NITRATE TRANSPORT AND ASSIMILATION IN
ASPERGILLUS NIDULANS

Naureen Akhtar

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Nitrate transport and assimilation in *Aspergillus nidulans*

Naureen Akhtar

This thesis is submitted in partial fulfilment for the degree of PhD at the University of St Andrews

May, 2012
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Abstract

In this study, several aspects of nitrate assimilation and transport have been studied using the filamentous fungus *Aspergillus nidulans*, which has been shown to be a safe laboratory organism as judged by its pathogenicity towards insect larvae. *In silico* analysis of the *A. nidulans* genome sequence, identified two putative genes designated *cnxL* and *cnxK* that might be involved in molybdenum cofactor biosynthesis as well as two putative nitrate reductases encoding genes *niaB* and *niaC*. All four genes are hitherto unknown. Although many features of these proteins provided clues of functionality, biochemical and genetical approaches employed in this present study failed to elicit expression of any of these four genes.

A NrtA protein structure model was developed based on residue homology with the *Escherichia coli* GlpT a protein, the structure of which has been solved. The results of thiol cross-linking of three double cysteine mutants in four NrtA essential residues, R87, R368, N168 and N459, indicated that the molecular distance between R87 and R368 is \( \sim 0.4 \, \text{Å} \), R368 and N168 \( \sim 6.2 \, \text{Å} \), R87 and N459 is \( \sim 2.2 \, \text{Å} \). Another important observation was the change in the confirmation of Tm 2 and Tm 8 in the presence of nitrate. This shift resulted in an increase of \( \sim 2 \, \text{Å} \) gap between the residues R87 and R368. Distances between amino acid residue pairs estimated using such molecular rulers contradicted the NrtA existing model. Cysteine-scanning mutagenesis studies were extended to the generation of a library of single cysteine mutants of NrtA residues spanning Tm 2 and Tm 8. The majority of single cysteine mutants possessed wild type NrtA protein expression levels but unfortunately most were found to be loss-of-function. Consequently, thiol chemistry of this crop of mutants was not perused.

Attempts were also made to overexpress and crystallise the bacterial nitrate transporters. In this regard, bacterial nitrate transporters, NarU (*E. coli*), Nar (*Bacillus cereus*), NarK1 and NarK2 (*Pseudomonas aeruginosa*) and NarK2 (*Thermus thermophilus*) fused with GFP were expressed in *E. coli* and used in crystallisation trials. Although this approach has proved successful for a number of membrane proteins, unfortunately was not helpful with regard to the purification of any of the above bacterial nitrate transporters to yield protein expression levels required for successful protein crystallography.

Finally, the effects of potential nitrate transport inhibitors were studied on net nitrate transport by NrtA and NrtB proteins of *A. nidulans*. The results indicated that chlorate had more of an inhibitory effect on NrtA net nitrate transport than that by NrtB. Chlorite and sulphite equally affected net nitrate transport by either NrtA or NrtB proteins while caesium strongly inhibited the net nitrate transport by NrtB transporter.

Although considerable information has been collected on several aspects of nitrate metabolism but there is a need to study this phenomenon in greater depth by further research.
Chapter One

General Introduction

1.1 Nitrogen

Nitrogen, the fifth most abundant element on earth and the key component of biomolecules, is an essential nutrient for all organisms. The major source of nitrogen is in the atmosphere, but here, nitrogen is present in the form of an inert gaseous molecule, \( \text{N}_2 \) that cannot be converted directly to organic nitrogen by living organisms. Therefore, this unavailable form of nitrogen needs to be converted first to an available form of nitrogen, ammonium. This is carried out by the process of nitrogen fixation by abiotic or biotic environmental factors. On the other side of the nitrogen cycle, considerable nitrogen is present in the form of complex organic nitrogenous matter that cannot be utilised directly for example by plants. First such complex molecules must be converted to inorganic forms, mainly ammonium, by degradation systems present particularly in the fungi. The resulting ammonium is oxidised to nitrite and then to nitrate by soil bacteria such as Nitrobacter and Nitrosomonas respectively (Falkowski, 1997; Hayatsu et al., 2008; Canfield et al., 2010; Dechorgnat et al., 2011). The activity of microorganisms in the nitrogen cycle has been summarised in Figure 1.1.

Figure 1.1: Schematic representation of microbiological processes in the nitrogen cycle.

(1) Nitrogen fixation, (2) bacterial, archaeal and heterotrophic nitrification, (3) bacterial, archaeal and fungal denitrification, (4) co-denitrification by fungi (5) co-denitrification by anammox (bacteria that convert nitrite and ammonium into dinitrogen gas under anaerobic conditions) and (6) ammonium oxidation. This figure and legends has been reproduced directly from Hayatsu et al. (2008).

The ever-increasing human population of the planet Earth (www.populationspeakout.org) results in increased demand for food and the use of nitrogen fertilisers to make up nitrogen deficiency in agricultural soils. The use of fertilisers has been increased greatly over the last five decades (Fixen and West, 2002; Fixen, 2009). The predominant forms of nitrogen fertilisers are nitrate, ammonium and urea (www.fertilizer.org). More than 50 % of the total fertilisers are used to grow just three
crucial crops, wheat, maize and rice (Raun and Johnson, 1999). The use of fertilisers not only increases the cost of crop productivity but also nitrogen from unused fertilisers is leached into the water and caused severe environmental and health problems (Good et al., 2004; Galloway et al., 2008; Diaz and Rosenberg, 2008; Hayatsu et al., 2008). There is a need for an effective management strategy to avoid (or minimise) the negative impacts of nitrogen fertilisers (Galloway et al., 2008). One way to overcome the extensive use of fertilisers is to improve the nitrogen utilisation efficiency by crop plants. This may be achieved by targeting enzymes, transporters or regulatory genes of nitrogen metabolism by molecular or genetic approaches (Fernandez and Galvan, 2008 and references therein).

1.2 Nitrate

Nitrate is the most available inorganic form of nitrogen and is an important nutrient of plants, fungi and bacteria, inhabiting most aerobic soils. The level of nitrate concentration in the soil varies with respect to the season and the activity of various biotic (type or population density of decomposers) and abiotic (type and amount of litter, temperature, humidity etc) environmental factors (Miller et al., 2007; Unkles et al., 2001; Forde, 2000; Crawford and Glass, 1998).

Nitrate is not only the major source of available nitrogen in the soil but also stimulates the growth of organisms by inducing genes encoding nitrate transporter proteins or enzymes involved in nitrate assimilation (Crawford and Glass, 1998). In addition nitrate also affects plant morphogenic processes for example root development (Zhang and Forde, 1998), root and shoot balance (Scheible et al., 1997a) and carbon metabolism (Scheible et al., 1997b). Finally the role of nitrate in gametogenesis has been reported in *Chlamydomonas reinhardtii* (Pozuelo et al., 2000).

Nitrate is the most preferred source of nitrogen that can be assimilated efficiently when sufficient supply of oxygen is available (Takasaki et al., 2004a; Takasaki et al., 2004b; Takaya, 2009). Most of the organisms obtain their nitrogen by reduction of nitrate to ammonium. Plants absorb nitrate from soil solutions by active transport through root cells. From the root cells, nitrate is transported to different parts of the plant in the phloem and then enters into the cells using channels or transporters and is often stored in the vacuoles of cells (Crawford and Glass, 1998).

1.3 Cellular transport systems

Transport systems are required by most living cells for the uptake of nutrients and excretion of unwanted or toxic substances. Such transport systems or permeases are lodged within cell membranes that act as barriers to separate cellular content from the outside environment and allow the movement of materials in and out of the cell (MacKinnon, 2004). Transporter membrane proteins present in living cells can be divided into channels or carriers (Saier, 2000; Busch and Saier, 2002).
Channels are pores that open only when substrate is present in the surroundings and allow the movement of substrate down an electrochemical gradient. Carrier proteins are integral membrane proteins that are involved in the movement of molecules by facilitated diffusion or active transport. Active transport of materials is the movement of substances against the concentration gradient and may be (i) primary active transport that uses the energy to translocate the materials against a concentration gradient or (ii) secondary active transport that uses an electrochemical potential gradient of one substance to translocate another substrate (Dahl et al., 2004).

Transmembrane transporter proteins are classified according to the Transporter Classification System (TC) which is based on combined transporter proteins functionality and phylogenetic information. According to the TC system (approved by the International Union of Biochemistry and Molecular Genetics, IUBMB), transporters are grouped into various hierarchical classification levels; class, subclass, superfamily or family, subfamily and finally the specific transporter (www.tcdb.org; Busch and Saier, 2002; Saier et al., 2006). Many transporter proteins belong to one of two major classes of transporters, namely the ATP-binding cassette (ABC) protein superfamily or the major facilitator superfamily (MFS) (Pao et al., 1998).

1.3.1 ABC superfamily

The ABC transporters are primary transporters as they utilise energy generated by ATP hydrolysis and hence allow the movement of materials against a concentration gradient. Downstream of the ATP binding domain(s), LSGGQ, a highly conserved signature sequence of the ABC protein superfamily is present (Szentpetery et al., 2004).

Compared to the MFS, ABC protein is a less studied group of transporter proteins and is involved in the influx of materials in prokaryotes or efflux in both prokaryotes and eukaryotes. The diversity of substrates of ABC transporters range from a single ion to a complete protein (Saier, 2000; Dahl et al., 2004; Hollenstein et al., 2007). The mechanism of transport by ABC transporters is not well understood. However it is postulated that conformational change due to ATP binding to the protein results in the formation of a translocation pathway, known as ‘powerstroke’. Finally the affinity of the substrate decreases with the protein binding site and substrate releases (Hollenstein et al., 2007).

1.3.2 Major facilitator superfamily (MFS)

MFS is the largest group of secondary active transporter proteins that transduce free energy stored in an electrochemical proton gradient into a substrate concentration gradient. MFS proteins are specific for their substrates and transport a high range of compounds including monosaccharide and oligosaccharide sugars, drugs, metabolites, amino acid peptides, nucleosides and, organic and inorganic ions (Pao et al., 1998). They comprise of approximately 10000 member proteins and are present in archaea, eubacteria and eukaryotes. Almost all the members of MFS have the same
topological structure. The length of the single peptide chained proteins belonging to this superfamily range from 400 - 600 amino acids and are arranged in 12 transmembrane helixes (Saier, 2000).

Being secondary active transporters, MFS transporter proteins translocate substrates using energy released by the downhill movement of a driver solute to energise the transport of the substrate. MFS proteins may either be (i) uniporters, that transport one type of substrate in one direction driven by a concentration gradient, (ii) symporters (eg. LacY, FucP) that translocate two or more different kinds of substrates in the same direction using electro-chemical gradient of one of the substrate and (iii) antiporters (eg. GlpT) that allow movement of two or more substrates in opposite directions through a membrane (Kaback et al., 2001; Law et al., 2008).

Unique to all MFS members is the MFS signature sequence with in loops between Tm 2 / Tm 3 and Tm 8 / Tm 9. As MFS proteins transport a vast variety of substrate therefore it is obvious that MFS signature sequence does not bind to a substrate. The possible roles of MFS signature described are gating mechanisms, conformational changes and / or solute accessibility (Jessen-Marshall et al., 1995; Pazdernik et al., 1997; Pazdernik et al., 2000).

1.4 Nitrate transport systems

Nitrate and nitrite are charged molecules and therefore should face difficulty in crossing cell membranes at a rapid rate against the membrane potential that is negative inside and also against a concentration gradient. Therefore the nitrate-utilising organisms have developed active transporter proteins for nitrate and nitrite transport (Clegg et al., 2002). Nitrate transporters belong to a subfamily of MFS, the nitrate / nitrite porter family (NNP) that includes nitrate / nitrite transporters from both prokaryotes and eukaryotes (www.tcdb.org; Pao et al., 1998).

