxA_1$ and $FoxA_2$ could be functional for transporting Fe-ferrioxamine in the wild-type cells, and $FoxA_2$ might be even more efficient given

that $FoxA_1$ could not fully compensate for the loss of $FoxA_2$ at low Fe-ferrioxamine concentrations (Fig. 4).

A number of possible mechanisms can be explored to explain the roles and regulation of the *N. europaea* $FoxA_1$ and $FoxA_2$ by using the extensive studies done in model species such as *E. coli*. Our results show that $FoxA_1$ is essential for the ferrioxamine uptake, but it may be an inefficient transporter since the *foxA*$_2$-disrupted mutant could barely grow in medium with 10 μM DFX and 0.2 μM Fe (Fig. 4). One possible reason for the lower efficiency of $FoxA_1$ in Fe-ferrioxamine uptake could be different specificities of $FoxA_1$ and $FoxA_2$ to different types of ferrioxamine. Several types of ferrioxamine such as B, E and G have been described, and different subspecies of *S. enterica* use different types of ferrioxamine (Kingsley *et al.*, 1999). Another possibility for the inefficiency of $FoxA_1$ for transporting Fe-ferrioxamine may be that it binds DFX (the unloaded form) more tightly than $FoxA_2$ does, and thus requires a higher ratio of Fe-loaded ferrioxamine to the total added DFX than $FoxA_2$ for effective transport of Fe-loaded ferrioxamine (Fig. 4). Binding of Fe-free siderophores by OM receptors has been observed in *E. coli* and *P. aeruginosa* (Hoegy *et al.*, 2005; Stintzi *et al.*, 2000; Yue *et al.*, 2003). The presence of excess Fe-free siderophores might interfere with the transport of Fe-loaded siderophores (Schalk *et al.*, 2001), and this may be the case with *N. europaea* $FoxA_1$. Comparison of the rates and efficiency of Fe-loaded ferrioxamine uptake cannot be made between the two single mutants because the *foxA*$_1$-disrupted mutant cannot grow in medium in which all Fe is sequestered by ferrioxamine.

The mRNA level of *foxA*$_2$ under the Fe-limited DFX-free condition was similar to that under Fe-replete condition (Table 2), i.e. *foxA*$_2$ was not induced by Fe limitation alone, suggesting that its expression requires a specific σ factor. It is also possible that $FoxA_2$ is not produced at initial growth stages, which means that *foxA*$_2$ alone cannot initiate the uptake of Fe-DFX complex, and thus cell growth. But RT-PCR detected *foxA*$_2$ mRNA in wild-type cells at early growth phase (OD_{600} 0.009) (data not shown). Thus, the results described above suggest that only $FoxA_1$ could turn on the expression of both *foxA*$_1$ and *foxA*$_2$, probably through the interaction of its N-terminal extension with an anti-σ factor that in turn activates a particular σ factor. This hypothesis is supported by protein sequence analysis. Alignment of the N-terminal extensions shows that out of the 14 conserved amino acid residues that are believed to be involved in the interaction with anti-σ factors (Schalk *et al.*, 2004), four are different in $FoxA_2$, compared with 13/14 that are conserved in $FoxA_1$ (Fig. 5). It is worth noting that these 14 AA residues are well conserved in eight different OM transducers across different species (Schalk *et al.*, 2004), and in *N. europaea* $FoxA_1$, but not as well conserved in $FoxA_2$. This difference might explain why $FoxA_2$ could not initiate the expression of the Fe-ferrioxamine uptake system even though it is required for the efficient uptake of Fe-ferrioxamine. The nucleotide sequences of the putative promoter regions of *foxA*$_1$ and *foxA*$_2$ (predicted with the program at http://www.fruitfly.org/seq_tools/promoter.html) are 84 % identical, suggesting the possibility of interaction with the same σ factor (the one activated by $FoxA_1$). Future studies using techniques such as site-directed mutagenesis and the bacterial two-hybrid system could help to dissect the specific interactions among components of the ferrioxamine and other siderophore uptake systems in *N. europaea*.

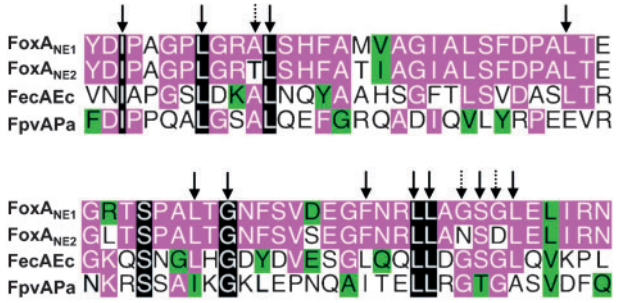

Fig. 5. Alignment of the N-terminal extensions of four OM siderophore transducers showing the 14 aa residues (indicated by arrows) involved in the interaction with the anti-σ factor; three amino acid residues are not conserved in *N. europaea* $FoxA_2$. FecAEc, FecA of *E. coli*; FpvAPa, FpvA of *P. aeruginosa*. See Schalk *et al.* (2004) for alignment of eight OM transducers showing the 14 conserved amino acid residues.

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