The nitrate concentration in soils is affected by seasonal and regional variations (Crawford and Glass, 1998; Hayatsu et al., 2008). Depending on the available concentration of nitrate in soil, two uptake systems have been identified, the low affinity transport system (LATS) also called NRT1, that operates when the concentration of available nitrate is more than 1 mM and the high affinity transport system (HATS) or NRT2 that functions if the concentration of nitrate in the surroundings is less than 1 mM (Glass and Siddiqi, 1995; Orsel et al., 2002). The expression of low affinity nitrate transporters is constitutive, whereas, the expression of high affinity nitrate transporter protein is nitrate inducible (Trueman et al., 1996 and references therein).
1.4.1 Nitrate transport systems in the plant model, Arabidopsis thaliana

Nitrate transport in Arabidopsis thaliana has been studied over a number of years and consequently there is a considerable body of knowledge for this process (Tsay et al., 1993; Tsay et al., 2007; Ho et al., 2009; Krouk et al., 2010b; Tsay et al., 2011). Transport of nitrate from soil to root cells is a highly efficient process in plants. Depending on the concentration of nitrate available, low affinity nitrate transporters NRT1 (operates at >1 mM nitrate) or high affinity nitrate transport systems, NRT2 (functional when concentration of nitrate is <1 mM) are functional (Glass and Siddiqi, 1995; Forde, 2000).

Low and high affinity nitrate transporter proteins in A. thaliana are encoded by the genes belonging to the gene family AtNRT1 and AtNRT2 respectively. Nitrate inducible AtNRT1.1 is a master gene and performs several important roles, (i) encodes a nitrate transporter protein, AtNRT1.1 (Tsay et al., 1993), (ii) acts as nitrate sensor and activates the expression of other nitrate metabolism related genes (Ho et al., 2009), (iii) involves in signalling processes that affect root development and seed germination (Ho and Tsay, 2010) and (iv) represses the lateral root growth by directing the transport of auxin from the roots (Krouk et al., 2010a). The AtNRT1.2 protein, another member of NRT1 protein family in A. thaliana is encoded by the gene AtNRT1.2 (Huang et al., 1999). In contrast to the AtNRT1.1 protein which is an inducible component of the low affinity nitrate transport system, expression of AtNRT1.2 protein is independent of the presence of nitrate and makes the constitutive component of low affinity nitrate transport system.

The A. thaliana AtNRT2 protein family belongs to the high affinity nitrate transport system and takes up nitrate when the concentration of available nitrate is low (Filleur et al., 2001; Orsel et al., 2004). AtNRT2.1, a member of the NRT2 protein family, carries out 72 % nitrate transport in A. thaliana. However NRT2.1 is unable to function alone and requires another protein NAR2 which has been found to be physically attached with the NRT2.1 to form a 150 kDa tetramer a functional stable form of the NRT2.1 nitrate transporter (Okamoto et al., 2006; Orsel et al., 2006; Yong et al., 2010). A disruption in the NAR2 gene results in complete loss of transport activity by inducible high affinity nitrate transport (Okamoto et al., 2006; Orsel et al., 2006). Such NRT2 and NAR2 complexes have also been reported in other plants, for example barley and rice (Glass, 2009; Ishikawa et al., 2009; Feng et al., 2011).

The AtNRT2.2 a functionally redundant protein of AtNRT2.1 makes a small contribution to nitrate transport but its activity increases when the AtNRT2.1 protein is lost or becomes non-functional (Li et al., 2007). Also within the NRT2 family five other gene members have been reported for A. thaliana, NRT2.3 to NRT2.7 although their interaction with NAR2 is unknown. Except for the AtNrt2.7 which was proposed to be involved in seed nitrate accumulation (Chopin et al., 2007), the other four proteins
NRT2.3 - NRT2.6 have not yet been characterised for their functional roles (Orsel et al., 2002; Okamoto et al., 2003). The $K_m$ value calculated by kinetic analysis for the A. thaliana high affinity nitrate transporters is $\sim 50 \mu M$ and that of low affinity transporters is $\sim 4 \text{mM}$ (Liu et al., 1999; Guo et al., 2002).

1.4.2 Nitrate transport systems in bacteria

Little is known about the nitrate transport mechanisms in bacteria. Moir and Wood (2001) suggested the involvement of ATP-dependent transporter proteins in bacteria that assimilate nitrate, and MFS nitrate transporter proteins, (homologues of NarK protein family) that transport nitrate usually for dissimilation under anaerobic conditions. It is unclear and controversial as to how the NarK protein functions (Moir and Wood, 2001; Jia et al., 2009).

In the eubacterium E. coli, two polytopic homologous redundant nitrate transporter proteins NarK and NarU encoded by the genes narK and narU respectively have been identified. Both NarK and NarU belong to the MFS superfamily and possess 12 transmembrane domains. In E. coli, both NarK and NarU proteins are capable of effluxing nitrate from the cell to support anaerobic growth (Clegg et al., 2002; Jia et al., 2009). The expression of E. coli NarK protein is induced by nitrate. Actively growing cells of E. coli show high expression of NarK. Similar to other members of narK genes, E. coli narK also forms a gene cluster with the nitrate reductase encoding gene, narGHJI. In contrast to E. coli NarK, the highest level of NarU expression was observed when the cells were in stationary growth phase or growing slowly, facing nutrient deficiency (Clegg et al., 2006). E. coli NarK and NarU not only transport nitrate but also catalyse the nitrite uptake and export (Jia et al., 2009).

In the thermophilic bacterium Thermus thermophilus two narK1 and narK2 genes that encode the nitrate transporter proteins NarK1 and NarK2 for anaerobic respiration have been reported. Both narK1 and narK2 form a gene clustered with the nitrate reductase (Ramirez et al., 2000). Similarly, in Pseudomonas aeruginosa, a denitrifying bacterium, two genes narK1 and narK2 encode nitrate/nitrite transporter proteins NarK1 and NarK2 respectively that cluster with nitrate reductase encoding gene, narGHJI (Sharma et al., 2006).

1.5 Application of the aspergilli to biological and medical research

Species of Aspergillus are potential experimental organisms and the availability of genome sequences for certain Aspergillus species have made these organisms even more attractive to researchers (Andersen and Nielsen, 2009). The various species of genus Aspergillus are ubiquitous and found as common inhabitants in the natural environment. In recent years, extensive research has been carried out on the genomics of the aspergilli resulting in the availability of fully sequenced genomes of a number of Aspergillus species with quite a few species in the sequencing pipeline. Aspergillus species that have been DNA sequenced so far are, A. fumigatus (Nierman et al., 2005; Fedorova et al., 2008),
A. flavus (Yu et al., 2005), A. oryzae (Machida et al., 2005), A. nidulans (Galagan et al., 2005) and A. niger (Pel et al., 2007).

Comparative analysis of A. nidulans genome sequence with the A. fumigatus, a serious human pathogen and A. oryzae, used in food industry to prepare miso, sake and soya sauce, revealed that over 5000 non-coding gene sequences were conserved in all the three Aspergillus species. It was also observed that similar to A. nidulans, A. fumigatus and A. oryzae also have potential sequences for a sexual life cycle although a sexual stage has not been yet observed in A. oryzae. Availability of the genome sequence of the model organism A. nidulans and other widely studied aspergilli has opened new horizons to study genome evolution and gene regulation (Galagan et al., 2005). The free open access to genome sequences of Aspergillus species resulted in diverse research efforts in the fields of transcriptome analysis, proteomics, metabolomics and metabolic modelling in Aspergillus species (Andersen and Nielsen, 2009 and references therein).

For instance, comprehensive research has been carried on proteomics and metabolomics of medically important Aspergillus species (Andersen and Nielsen, 2009 and references therein). In this regard, the aspergilli produce a number of mycotoxins (secondary metabolite) that are not thought to be necessary for their own survival. The most common mycotoxins concerned with human health are aflatoxin, sterigmatocystin, ochratoxin and gliotoxin (www.aspergillus.org.uk; Bennett and Klich, 2003 and references therein). It has also been reported that out of 250 known species of genus Aspergillus only 40 species cause infection in humans although this figure is increasing (Klich, 2006) probably with the increase in number of patients with reduced immunity.

The aspergilli (including A. nidulans) are not specialised as human pathogens rather they are opportunistic and are able to cause diseases in immune-suppressed patients (Kim et al., 1997; Latge, 1999; Lucas et al., 1999; Latge, 2001; Khanna et al., 2005; Bennett, 2009). A. fumigatus, A. flavus and A. terreus are the most significant infection-causing species of genus Aspergillus for humans (Latge, 2001; Hedayati et al., 2007; Person et al., 2010). Although A. nidulans is generally thought to have no or a very low pathogenicity, a few human A. nidulans infections have actually been reported (Kim et al., 1997; Chakrabarti et al., 2006). Details of human health risk from aspergilli will be discussed further in Chapter 3, Section 3.1.1.

1.5.1 Aspergillus nidulans - a model eukaryotic organism

Aspergillus nidulans, a filamentous Ascomycetes, is a well-established fungal model system and has been used frequently in genetics and cell biology. A. nidulans is easy to grow on solid as well as liquid minimal medium supplemented with one of a wide range of carbon and nitrogen sources. In addition, A. nidulans can grow asexually to produce conidia as well as sexually (Emericella nidulans) to generate the ascospores (Clutterbuck, 1974). Moreover, A. nidulans is a homothallic, haploid
fungus and can also be induced to carry out heterokaryosis with the formation of heterokaryons. Also genetic transformation of *A. nidulans* is now routine (Tilburn *et al.*, 1983; Yelton *et al.*, 1984; Ballance and Turner, 1985).

A number of physiological and metabolic processes have been successfully studied using *A. nidulans* as a eukaryotic model organism. For example the process of mitosis, the formation of tubulin and microtubules (Oakley, 2003; Szewczyk and Oakley, 2011) and carbon (David *et al.*, 2006; Mogensen *et al.*, 2006), nitrogen (Cove and Pateman, 1963; Pateman *et al.*, 1964; Pateman *et al.*, 1967; Cove, 1976; Brownlee and Arst, 1983; Unkles *et al.*, 1991; Unkles *et al.*, 2001) and sulphur metabolism. Currently, many laboratories all over the world from the USA to Japan are conducting research into the biology, biochemistry, genetics and applications of *A. nidulans*. Some of these laboratories are working jointly in international terms to promote and disperse knowledge derived from *A. nidulans* (www.broadinstitute.org).

Genetically, *A. nidulans* is very close to certain medically and industrially important *Aspergilli*, for example, *A. fumigatus*, *A. flavus*, *A. niger* and *A. oryzae* (*Aspergillus* Genome Database, AspGD; www.aspergillusgenome.org). Also it is quite straightforward to induce chemical and site-directed mutations in *A. nidulans* (Unkles *et al.*, 2004a; Kinghorn *et al.*, 2005 and references therein). All the above features made this fungus very attractive for scientific studies.

1.6 Nitrate transport systems in *A. nidulans*

*A. nidulans* has been used extensively to study nitrate transport and metabolism for the last six decades resulting in our fundamental understanding of this pathway. The first regulatory and structural gene involved in nitrate assimilation of *A. nidulans* was identified in 1963 by Cove and Pateman. Most of the genetic and biochemical aspects of nitrate assimilation pathway in the lower eukaryotic fungus, *A. nidulans* are similar to the higher plants so that the information can be transferred to plants. However, on the other hand nitrate reductase activity is required for nitrate transport in fungi but not for plants, highlighting a major difference between fungi and plants at least as shown in *Aspergillus* and *Arabidopsis* (Unkles *et al.*, 2004b).

The earlier conventional physiological studies of growth of *A. nidulans* mutant strains on nitrate characterised the main components of nitrate assimilation, including its regulation and inhibition by chlorate (Cove and Pateman, 1963; Pateman *et al.*, 1964; Pateman *et al.*, 1967; Cove, 1976). The gene *nrtA* (originally designated *crnA*) was identified that encodes a nitrate transporter protein, NrtA in *A. nidulans* (Brownlee and Arst, 1983; Unkles *et al.*, 1991; Unkles *et al.*, 2001).
During the asexual cycle of *A. nidulans*, unicellular haploid spores, conidia or conidiospores are produced. Upon germination, conidia form haploid homokaryons. Two homokaryons may fuse with each other to form a heterokaryon. During the parasexual cycle, nuclei of heterokaryons fuse to form a diploid. In the sexual life cycle, ascospores are formed within the asci which are enclosed in the fruiting body, the cleistothecium. This figure has been taken directly from Casselton and Zolan (2002).
It was also observed that mutant strains in the nrtA gene continue to grow on nitrate provided as the sole source of nitrogen. Also the net nitrate uptake kinetics was not straight forward. Both observations leading to the prediction of the presence of more than one nitrate uptake system. Subsequently, another nitrate transporter, NrtB, encoding gene in A. nidulans, nrtB was observed, cloned and characterised (Unkles et al., 2001). NrtA and NrtB belong to the high affinity nitrate transporter protein family and mutation in one gene does not affect the growth on nitrate as a sole source of nitrogen whereas the mutant in both genes abolishes the growth on nitrate even in the presence of up to concentrations of 200 mM nitrate. From mutant growth results and BLAST searches, it is suggested that nrtA and nrtB are the only genes involved in the nitrate transport in A. nidulans, although NrtA and NrtB are regulated in a similar manner and perform the same function.

The rate of transport and affinity of nitrate by NrtA is approximately three times higher than NrtB suggesting ecological plasticity of nitrate transport (Unkles et al., 2001).

Brownlee and Arst (1983) provided fundamental kinetic data for nitrate transport in A. nidulans and described interesting and exciting new facts regarding nitrate transport. The conclusions drawn by Brownlee and Arst were (i) nrtA1 (crnA1) mutant has several fold reduction in net nitrate uptake in conidia and young mycelium but not in older mycelium, (ii) uptake of nitrate by nrtA1 mutant suggested the presence of more than one uptake system, (iii) net nitrate uptake is strongly inhibited by chlorate, a potential structural analogue of nitrate, (iv) uncouplers inhibit nitrate uptake probably due to lack of proton gradient across the membrane, (v) net nitrate uptake and nitrate reductase are sensitive to the metabolic inhibitors, cyanide, azide and N-ethylmaleimide.

1.6.1 General characteristics of NrtA protein

nrtA, encoding NrtA protein, was the first eukaryotic high affinity nitrate transporter gene to be sequenced (Unkles et al., 1991). A 57 kDa (507 amino acids) NrtA protein, TC 2.A.1.8.5 (www.tcdb.org), belongs to the high affinity nitrate transporter family of major facilitator superfamily, MFS (Unkles et al., 1991; Forde, 2000; Unkles et al., 2001). nrtA is located on chromosome VIII forming a gene cluster with niaD (nitrate reductase encoding gene) and niiA (nitrite reductase encoding gene) in a gene order nrtA-niiA-niaD (Tomsett and Cove, 1979; Johnstone et al., 1990). Similar to other members of the MFS, the NrtA protein contains 12 putative transmembrane domains (Tms). These hydrophobic Tms of NrtA are present in the form of α-helices that pass through the membrane in a zigzag manner and are connected by hydrophilic loops. Both C- and N-terminal ends of NrtA are present most likely inside the cytoplasm. It was also proposed that the Tms form the boundary of hydrophilic cavity for the transport of nitrate (Unkles et al., 1991; Trueman et al., 1996; Kinghorn et al., 2005).
The MFS signature motif, GXXXNXXGXR, is present in NrtA located near the loop between Tm 2 and Tm 3 with another copy around loop between Tm 8 and Tm 9. The NrtA protein has another conserved motif, AAGXGNXGGG, unique to all members of the high affinity nitrate transporters and referred to as the nitrate signature sequence (NS). Two copies of nitrate signature are present in NrtA protein, NS1 located in Tm 5 (amino acid residues 163-172) and NS2 in Tm 11 (Trueman et al., 1996; Forde, 2000). The occurrence of the two copies of MFS motif and the nitrate signature, one copy of each in the first six Tms and the second in second six Tms in particular positions, support the notion that duplication of the first six Tms took place during the course of evolution (Pao et al., 1998). These two halves of NrtA proteins are joined by a large substantial central loop between Tm 6 and Tm 7 (Kinghorn et al., 2005) (Figure 1.3). However it has also been observed that when only one half of the protein was made (6 Tms) no transport of nitrate was recorded.

Besides conserved MFS and nitrate signature motifs, a number of amino acid residues and motifs are highly conserved between nitrate transporter proteins. Kinghorn and colleagues (2005) identified sixteen highly conserved amino acid residues among 52 nitrate transporters from archaeabacteria to plants and A. nidulans NrtA. The residues that were conserved among all 52 protein sequences are R87 (Tm 2), F140 (Tm 5) G157 (Lp Tm 4/ Tm 5), G170 (Tm 5), Y323 (Tm 7) G328 (Tm 7), R368 (Tm 8), G371 (Tm 8), G372 (Tm 8), D376 (loop Tm 8 / Tm 9) and G452 (Tm 11). Among these highly conserved residues, R87 and R368 are essential for substrate binding (Unkles et al., 2004a). Many residue alterations of NrtA identified residues that play an important role in nitrate transport (Kinghorn et al., 2005). Mutational analysis of the nrtA gene helped us to understand its structure and/or function. Whereas NrtA alteration in certain amino acid residues showed wild type growth and NrtA wild type protein expression levels, changes in other residues resulted in loss-of-function. For example when highly conserved arginine residues, R87 (Tm 2) and R368 (Tm 8) were replaced with a number of amino acids (Unkles et al., 2004a; Kinghorn et al., 2005), only a lysine replacement in R386 was tolerated with the mutant R368K having a significant decrease in $K_m$ value for nitrate (Unkles et al., 2004a).

1.6.2 The NrtB nitrate transporter

The 53.7 kDa NrtB transporter protein (497 amino acid residues) encoded by the gene nrtB is also a member of MFS high affinity nitrate transporter protein family with 12 putative $\alpha$-helical transmembrane domains. The genomic DNA sequence of nrtB identified four putative introns out of which three are on the same positions as on nrtA gene however one intron is unique to nrtB. The NrtB protein is 76% similar and 61% identical to NrtA (Figure 1.4). One potential NirA (nitrate induction protein) binding sequence (CCGCAGGAG) and 10 possible AreA (nitrogen metabolite repression protein) binding sequence (GATA) are present. The kinetics characterisation of NrtB for nitrate
transport shows that this protein has $V_{max} \sim 150$ nmol/mg DW/h and the $K_m \sim 10$ µM (Unkles et al., 2001), a very different to NrtA.

Figure 1.3: Putative secondary structure of *A. nidulans* NrtA.

Yellow dashes enclose the MFS signature motifs and blue surround the nitrate signature sequence. The residues highlighted in red are highly conserved as predicted by the alignment of 52 available NrtA homologue sequences. The figure and legends have been adapted from Kinghorn et al. (2005).

1.7 Nitrite transport by *A. nidulans*

Interestingly, although the double mutant (*nrtA, nrtB*) results in growth abolition on nitrate as the sole source of nitrogen, the double mutation did not affect growth on nitrite thus suggesting the presence of nitrite transporter(s) and that it is incapable of nitrate transport. The NitA protein, encoded by the *nitA* gene, was identified as a sole nitrite transporter in *A. nidulans* ($K_m \sim 4.2$ µM and $V_{max} \sim 170$ nmol/mg DW/h).

Standard growth tests of a *nitA* mutant (*nitA16*) on nitrite provided a clue as to whether or not NrtA, NrtB participates in nitrite transport. No growth of the triple mutant (*nrtA, nrtB, nitA*) on nitrate as well as on nitrite supported the hypothesis that NrtA, NrtB and NitA are the only proteins that constitute the nitrate / nitrite transport system in *A. nidulans*. Therefore in summary, NrtA and NrtB are nitrate transporters and both or at least one of them, NrtA and NrtB, had the ability to transport nitrite transport and that NitA acts as an exclusive nitrite transporter. All three transporters are nitrate inducible and ammonium repressible (Unkles et al., 2001; Wang et al., 2008a).
Figure 1.4: The amino acid sequence alignment of *A. nidulans* NrtA and NrtB nitrate transporter proteins.

| **NrtA** | MDFAKLVSPEVNPNNRKLALTIPVLMNPFDFL1GRVFFSWSWFLAIRLAFSPLVS 57 |
| **NrtB** | MKPTQVLAVAAPVQTRKARSIPVLMNPFDFL1GRVFFSWSWFLAIRLAFSPLVS 60 |

**: :** :***:*::****:**::*:*** :********:********:**::*******:**:::******* : 64

| **NrtA** | TVTIRDDLMSQTQIANSNIIALLATLLVRICGPLCDRFGPRLVFIGLLILGAVPTAMA 117 |
| **NrtB** | MKPTQVLAVAAPVQTRKARSIPVLMNPFDFL1GRVFFSWSWFLAIRLAFSPLVS 120 |

**: :** :***:*::****:**::*:*** :********:********:**::*******:**:::******* : 123

| **NrtA** | GLVTSPQGLIALRFFIGILGGTFVPCQVWCTGFFDSIVGTANSLAGCGAGGITYFV 177 |
| **NrtB** | GLVTSPQGLIALRFFIGILGGTFVPCQVWCTGFFDSIVGTANSLAGCGAGGITYFV 180 |

**: :** :***:*::****:**::*:*** :********:********:**::*******:**:::******* : 183

| **NrtA** | MPAIFDSLIRDPQLPAHRVAVVFILVAAALQMLFTCDPTPGKWERMKEVW 237 |
| **NrtB** | MPAIYDSFVHGRLTHKAVSVYIFVIIIVSIALMLFTCPDPTPGKWADR-------- 233 |

**: :** :***:*::****:**::*:*** :********:********:**::*******:**:::******* : 239

| **NrtA** | TVTAKSNIVDLSSQGSSRPSSPSIIIAYAIIPDVEKGTETPLGQSAIQGFDARAN 297 |
| **NrtB** | -EKTSGQSIVDLSSTPNASSN-SINISSDEKKHVPEVTDESEQVNHRA-QUESSS- 289 |

**: :** :***:*::****:**::*:*** :********:********:**::*******:**:::******* : 299

| **NrtA** | AVATPSKRSKAVNVSPLATVACVAYCSFGEILSISILGAYLLNFPPLGQTQGTA 357 |
| **NrtB** | VIEAPTIKRLSALDSALVAVYPCSFGEALAINSILGAYLLNFPPLGQTQGTA 349 |

**: :** :***:*::****:**::*:*** :********:********:**::*******:**:::******* : 359

| **NrtA** | AMPFLNIVCPSAGFLADFLYRNTPWAKLKLSSFLVVMGAMAFAMGFSDKSEMT 417 |
| **NrtB** | SMFLNIVCPSAGFLADFLYRNTPWAKLKLSSFLVVMGAMAFAMGFSDKSEMT 409 |

**: :** :***:*::****:**::*:*** :********:********:**::*******:**:::******* : 423

| **NrtA** | FGVTAGAFFLESCNAGIFSLLVPHVHYANGIVSGMGGFQGLGGIIIPAFRYPHYSDH 477 |
| **NrtB** | FGLVVMAPFAIAAGANFAIPVPHVPSANGIVSGYTQGMNFGGIIIPAFRYPHYSDH 469 |

**: :** :***:*::****:**::*:*** :********:********:**::*******:**:::******* : 483

| **NrtA** | RGIWILGVISMAVFVSWSWFRPVPKQNYRE 507 |
| **NrtB** | RSLWIGFIIILGCTLLFSWSRVPVPKQNYRE 497 |

The amino acid sequences of the two proteins encoded by *nrtA* and *nrtB* genes were aligned using the European Bioinformatics Institute (EBI) protein alignment tool ClustalW (www.ebi.ac.uk). The numbers on the right side represent the amino acid residues. The amino acid residue motifs highlighted in blue denote nitrate signature sequence and those highlighted with yellow are the copies of MFS motif. Conserved arginine residues (R87 and R368 in NrtA) are highlighted in pink. ‘*’ is used for the conserved residues, ‘+’ indicates the conserved substitution of residues and ‘.’ is for semi conserved substitution of residues.
Recently *A. nidulans* NitA, the high affinity nitrite transporter and a member of the formate / nitrite transporter (FNT) family has been characterised using the tracer $^{13}$NO$_2^-$ (Unkles *et al.*, 2011). Kinetic studies of NitA indicated that the $K_m$ is $4.8 \pm 8$ µM and $V_{\text{max}}$ $228 \pm 49$ nmol/mg DW/h for nitrite. Unkles and colleagues also presented two and three dimensional models of NitA protein. In this respect, asparagine residues, N122 (Tm 3) and N246 (Tm 6) are highly conserved in the FNT family while N173 (Tm 4) and N214 (Tm 5b) were found to be present in the 80 % homologues of NitA (Finn *et al.*, 2008). Mutational analysis of conserved asparagine residues N122 (Tm 3), N173 (Tm 4), N214 (Tm 5b) and N246 (Tm 6) revealed that N122 and N246 are irreplaceable. However the replacement of asparagines at position 214 with other negatively charged residue, aspartate resulted in wild type growth. N173 was found to be tolerant to smaller side chain amino acid replacements (Unkles *et al.*, 2011).

1.8 Nitrate assimilation

Fungi play a critical role in the nitrogen cycle in the soil and are able to assimilate both organic (amino acids and nucleotides) and inorganic (nitrate, nitrite and ammonium) nitrogen compounds present in the soil. Fungi have well developed metabolic systems for the assimilation of nitrate (as well as nitrite) to ammonium and then ammonium assimilation to glutamine and glutamate (reviewed in Cove, 1979 and references therein). Once nitrate is transported into the cell by nitrate transporters, it is reduced to nitrite by nitrate reductase and then to ammonium by nitrite reductase. In organisms that perform nitrate assimilation, nitrate induces the expression of nitrate reductase and nitrite reductase encoding genes in fungi and plants (Quesada and Fernandez, 1994; Perez *et al.*, 1997; Krapp *et al.*, 1998; Zhuo *et al.*, 1999; Vidmar *et al.*, 2000; Unkles *et al.*, 2001; Clegg *et al.*, 2002).

The genes encoding the nitrate transporter(s), nitrate reductase and nitrite reductase may be present in operon(s) in prokaryotes (Omata *et al.*, 1993; Lin *et al.*, 1994). In certain lower eukaryotic organisms; for example, *Aspergillus nidulans* (Johnstone *et al.*, 1990), *Hansenula polymorpha* and *Chlamydomonas reinhardtii* (Perez *et al.*, 1997) the genes are clustered but do not form operons. And thus far there is no clear evidence of nitrate assimilation gene clustering in plants.
Figure 1.5: Secondary and tertiary structure models of *Aspergillus nidulans* nitrite transporter, NitA.

(a) Secondary structure model of NitA based on crystal structure of *E. coli* formate transporter, FocA. Tms are rainbow coloured from blue (N-terminal) to red (C-terminal). Conserved asparagine residues are outlined in black. (b &c) tertiary structure model of NitA viewed from the plan of membrane (c) same tertiary structure model of NitA showing relative positions of conserved asparagine residues. Figure and legends have been taken directly from Unkles et al. (2011).

1.9 Dissimilatory nitrate reduction

Most of the eukaryotic organisms are aerobic and require oxygen for their growth and survival. However, in contrast with higher eukaryotes, some yeasts and filamentous fungi have evolved to anaerobic environments (Tsuruta *et al*., 1998; Takaya, 2009 and references therein). In such organisms a set of genes is expressed under the hypoxic or anoxic condition to support anaerobic growth and survival (Zitomer and Lowry, 1992; Zhou *et al*., 2002; Takasaki *et al*., 2004b). Dissimilation of nitrate usually occurs in filamentous fungi either by denitrification or ammonium fermentation under oxygen stress conditions to produce energy. *Fusarium oxysporum* (Zhou *et al*., 2002) and *A. nidulans* (Takasaki *et al*., 2004b) are fungal example that such organisms can respire...
nitrogen in anaerobic environments. Dissimilation of nitrate is unusual for eukaryotes and such processes are typically present in bacteria (Nakahara et al., 1993).

1.9.1 Fungal denitrification
Denitrification originally discovered and characterised in bacteria, is a sequential anaerobic process composed of a series of reduction reactions under hypoxic or low oxygen conditions resulting in generation of ATP. During denitrification, nitrate or nitrite is reduced to gaseous nitrogen such as nitrous oxide (N\textsubscript{2}O) or dinitrogen (N\textsubscript{2}). The reduction process of nitrate to nitric oxide occurs in mitochondria (Takasaki et al., 2004a; Takasaki et al., 2004b; Takaya, 2009).

The reduction of nitrate in fungal denitrification occurs by ubiquinol-Nar, nitrate reductase. This process takes place in mitochondria and is coupled to ADP phosphorylation. Fungal Nar shares the similar properties with the bacterial Nar, for example inhibition by tungstate, an inhibitor of the molybdenum enzyme, nitrate reductase (Uchimura et al., 2002). However fungal Nar is evolutionary distant from the bacterial Nar and also bacterial Nar is localised in cell membrane. However it is noteworthy that most denitrifying fungi lack Nar and are unable to reduce nitrate. Instead the denitrification substrate for such fungi is nitrite which is reduced to nitric oxide by Nir, nitrite reductase (Tsuruta et al., 1998). The steps involved in fungal denitrification are shown in Figure 1.6. The enzyme that catalyses each step is indicated.

![Figure 1.6: Schematic representation of fungal denitrification.](image)

Nitrate (NO\textsubscript{3}\textsuperscript{−}) is reduced to nitrite (NO\textsubscript{2}\textsuperscript{−}) by nitrate reductase (Nar), the resulting nitrite is reduced to nitric oxide (NO) by nitrite reductase (Nir), and by nitric oxide reductase (Nor) finally reduced to nitrous oxide.

1.9.2 Fungal ammonium fermentation
While denitrification occurs under hypoxic conditions, ammonium fermentation takes places in the complete absence of oxygen (Lockington et al., 1997). Zhou et al. (2002) studied enzymes involved in fungal ammonium fermentation. Alcohol dehydrogenase (Adh), aldehyde dehydrogenase (Add) and acetate kinase (Ack) were identified for the oxidation of ethanol to acetate. This catalytic step is coupled with ATP generation and the reduction of nitrate to ammonium. The reaction steps in fungal ammonium fermentation with their catalytic enzymes are summarised in Figure 1.7.

Takasaki and colleagues (2004b) identified ammonium fermentation in \textit{A. nidulans}. In \textit{A. nidulans} during ammonium fermentation ethanol is used as the carbon source. This ethanol oxidation results in the formation of acetate with the generation of ATP (Figure 1.7). It was also observed that the strains
mutant in either *niaD* or *niiA* gene failed to oxidise the ethanol. Therefore it was concluded that *A. nidulans* has adapted dissimilation process utilising assimilatory enzymes.

Figure 1.7: Schematic representation of fungal ammonium fermentation.

\[
\begin{align*}
\text{NO}_3^- & \xrightarrow{\text{NiaD}} \text{NO}_2^- & \text{NADH} & \rightarrow \text{NAD}^+ \\
\text{NO}_2^- & \xrightarrow{\text{NiiA}} \text{NH}_4^+ & 3\text{NAD}^- & \rightarrow 3\text{NAD}^+
\end{align*}
\]

Enzymes involved in ethanol fermentation by *A. nidulans* are alcohol dehydrogenase (Adh), aldehyde dehydrogenase (Add) and acetate kinase (Ack). This figure is adapted from Takaya, (2009).

1.10 Nitrate assimilation and *A. nidulans*

*Aspergillus nidulans* has been the host for over five decades to study nitrogen assimilation and such investigations have contributed significantly to our knowledge regarding the genes, enzymes and transporters involved in nitrogen assimilation (Figure 1.8). In the cytoplasm, nitrate is reduced to nitrite by nitrate reductase (NR) and nitrite reductase (NiR) to ammonium. These two enzymes, NR and NiR, are encoded by the genes *niaD* and *niiA* respectively (Cove and Pateman, 1963; Pateman et al., 1964; Pateman et al., 1967; Johnson et al., 1980; Brownlee and Arst, 1983; Millar et al., 2001; Unkles et al., 1997; Unkles et al., 2001; Heck et al., 2002; Unkles et al., 2004b; Wang et al., 2008a; Takaya, 2009; Schinko et al., 2010; Unkles et al., 2011).

Figure 1.8: A simplified illustration of the nitrate assimilation pathway in *A. nidulans*.

\[
\begin{align*}
\text{NO}_3^- & \xrightarrow{nrtA} \text{NO}_3^- & \xrightarrow{nrtB} \text{NO}_3^- & \xrightarrow{niaD} \text{NO}_2^- & \xrightarrow{niiA} \text{NH}_4^+
\end{align*}
\]

Nitrates permeates into the cell by two transporters NrtA and NrtB encoded by *nrtA* and *nrtB* genes respectively. Inside the cell, nitrate is reduced to nitrite by nitrate reductase which is encoded by *niaD* gene (Cove and Pateman, 1963) and the resulting nitrite is reduced to ammonia by the gene product of *niiA* (Pateman et al., 1967), the nitrite reductase. This figure has been adapted from Kinghorn and Unkles, (1994).
Two further genes, \textit{nirA} and \textit{areA} are crucial for the regulation of structural genes \textit{niaD}, \textit{niiA}, \textit{nrtA}, \textit{nrtB} and \textit{nitA} of nitrate assimilation. The regulatory gene \textit{nirA} encodes the NirA protein that is involved in nitrate induction for the expression of \textit{niaD}, \textit{niiA}, \textit{nrtA}, \textit{nrtB} and \textit{nitA} genes. The expression of NirA itself is constitutive and therefore does not depend on the presence (or absence) of nitrate (or any other nitrogen source) \cite{Burger1991, Strauss1998}. The protein AreA, encoded by the regulatory \textit{areA} gene, is involved in ammonium repression (or nitrogen metabolite repression) and represses \textit{niaD}, \textit{niiA}, \textit{nrtA}, \textit{nrtB} and \textit{nitA} expression (and many other genes involved in nitrogen break down pathways) if ammonium or glutamine is present in the medium to conserve energy \cite{Kudla1990, Bloom1992, Strauss1998, Schinko2010 and references therein}.

The NirA protein encoded by \textit{nirA} gene exerts positive regulatory control mechanism. The \textit{nirA} binding sequence is CTCCGTGG in structural gene promoters \cite{Punt1995}. The positively acting AreA protein encoded by a regulatory \textit{areA} gene binds to GATA-binding sequences \cite{Punt1995, Marzluf1997 and references therein}.

\subsection*{1.11 Molybdenum cofactor biosynthesis in \textit{A. nidulans}}

The molybdenum cofactor is the essential and common component of several enzymes that catalyses one of a number of biochemical reactions in carbon, nitrogen and sulphur metabolism. Molybdenum cofactor is ubiquitous from bacteria to humans \cite{Rajagopalan1996, Heck2002, Schwarz2009}.

For the first time, in \textit{A. nidulans}, such a cofactor was reported by Cove and Pateman in 1963 \cite{Pateman1964} when they isolated a number of nitrate reductase defective mutants and observed that certain mutants simultaneously lost xanthine dehydrogenase activity. These results were interpreted by the presence of a common cofactor for nitrate reductase and xanthine dehydrogenase and the strains referred to as ‘\textit{cnx}’ mutants.

A number of genes, \textit{cnxABC}, \textit{cnxE}, \textit{cnxF}, \textit{cnxG} and \textit{cnxH} were identified in \textit{A. nidulans} by conventional genetic analysis \cite{Cove1979 and references therein}. After the advent of DNA sequencing methods, and with biochemical reference in certain cases to \textit{E. coli}, the \textit{A. nidulans} genes and intermediates were elucidated by Kinghorn and colleagues. The complex protein CnxABC was reported to be involved in the early step of cofactor biosynthesis and catalysing the formation of an intermediate molecule designated as precursor Z \cite{Unkles1997}. While CnxF, CnxG and CnxH catalyse several intermediate steps \cite{Appleyard1998, Unkles1999} and finally CnxE being involved in final conversion of molybdopterin to the molybdenum cofactor with the addition of molybdenum \cite{Millar2001} (Figure 1.9). Another gene, \textit{cnxJ} that encodes the
protein CnxJ has also been reported associated with regulation of the level of molybdenum cofactor in *A. nidulans* (Arst *et al.*, 1982 and references therein).

*A. nidulans* CnxE (encoded by *cnxE* gene) consists of two domains, one has high similarity with the *E. coli* protein MoeA and other one with MogA (Millar *et al.*, 2001; Heck *et al.*, 2002). The CnxE protein has homologues in *Drosophila melanogaster* (cinnamon), rat (gephyrin) and *Arabidopsis thaliana* (Cnx1). Similar to CnxE, all orthologues have fused MogA-like and a MoeA-like domains. The orientation of MogA-like and MoeA-like domains is reverse in Cnx1 as compared to CnxE, cinnamon and gephyrin (Stallmeyer *et al.*, 1995; Stallmeyer *et al.*, 1999; Heck *et al.*, 2002; Schwarz *et al.*, 2009). The illustration in Figure 1.10 represents the comparison of molybdenum cofactor biosynthesis genes and steps involved in the different groups of organisms.

Figure 1.9: Schematic overview of molybdenum cofactor biosynthesis pathway in *A. nidulans*.

![Diagram of molybdenum cofactor biosynthesis pathway](image)

Steps in cofactor biosynthesis catalysed by *cnx* genes. This figure has been taken directly from Millar *et al.* (2001).
This is a generalised scheme of molybdenum cofactor (MoCo) synthesis pathway. In eukaryotes, MoCo synthesis is a four steps pathway. In \textit{E. coli} (prokaryotes) an additional step occurs in which with the addition of GTP to MoCo, molybdenum guanine dinucleotide (MGD) is formed. Proposed (first) step and partially characterised (second) step intermediates are indicated in parenthesis. Different proteins that catalyse the each step are highlighted with different colours while similar colour is applied to proteins / domains involved in nucleotide transfer. Homologous protein nomenclature is as, cnx, plants; Mo, \textit{E. coli} and MOCS, gephyrin. The grey box encloses the separate pathway of molybdopterin (MPT) synthesis. The figure and legends were reproduced from Schwarz \textit{et al.} (2009).
1.12 Structures and mechanisms of membrane transporter proteins

Uhl and Hartig, (1992) used the term ‘transporter explosion’ for the increasing knowledge of transporter proteins and these words proved to be accurate with the availability of sequenced genomes of a number of organisms. Phylogenetic analysis demonstrated the independent evolution of membrane transporter proteins (Saier, 2000; Busch and Saier, 2002). However the available crystal structures of MFS membrane transporter proteins show that they share the same architecture (Abramson et al., 2004). If the folding pattern of a protein of unknown crystal structure is the same with that of a protein with known structure, a three dimensional model of protein can be generated on the basis of known protein structure as the template (Dahl et al., 2004).

In comparison with water soluble proteins, the number of membrane proteins that have been crystallised so far is low because of two main reasons (i) their hydrophobic nature and (ii) low expression in nature. Recent advances in protein overexpression techniques have been encouraging. However crystallisation of membrane proteins due to overexpression has been successful in bacteria.

Purification and crystallisation of membrane proteins is especially challenging. Only a few membrane transporter proteins from MFS group have been crystallised. The proteins for which crystal structures are available include E. coli lactose transporter, LacY in inward facing conformation (Abramson et al., 2003; Guan et al., 2007; Chaptal et al., 2011), antiporter of inorganic phosphate and glycerol-3-phosphate, GlpT of E. coli (Huang et al., 2003), the multidrug transporter protein, EmrD also in occluded state (Yin et al., 2006). The crystal structure of E. coli FucP, the fucose/H+ symporter has also been solved in an outward open conformation (Dang et al., 2010). The crystal structure of PepT_So, the mammalian homologue of the peptide transporter protein in Shewanella oneidensis has solved in occluded state (Newstead et al., 2011). Finally the two dimensional structure of Oxalobacter formigenes oxalate-formate transporter, OxIT (Hirai et al., 2002).

From the crystal structures available, it was obvious that members of MFS proteins share similar even super-imposable structures. Another architectural feature is the presence of a central hydrophilic pore that harbours the substrate binding site. Access to this central cavity alternates from the outside to the inside of the cell and vice versa, is made possible by flexible movement of the transmembrane domains. This mechanism of substrate translocation in and out of the cell is called the alternating access model and is illustrated in Figure 1.11 (Huang et al., 2003; Abramson et al., 2004; Law et al., 2008).
Figure 1.11: The alternating access model of substrate translocation.

Schematic representation of the alternating access model for a MFS antiporter. (a-e) represent the stages of translocation of two different substrates moving in opposite directions. The N-terminal six Tms bundle of the membrane protein is indicated with blue coloured barrels while C-terminal half of transporter protein is represented as red barrels. Both N- and C-terminal halves are connected by the cytoplasmic loop. The two different substrates are represented as small black and yellow circles. (a) the C- and N-terminal bundles move away from each other and open towards periplasmic side, (b) substrate molecule (black) enters in the central cavity and both halves of protein close the central cavity from the periplasmic side (c) both bundles now open towards cytoplasmic side and the substrate enters in the cytoplasm, (d) the other substrate (yellow) enters in the central cavity, (e) Tm bundles close the cavity from the cytoplasmic side and (e) the cavity opens towards the periplasm to transport the substrate out of the cell. This figure is based on Huang et al. (2003) and Abramson et al. (2004).

The lactose permease (LacY) catalyses the coupled translocation or symport of β-galactosidase and H⁺ in E. coli. LacY is encoded by gene LacY, the first membrane transport protein encoding gene that was DNA sequenced (Buchel et al., 1980). LacY, the most extensively studied MFS protein has proved to be a model to study transport mechanisms in other member proteins of this superfamily. The LacY contains 417 amino acids with a 46 kDa molecular weight.

Crystal structures of (i) wild type LacY (Guan et al., 2007) and mutant LacY (Abramson et al., 2003; Mirza et al., 2006; Chaptal et al., 2011) has been determined (Figure 1.12). These crystal structures of LacY show that it consists of 12 α-helical transmembrane domains arranged in two pseudosymmetrical bundles, N-terminal and C-terminal bundles, each with six helices. These N- and C-terminal bundles surround a large central cavity. Most of the residues involved in the sugar binding (E126 and R144) are present in the N-terminal bundle and residues important for the translocation of H⁺ (E269 and R302, H322 and E325) are present in the C-terminal bundle (Abramson et al., 2003).

Glycerol-3-phosphate is an antiporter (GlpT) of inorganic and organic phosphate while the energy provided by an inorganic phosphate ion gradient. The 3.3Å crystal structure of GlpT (Figure 1.13) in
the absence of substrate, has contributed to answering many fundamental questions especially with regard to the characteristics of substrate binding sites and conformational changes that take place due to substrate binding and translocation. Similar to the structure of LacY, both C-terminal and N-terminal halves of GlpT consists of six helices that are connected by a central loop, again between Tm 6 and Tm 7 (Huang et al., 2003).

The crystal structure of *E. coli* FucP, the fucose/H⁺ symporter transporter, at the resolution of 3.1 Å has been solved and in an outward open conformation (Figure 1.10). Although both LacY and FucP are sugar transporters, both these proteins share very low residue sequence similarity. However the crystal structure of both transporters can be superimposed indicating similar basic protein architecture. Similar to other MFS proteins, FucP transporter protein can be considered as a protein of two halves, with N- and C-terminal halves with both N- and C-termini located in the cytoplasm. Interestingly and contrary to other MFS proteins, FucP has been crystallised in the outside open conformation. A central cavity, which appears to be the translocation site of substrate, is approximately 20 Å in depth and 10 Å in diameter at the periplasmic side. The central cavity is surrounded by Tms 1, 2, 4, 5 of the N-terminal half and Tms 7, 8, 10, 11 of the C-terminal half of FucP protein (figure 1.14) (Dang et al., 2010).

Figure 1.12: Overall structure of LacY

This figure is based on the crystal structure of the C154G mutant LacY. (a) Ribbon representation of LacY is parallel to membrane. The N-terminal Tms are shown in blue and C-terminal domains in red. The substrate β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG) is represented by black spheres. (b) A ribbon representation of LacY as viewed from the cytoplasmic side. The Tms are numbered and coloured according to rainbow pattern from Tm 1 purple to Tm 12 pink. This Figure and legends are reproduced directly from Abramson et al. (2004).
Figure 1.13: Overall structure of GlpT.

(a) Ribbon representation of GlpT viewed parallel to the membrane. The Tms in the N-terminal half of the protein are shown in blue and that of in C-terminal domain in red. (b) Ribbon representation of GlpT viewed from cytoplasmic side. The Tms are numbered and are coloured with rainbow scheme from Tm 1 purple to Tm 12 pink. The Figure and legends are taken directly from Abramson et al. (2004).

Figure 1.14: Overall structure of FucP.

(a) A ribbon representation of FucP. (b) FucP viewed in surface electrostatic potential to expose the central cavity. (c) FucP viewed from the periplasmic side in surface electrostatic potential. The figure and legends reproduced directly from Dang et al. (2010).
1.13 Cysteine scanning mutagenesis

In the absence of a crystal structure as a consequence of difficulties experienced in membrane proteins purification and crystallisation, biochemical methods combined with biophysical studies have been used with some success to explore the structure as well as functional mechanism of such polytopic membranes (Voss et al., 1997).

Biochemical studies may be used to identify the accessibility of intramembrane residues to the lipid phase of membrane (Yan and Malony, 1995) and the arrangement of transmembrane domains (Falke et al., 1997). Cysteine scanning mutagenesis can be applied successfully to study static as well as dynamic aspects of protein structure and function. Such scanning of cysteine mutants can only be performed in a cysteine-less protein background. Few proteins are devoid of cysteine and therefore natural cysteine residues must be altered to other acceptable residues i.e. do not lead to the abolition of activity. Small neutral amino acids such as alanine or serine are usually assessed initially. In this cysteine-less backbone, cysteines are then introduced one by one to create a cysteine strain library. Such a library of single cysteine mutants allows the study of accessibility of cysteine residues, at known position in the membrane protein, to thiol-reactive reagents. In this respect a library of single cysteine mutants has been used for structure and functional experimentation of transporter of interest (Frillingos et al., 1998; Kaback et al., 2001; Ermolova et al., 2003; Zhang et al., 2003; Ermolova et al., 2006; Guan and Kaback, 2007; Wang et al., 2008b).

1.13.1 Cysteine scanning mutagenesis of LacY

Before the various crystal structures of LacY have been resolved, biochemical and biophysical studies on various mutants of LacY have provided the significant information about the structure as well as function of this sugar transporter.

In the absence of a crystal structure, extensive cysteine scanning mutagenesis of almost every residue position in LacY was carried out (Frillingos and Kaback, 1996; Frillingos and Kaback, 1997; Venkatesan et al., 2000a; Venkatesan et al., 2000b; Venkatesan et al., 2000c; Kwaw et al., 2001; Zhang et al., 2003; Ermolova et al., 2003; Ermolova et al., 2006). The findings resulted in the creation of a three dimensional model (that now is in agreement with the subsequent crystal structure of LacY protein) with alternating access model (Abramson et al., 2003). Following the successful cysteine scanning mutagenesis attempts for LacY, this powerful technique was extrapolated to other MFS transporter proteins including NrtA.
1.13.2 Cysteine scanning of NrtA

Unkles et al. (2005) generated a cysteine-less but functional construct of *A. nidulans* NrtA protein by site-directed mutagenesis. They replaced all the eight native cysteine residues after determining the essentiality of each cysteine in nitrate transport by NrtA protein. These eight cysteine residues were C90, C94 (Tm 2), C143, C147 (Tm 4), C219 (Tm 6), C325 (Tm 7), C367 (Tm 8) and C431 (Tm 10). The cysteine-less construct with replacements C90A, C94A, C143A, C147T, C219S, C325S, C367S, and C431S was selected from a series of constructs with various combinations of mutations, on the basis of the highest nitrate transport activity (40% of wild-type) with protein expression levels approximately similar to the wild-type strain. This construct was also driven by the *A. nidulans* highly expression promoter from GPD and was designated as pGPDAAT. The kinetic analysis of nitrate transport by cysteine-less transformants showed that the *K*ₘ for nitrate of cysteine-less strain was very similar to the wild type strain. However and as expected, the *V*ₘₐₓ was decreased to one third of the wild type strain velocity.

1.14 Thiol cross-linking of double cysteine mutants

To obtain insights into the mechanism of protein transport, it is important to determine (i) helix packing, (ii) identify critical residues involved and (iii) inter-relationship including the role of essential (critical) in transport (Guan et al., 2001). To study membrane proteins helix packing, the thiol cross-linking method can be employed (Sorgen et al., 2002).

Disulfide bond formation or homobifunctional thiol cross-linking between cysteine residues of double cysteine mutant is a useful method for determining tertiary and quaternary configuration of polytopic membrane proteins (Fasold et al., 1971; Peters and Richards, 1977; Yu et al., 1995). Bifunctional chemical cross-linkers of known arm lengths are actually molecular rulers that can be used as a tool for distance determination between cross-linked cysteine residues. Such intra-molecular and intermolecular cross-linking of proteins is used in the structural and functional characterisation of proteins. Such information is especially valuable to map protein topography when the crystal structure of a protein is not available (Swaney, 1986; Kwaw et al., 2000).
Figure 1.15: Structures of some homobifunctional thiol cross-linkers.

(a) Bis (maleimido) ethane, BMOE; (b) bis (maleimido) hexane, BMH; (c) 4-di-(3’ –[2’ – pyridyldithio] propionamido) butane, DPDPB. The distance between the thiol reactive groups on either end of the molecule has been shown in Å. All these three examples of homobifunctional thiol cross-linkers are flexible. These structures of thiol specific cross-linkers have been reproduced from www.piercenet.com, the supplier of cross-linkers used in this present study.

1.14.1 Thiol-cross-linking studies in LacY

Thiol cross-linking combined with factor Xa proteolysis has been applied to a number of proteins including LacY to determine helix packing, tilts and conformational changes due to substrate binding (Kwaw et al., 2000; Kaback et al., 2001; Sorgen et al., 2002). Thiol cross-linking was used to identify (i) residues that were exposed to the LacY lipid bilayer (Guan et al., 2001) and to confirm (ii) charge pairing of certain residues (Wolin and Kaback, 2000; Zhang et al., 2002). A further study of LacY, provided evidence that during substrate binding, the loops between Tm 4 / Tm 5, Tm 8 / Tm 9 and Tm 10 / Tm 11 undergo conformational alterations (Kwaw et al., 2000) in movement of Tm 6 and Tm 8, Tm 5 and Tm 8. These Tms come closer upon substrate binding without affecting the relative distance between Tm 5 and Tm 6 (Guan et al., 2001).

X-ray crystallography of LacY permease (inward conformation) revealed the completely closed central cavity on the periplasmic side. It was also demonstrated that essential residues for sugar and H⁺ transport are located on the apex of the central cavity and Tms 1 and Tm 2 from the N-terminal and Tm 7 from C-terminal half of the protein seal the central cavity from the periplasmic side. Zhou and colleagues (2008) studied thiol cross-linking of three double cysteine functional mutants
(I40C/N245C, T45C/N245C and I32C/N245C) present at the interface of the Tm 1 / Tm 2 and Tm 7 at the periplasmic side of permease. The results indicated that after cross-linking with rigid homobifunctional thiol reagents less than 15 Å in length, completely blocked transport activity. In contrast flexible cross-linking reagents longer than 15 Å in length retained partial or full transport activity. These results gave roots to the theory of opening and closing of a large periplasmic cavity during substrate translocation in LacY permease.

Distances approximated from the thiol cross-linking of double cysteine mutants also provided information on the mechanism of LacY translocation via the alternating access model. Finally it was reassuring that most distances between residues, determined by thiol cross-linking of double cysteine mutants in LacY agreed with the results obtained by X-ray crystallography (Abramson et al., 2003).

1.15 Membrane protein crystallisation

Membrane proteins are the sites of certain fundamental biological processes that catalyse the transport of materials across the cells and may also act as signal receptors in living organisms (Michel, 1983). Therefore it is essential for academic and fundamental research to have detailed structural information of membrane proteins (Leviatan et al., 2010).

1.15.1 Detergent screening for membrane protein crystallisation

One major obstacle to membrane protein crystallisation is low expression (Tate, 2001). A number of protein overexpression systems have been developed for bacterial membrane proteins. Another problem is purification since membrane proteins lose their stability and natural folding when free of lipid layer of membrane. Membrane proteins are purified usually in detergents since detergents often have the ability to maintain the isolated membrane protein in its natural folded form (Prive, 2007; Newstead et al., 2008). Notwithstanding there is in fact a key difference between the lipid bilayer and detergents. In this regard, and in contrast to the lipid bilayer, detergents form micelles, small well defined structures that surround extracted membrane proteins.

n-dodecyl-β-D-maltopyranoside (DDM), n-decyl-β-D-maltopyranoside (DM), n-octyl-β-D-glucopyranoside (OG), and n-dodecyl-N-N-dimethylamine-N-oxide (LDAO) are the most commonly used detergents for membrane protein purification (Prive, 2007). The solubility of detergents in water is limited due to their amphipathic nature. Detergents can form micelles when present in a certain concentration. Critical micelle concentration (CMC), the minimum concentration of detergent required for the formation of micelles, is the characteristic property of a detergent. But the amount of detergent required to disrupt the membrane structure to obtain the protein, termed the critical solubilisation concentration (CSC), is often much higher than the CMC. For example the CMC of DDM is 0.009 % but its CSC is 1-4 %. In detergent solution, solubilised membrane proteins form
complexes with detergent called protein-detergent complex (PDC). The stability of the PDC is essential for well-ordered crystals.

In a crystal lattice of membrane protein, three components are present; detergent, protein and buffer. Unfortunately protein-detergent complexes are not always stable and disintegrate resulting in aggregation and misfolding of proteins. The larger the size of the PDC, the less stable is the crystal. Smaller micelle sized detergents, for example OG and LDAO, allow more protein-protein contacts, hence form stronger crystal lattice (Prive, 2007; Sonoda et al., 2010; Sonoda et al., 2011).

1.15.2 Solubility screening of protein detergent complex
Size exclusion chromatography (SEC) not only separates the molecules from a mixture of different molecules on the basis of their hydrodynamic volumes but also has other applications for example (i) protein purification, (ii) assessment of protein monodispersity in detergent solution (iii) determination of the molecular mass of protein and (iv) determination of protein-detergent micelle dimensions. Such characteristics make this method successful in protein purification and crystallisation (Kunji et al., 2008).

The monodispersity of protein in detergent containing solution is a reflection of its structural integrity. SDS-PAGE is the simplest technique to identify the aggregated proteins. If the protein is in an aggregated form, it should not be soluble in SDS buffer and the band of expected size would be missing or sometimes in oligomers of protein on the stained gel. However, size exclusion chromatography is the most powerful and quantitative technique to differentiate monodispersed or aggregated protein in a sample. The monodispersed membrane protein in solution tends to form a single symmetrical peak on size exclusion column (Kawate and Gouaux, 2006; Kunji et al., 2008; Sonoda et al., 2010). Fluorescence based size exclusion chromatography (FSEC) of membrane transporter protein fused with the green fluorescent protein (GFP) is an emerging technique. FSEC not only monitors the level of expression but more importantly monodispersity of detergent soluble protein (Drew et al., 2001; Drew et al., 2005; Kawate and Gouaux, 2006; Hsieh et al., 2010; Sonoda et al., 2010; Sonoda et al., 2011).
Figure 1.16: Flow chart of pre-crystallisation detergent screening of GFP fusion protein by FSEC.

The amplified gene of interest is cloned to a GFP fusion vector. The TEV protease site is in between the GFP moiety and the gene of interest for cleavage. At the other end of GFP, a His-tag is present for detection of protein by immunoblotting. The fusion construct is transformed and overexpressed in bacterial cells. Protein is extracted and solubilised in detergent containing buffer. (b) Solubilised crude membranes are loaded to SEC column which is attached to a fluorometer, UV detector and a fraction collector. The chromatograph labelled as fluorescence is a typical FSEC profile of elution protein. The UV absorbance is a representative of UV absorbance pattern in a crude cell lysate. This figure is based on Kawate and Gouaux, (2006).
1.16 Insects as infection models

Insects have been used to study the microbial pathogenicity and have replaced the use of vertebrate infection models due to similar response to infection by innate immune system (Ratcliffe, 1985; Salzet, 2001). The cuticle of the insect, a functional analogue of mammal’s skin is the first line of defence that prevent or retard the entry of pathogen in the haemocoel (body cavity) of insect. The outer waxy layer of cuticle that is composed of lipids, fatty acids and sterols is also antimicrobial in nature (Lecuona et al., 1997).

The haemolymph present in the haemocoel of an insect is the functional analogue of blood in mammals. Similar to blood, the haemolymph transports nutrients, waste materials and signal molecules but has no role in insect respiration system (Matha and Mracek, 1984). The haemolymph the main site of immune response also contains circulating cells, haemocytes, functional analogue of mammalian phagocytes and anti-microbial peptides that trap and kills invading pathogens (Morton et al., 1987; Vilmos and Kurucz, 1998; Kavanagh and Reeves, 2004).

The larvae of the Greater wax Moth, *Galleria mellonella* have been successfully used to study the pathogenicity capability of a number of microorganisms (Jander et al., 2000; Salamitou et al., 2000; St. Leger et al., 2000; Brennan et al., 2002; Reeves et al., 2004; Fuchs et al., 2010a; Fuchs et al., 2010b). *G. mellonella* belongs to insect order Lepidoptera, family Pyralidae. The significance of this insect larvae as an infection model will be discussed in detail in Chapter 3.
Chapter Two

Materials and methods

2.1 Introduction

This chapter describes the detailed procedures and methods used during this present study.

2.2 Media and supplements for Aspergillus growth

*Aspergillus* growth media and incubation conditions were used as described by Clutterbuck, 1974. *Aspergillus* strains were grown either on solid complete medium or solid / liquid minimal medium (as required). The complete agar medium was prepared by 1 % (w/v) glucose, 0.2 % (w/v) peptone, 0.1 % (w/v) yeast extract, 17.5 mM KCl, 5 mM MgSO₄, 7H₂O, 30 mM KH₂PO₄, 1 x trace elements solution (1 L 1000 x trace element solution contained, 0.4 g MnCl₂. 4H₂O, 1 g ZnSO₄, 0.5 g CuSO₄, 1.1 g Na₂MoO₄. 2H₂O, 0.5 g CoCl₂. 6H₂O, 0.5 g FeSO₄. 7H₂O, 1 g HBO₃, 3.72 g citric acid, pH 6.5). 1 x vitamin solution was also added to complete medium (the stock of 1 L 1000 x vitamin solution was prepared by 0.1 g *p*-amino benzoic acid, 0.5 g pyridoxine, 0.002 g biotin, 0.2 g pantothenic acid and 1 g riboflavin), 0.1 % (w/v) casamino acids, 1.2 % agar.

*Aspergillus* minimal growth medium (Cove, 1966) was prepared from 1 % (w/v) glucose, 1 x trace elements solution, 17.5 mM KCl, 5 mM MgSO₄, 7H₂O, 30 mM KH₂PO₄ and in case of solid medium 1.2 % agar, pH adjusted to 6.5. Nitrogen source was added to minimal medium as required.

2.3 Pathogenicity testing

The caterpillars of greater wax moth, *Galleria mellonella* were purchased from the supplier (Livefood, Somerset, UK) and used on arrival. A group of 30 healthy larvae of approximately similar size and without any grey or black markings (showing ill health or damage) on the body was selected for each treatment and transferred to a plastic Petri dish. Details of the *Aspergillus* strains tested for their pathogenesis in this present study are listed in Table 2.1.
Table 2.1: *Aspergillus* species examined for pathogenicity.

<table>
<thead>
<tr>
<th>Aspergillus species</th>
<th>Strain number</th>
<th>Origin</th>
<th>Source</th>
</tr>
</thead>
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<td>STA171</td>
<td>University of St Andrews, UK</td>
<td>Environment</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td>C51</td>
<td>School of Biological Sciences, University of Birmingham, UK</td>
<td>Environment</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td>C49</td>
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<td>Environment</td>
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<td>Department of Medical Mycology, University of Glasgow, UK</td>
<td>Human infection</td>
</tr>
<tr>
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<td>N/A</td>
<td>Department of Medical Mycology, University of Glasgow, UK</td>
<td>Human infection</td>
</tr>
<tr>
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<td>International Mycological Institute, UK</td>
<td>Koji</td>
</tr>
<tr>
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<td>Koji</td>
</tr>
<tr>
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<td>Koji</td>
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<td>Environment</td>
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<td>N/A</td>
<td>Department of Medical Mycology, University of Glasgow, UK</td>
<td>Human infection</td>
</tr>
</tbody>
</table>

All *Aspergillus* species used to study the pathogenicity were prototrophic.

2.3.1 Inoculum preparation and survival assay

*Aspergillus* strains revived from silica gel preservation by growth on complete medium for one week at 30 °C. Inocula were prepared by harvesting the spores in saline Tween 80 (0.9 % (w/v) NaCl, 0.1 % (v/v) Tween 80). The spore number was adjusted by microscopic means using a haemocytometer (Improved Neubauer, Philip Harris Scientific, Staffordshire, UK). The number of colony forming units (CFU) or viable spores was determined by plating out the known dilution of spore suspension in triplicate. An aliquot of 10 µl of the spore inoculum was injected directly into the haemocoel through the last pro-leg of *G. mellonella* larva (Fuchs et al., 2010a) using the Hamilton syringe. The spores of highly virulent strains were also injected after heating at 70 °C for 45 min (heat killed spores). Inoculated larvae were first transferred to a Petri dish containing a Kimwipe on the bottom of the plate, to observe haemolymph leakage and then incubated at 37 °C in fresh Petri dish. Syringe was sterilised with ethanol and sterile distilled water in between the treatments. One group of unmanipulated larvae and the other injected with 10 µl saline Tween 80 only were also incubated with the inoculated larvae as controls. Larvae were scored daily for their survival up to 168 h post-inoculation. Pupae were scored as being alive. Larvae showing no response to touch were considered as dead and removed from the Petri dish.

Survival probabilities for larvae after injecting the spores were plotted by Kaplan-Meier method (Bland and Altman, 1998) and statistically analysed by Logrank test (Bland and Altman, 2004).
2.4 Study of the role of novel and uncharacterised genes in A. nidulans nitrate metabolism

2.4.1 In silico studies

Two novel nitrate reductase encoding genes, niaB and niaC, and two novel molybdenum cofactor biosynthesis genes in A. nidulans, cnxK and cnxL were discovered recently. Using the Aspergillus Genome Database, AspGD (www.aspergillusgenome.org) and European Bioinformatics Institute website (www.ebi.ac.uk) different bioinformatics tools were employed to acquire the information regarding the position and location of these genes on chromosomes; their sequence; number, position and sizes of introns etc. Homology and alignment of the proteins encoded by these newly discovered cnx and nia genes with each other, with other species in Aspergillus group as well as similar proteins from other organisms were also studied.

2.4.2 Selection of A. nidulans mutant strains

In the present study a variety of mutants with respect to nitrate metabolism were used to identify the possible role of proteins encoded by the genes niaB, niaC, cnxL and cnxK in nitrate metabolism of A. nidulans. Details of these mutants is summarised in Table 2.2.

2.4.3 Anaerobic studies

Strains (listed in Table 2.2) were grown from single point inoculum on complete agar medium at 37 ºC. Spores harvested and suspended in saline Tween 80 were used to inoculate 200 ml of liquid minimal medium supplemented with 5 mM urea as the sole source of nitrogen in 1 L flasks. Such flasks were incubated at room temperature at 250 rpm in an orbital shaker. Upon germination (16 – 20 h) under aerobic conditions, mycelia were harvested and divided among three screw capped 500 ml flasks each containing 200 ml liquid minimal medium supplemented with 10 mM NaNO₃ as a sole source of nitrogen and with one of the three different carbon sources from 1 % glucose, 3 % acetate (sodium) or 3 % ethanol. Two sets of each treatment were prepared; one set for 5 h and other set for 24 h incubation period.

Immediately after inoculation, each flask was subjected to anaerobic conditions by passing argon gas at the rate of 0.5 L / min for a period of 2 min (i.e.1 L volume of gas) to displace oxygen from the flask and the flask incubated at room temperature with orbital shaking at 250 rpm. A second gassing was given at the middle of the incubation period i.e. after 2.5 or 12 h for 5 and 24 h incubation periods respectively. Mycelia were harvested using ethanol sterilised Miraclth (Calbiochem, Darmstadt, Germany), aliquoted into 100 mg packages, quickly frozen in liquid nitrogen and stored at -80 ºC until used for total RNA extraction.
Table 2.2: *A. nidulans* structural and regulatory mutants used in northern blotting experiments for the presence of gene transcripts.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Growth on nitrate</th>
<th>Mutation resulted in</th>
<th>Reference/origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>G01</td>
<td>Wild type (WT)</td>
<td>Yes</td>
<td>N/A</td>
<td>Dept. of Genetics University of Glasgow</td>
</tr>
<tr>
<td>G817</td>
<td>niaD17</td>
<td>No</td>
<td>Loss-of-function in the nitrate reductase structural gene</td>
<td>Dept. of Genetics University of Glasgow</td>
</tr>
<tr>
<td>G834</td>
<td>nirA1</td>
<td>No</td>
<td>Loss-of-function in the nitrate inducer control protein, NirA</td>
<td>Reviewed in Cove, 1979</td>
</tr>
<tr>
<td>G015</td>
<td>nirA&lt;sup&gt;C&lt;/sup&gt;1</td>
<td>Yes</td>
<td>Constitutive expression of the nitrate assimilation structural genes, in the absence of nitrate</td>
<td>Cove, 1979</td>
</tr>
<tr>
<td>L202</td>
<td>areA300</td>
<td>No</td>
<td>Altered ammonium repression of <em>niaD</em></td>
<td>Shaffer and Arst, 1984</td>
</tr>
<tr>
<td>N481</td>
<td>creA&lt;sup&gt;d1&lt;/sup&gt;</td>
<td>Yes</td>
<td>Expression of genes under carbon catabolite repression, in the presence of glucose</td>
<td>Bailey and Arst, 1975</td>
</tr>
<tr>
<td>JK16</td>
<td>cnxE16</td>
<td>No</td>
<td>Loss-of-function of the molybdenum cofactor synthetic protein, CnxE</td>
<td>Heck <em>et al.</em>, 2002 and references therein</td>
</tr>
<tr>
<td>JK163</td>
<td>cnxE163</td>
<td>No</td>
<td>Loss-of-function of the molybdenum cofactor synthetic protein , CnxE</td>
<td>Millar <em>et al.</em>, 2001 and references therein</td>
</tr>
<tr>
<td>BC090</td>
<td>xprD1</td>
<td>No</td>
<td><em>areA</em> ammonium de-repression of <em>niaD</em> and other enzymes under ammonium repression</td>
<td>Cohen, 1972</td>
</tr>
<tr>
<td>T110</td>
<td>nrtA, nrtB</td>
<td>No</td>
<td>Lack of nitrate uptake</td>
<td>Unkles <em>et al.</em>, 2001</td>
</tr>
</tbody>
</table>

A defect in nitrate metabolism is listed along with the relevant genotype of *A. nidulans* mutant strain. These strains were in silica gel storage at School of Biology, University of St Andrews, UK. N/A denotes, not applicable.

2.4.4 Synthesis of gene probes

Using the DNA of *A. nidulans* mutant strain T110 as template, the coding regions for *niaB* (251 bp), *niaC* (488 bp), *cnxL* (250 bp) and *cnxK* (300 bp) genes were amplified by polymerase chain reaction (PCR). The sequences of the primers used to amplify the genes are listed in Table 2.3.

25 µl of the PCR reaction was carried out (1 x ready mix (enzyme+buffer) KAPA HiFi polymerase (KAPA Biosystems, Woburn, USA), 100 ng template DNA, 0.5 µM of each primer and 0.2 mM
PCR reaction was carried out according to the programme; 1 cycle of 2 min at 95 °C and followed by 30 cycles each with 20 sec denaturation at 98 °C, 15 sec annealing at 60 °C and 10 sec elongation at 72 °C. PCR product was run on 1.5 % agarose gel with molecular size marker, Hyperladder I (Bioline, London, UK). DNA fragment was purified by KeyPrep Nucleic Acid Extraction Kit (Anachem, Luton, Bedfordshire, UK).

Table 2.3: Sequences of the primers used to amplify the gene probes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>niaB</td>
<td>CTG GTG GAT ACC CCG GAC AC</td>
<td>GAG ACG GTC GGG ATG GTT GAT C</td>
</tr>
<tr>
<td>niaC</td>
<td>GTT GAA AGA GGA GAG CTC CTT AC</td>
<td>GTT ATC CGG CGT CAT CTG GTC</td>
</tr>
<tr>
<td>cnxL</td>
<td>CGG TCG TTC TAC GCG CAT G</td>
<td>CTG GAC ACC GTC TGC TCT G</td>
</tr>
<tr>
<td>cnxK</td>
<td>CAG AGA GAG CGA TGT TCG ATC</td>
<td>GTT TCT GTC TGC AAA CCA GCC</td>
</tr>
</tbody>
</table>

These primer sequences were selected on the basis of sequence of these genes given by the Aspergillus Genome Database (www.aspergillusgenome.org) and were acquired from Eurogentec (Southampton, UK).

2.4.5 Extraction and gel electrophoresis of total RNA

Using sterile pestle and mortar, 100 mg of mycelia was ground to fine powder in liquid nitrogen. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, West Sussex, UK) by following the manufacturer’s instructions. The concentration of RNA was determined using the Nanodrop spectrophotometer (ND-1000, Thermo Scientific, Northumberland, UK).

The gel electrophoresis and northern blotting of RNA was carried out using the method described by Davis et al. (1986). The quality of RNA was verified by electrophoresis on 1 % agarose gel in 1 x MOPS (20 mM 3-[N-morpholino] propane sulphonic acid, 5 mM sodium acetate and 1 mM EDTA), 5.4 % (v/v) formaldehyde and 1.5 µl of 10 mg / ml ethidium bromide. 5 µg of non-degraded RNA samples, after heating at 95 °C samples were run approximately for 5 h on 1 % agarose gel (as described above) at 80 volts for northern blot analysis with RNA size marker, RNA Millennium (Ambion, Foster City, USA). G01 (Table 2.2), the wild type strain for NrtA protein, grown aerobically and induced with 10 mM NaNO₃ was also run with the samples as control. RNA bands in the gel were visualised under UV light, excessive gel on the sides as well as comb was removed and a small cut was made at the right top corner of the gel for orientation. Gels were washed twice with 10 x SSC (1.5 M NaCl, 150 mM sodium citrate, pH 7).
2.4.6 Northern blot analysis and $^{32}$P labelling of genes

Filters were set on a Perspex raised stand placed in a glass dish containing 10 x SSC. Two long pieces of 3 MM Whatman paper (Kent, UK) soaked in 10 x SSC were placed over the stand so that their sides should remain dipped in the 10 x SSC in glass dish. On the top of this bridge, gel with RNA side up with cut end at the right corner was placed. Over the gel, Hybond-N nylon membrane (GE Healthcare, Amersham) of the same size with the cut end on the gel’s cut end was carefully laid avoiding any air bubble. Over the membrane, three gel sized 3 MM Whatman papers soaked with 10 x SSC were placed over the wet papers, three dry papers were placed. Atop of this set up, a bundle of paper towel and finally a weight was placed to allow RNA transfers to membrane. Blotting was carried out overnight at room temperature. On the following day, membrane removed, washed with 1 x SSC, dried and placed in the cross-linker (Spectrolinker XL-1500 UV Crosslinker) to cross-link the RNA to the Hybond-N nylon membrane.

Before starting the hybridisation procedure, the nylon membranes were soaked thoroughly in distilled water in a plastic container, water was then drained and the RNA hybridisation solution (for 1 L hybridisation buffer: 300 ml 20 x SSPE (3 mM NaCl, 0.2 M NaH$_2$PO$_4$, 20 mM EDTA), 50 ml 100 x Denhardt’s solution (2 % (w/v) gelatin, 2 % (v/v) polyvinylpyrrolidone (PVP), 2 % (v/v) Ficoll 400), 1 % (w/v) SDS, 50 % (v/v) deionised formamide, 50 ml of 5 mg / ml herring sperm DNA) was added and incubated at 42 ºC for 1 h in DNA hybridiser (HB-1D, Techne, Taylor Scientific, St. Louis, MO, USA) at 80 rpm.

Radiolabelling was carried out by $\alpha$ $^{32}$P dCTP following the method of Feinberg and Vogelstein (1983). 50 ng of the DNA probe was boiled for 2 min to denature the double stranded DNA and incubated on ice to prevent reannealing of the strands. To this denatured DNA, 0.2 mM dCTP, 1 x labelling buffer (Promega, Hampshire, UK), 2.5 units Klenow fragment (Fermentas, Yorkshire, UK) and finally 2.5 µl of $\alpha$ $^{32}$P was added. After gentle mixing, reaction mixture was incubated at 37 ºC in a heating block for 30 min. Radiolabelled gene probe was isolated from the mixture by size exclusion chromatography performed using a NICK gel filtration column (GE Healthcare, Buckinghamshire, UK). A 4 µl aliquot of a saturated solution of Blue dextran dye was added to the reaction mixture which then was loaded on to the NICK column that was washed and equilibrated with the TE buffer. The blue coloured labelled probe was collected in an Eppendorf tube, boiled for 5 - 10 min, incubated on ice for 5 min, added to the hybridisation buffer in the container of membranes and left overnight at 42 ºC inside the DNA hybridiser at 80 rpm.
The following day, the membranes were washed with decreasing strength of SSC (5 x, 3 x, 1 x) for periods of 30 min each time at 42 °C and 80 rpm orbital shaking inside the DNA hybridiser until background radiation was negligible. Excessive hybridisation buffer was removed; membranes were sealed in plastic bags and exposed to phosphor screens (Kodak Imaging Screen K, Bio-Rad) in phosphony case for 2-3 days. Such screens were developed by FX Molecular Imager (Bio-Rad) and results were analysed using the Quantity One software version 4.3.1 (Bio-Rad).

Same blots were washed with 0.1 % SDS at 42 °C and 80 rpm orbital shaking for 30 min and re-probed using the A. nidulans actA (actin) gene which is a 0.83-kb NcoI-KpnI fragment (Fidel et al., 1988).

2.4.7 Generation and characterisation of knock-out mutants

cnxL, cnxK and niaB genes were found to form a cluster on chromosome VII of A. nidulans. Knock-out mutants of the gene cluster, cnxL-cnxK-niaB were generated to study the possible role of these novel genes in nitrate metabolism. Transformant strain T12101 (nrtB110, nkuA, pyroA4) was used to generate knock-out mutants and made by Dr. S E Unkles. Tranformants were selected on the basis of prototrophic repair of the pyroA4 vitamin auxotrophy and screened for the knock-out phenotype. The mutants were grown on minimal medium supplemented with different concentrations of various nitrogen and carbon sources, aerobically and under semi-anaerobic conditions. Detail of the knock-out mutants generated for this present study is given in the Table 2.4.

<table>
<thead>
<tr>
<th>Gene knock-out</th>
<th>Transformant No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>∆niaB</td>
<td>T12320-T12328, T12380-T12388</td>
</tr>
<tr>
<td>∆cnxL</td>
<td>T12360-T12367</td>
</tr>
<tr>
<td>∆cnxK</td>
<td>T12400-T12407</td>
</tr>
<tr>
<td>∆nia, ∆cnxK, ∆cnxL</td>
<td>T12340-T12348</td>
</tr>
</tbody>
</table>

Genes coding for CnxL, CnxK and NiaB proteins were knocked-out individually and in one mutant complete gene cluster was deleted.

2.5 Inhibition of growth and net nitrate / nitrite transport in A. nidulans

The effect of structural analogues of nitrate on growth and net nitrate / nitrite transport of A. nidulans mutant strains was studied. These strains were different from each other with respect to the presence or absence of one or both of the nitrate transporter proteins, NrtA and NrtB. The information in Table 2.5 summarises details of these mutant strains.
Table 2.5: *A. nidulans* mutant strains used to study the effect of inhibitors on growth and net nitrate / nitrite transport.

<table>
<thead>
<tr>
<th>Mutant/strain</th>
<th>Functional transporter(s)</th>
<th>Relevant mutation</th>
<th>Reference/origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>NrtA, NrtB, NitA</td>
<td>N/A</td>
<td>Department of Genetics, University of Glasgow, UK</td>
</tr>
<tr>
<td>T110</td>
<td>NitA</td>
<td>nrtA747, ∆nrtB</td>
<td>Unkles et al., 2001</td>
</tr>
<tr>
<td>nrtA1</td>
<td>NrtB, NitA</td>
<td>nrtA1</td>
<td>Arst and Cove, 1973</td>
</tr>
<tr>
<td>nrtB110</td>
<td>NrtA, NitA</td>
<td>∆nrtB</td>
<td>Unkles et al., 2001</td>
</tr>
<tr>
<td>T12048</td>
<td>NrtB</td>
<td>nrtA747, ∆nitA26</td>
<td>Present study</td>
</tr>
<tr>
<td>T12200</td>
<td>NrtA</td>
<td>∆nrtB, ∆nitA26</td>
<td>Present study</td>
</tr>
</tbody>
</table>

T denotes transformant. Wild type is *biA1* (biotin auxotroph).

2.5.1 Determination of vitamin auxotrophic markers within mutants

The additional genotypic marker(s) contained within mutant strains was determined. The mutant strains were grown on minimal agar medium supplemented with 5 mM ammonium and one of the vitamins from biotin (bio), *p*-aminobenzoic acid (paba) or pyridoxine (pyro). All possible combinations of these three vitamins were also tested to identify if any of the mutant(s) needed more than one vitamin for growth. All strains grew in the presence of all three vitamins.

2.5.2 Growth tests

The mutant strains were maintained on complete agar medium. The growth inhibition tests by carbonate, bicarbonate, formate, chlorite and sulphite (sodium salts); chlorate (potassium salt); malonate (malonic acid); oxalate (oxalic acid) and caesium (chloride) were carried out on solid minimal medium supplemented with nitrate / nitrite (NaNO₃ or NaNO₂), as required. Minimal medium supplemented with proline, as the sole source of nitrogen was used to test the resistance or sensitivity of mutant strains for these inhibitors. Aqueous solutions of the inhibitors, pH adjusted to 6.5, were prepared fresh when required. Nitrogen source (nitrate / nitrite / proline) was added to molten minimal medium and finally the inhibitor solution was added to provide the final concentration as required. Inoculated Petri dishes were incubated at 37 °C for 48 h or so and scored for growth response.

2.5.3 Net nitrate / nitrite transport assays

Effect of potential inhibitors on net nitrate / nitrite transport, was determined by measuring the depletion of nitrate / nitrite from the assay medium (Brownlee and Arst, 1983). The mutant strains were grown on complete agar medium from single point inoculum at 37 °C. Conidia from half of the Petri dish were harvested in saline Tween 80, vortex thoroughly and used to inoculate 200 ml of liquid minimal medium supplemented with 5 mM urea as sole source of nitrogen. Such flasks were
incubated at 200 rpm with orbital shaking at 37 °C for 6.5 h. 10 mM NaNO₃ was added to induce the protein(s) 100 min prior the harvest.

A 50 ml aliquot containing mycelium was filtered through the pre-weighed Whatman GF/C glass fibre filter under vacuum and oven dried at 80 °C overnight to measure the dry weight of the mycelium. A further 50 ml aliquot was taken and filtered rapidly under vacuum through a cellulose acetate filter avoiding compaction of mycelium. The mycelium was washed with 50 ml of pre-warmed distilled water at least 4 times. The filter containing mycelium was transferred to a 50 ml of pre-warmed minimal medium in a 250 ml conical flask supplemented with 500 µM NaNO₃ (for net nitrate transport) or 100 µM NaNO₂ (for net nitrite transport) and appropriate concentration of inhibitor. The flask was shaken rapidly to suspend the mycelium in the medium. An aliquot of 3 ml was filtered rapidly through a Whatman GF/C glass fibre filter under vacuum. That was the zero time reading. The flask was then incubated at 37 °C and at 200 rpm and a further aliquot of 3 ml was taken after 20 min and processed as before. To determine the Kₘ of transporter proteins for nitrite, net nitrite transport was carried out using a range of nitrite concentrations.

For net nitrate transport, an aliquot of 50 µl of the filtrate was added to 950 µl of 5 % perchloric acid and absorbance was recorded at 204 nm by UV visible spectrophotomer (Camspec, Leeds, UK). For net nitrite transport 100 µl of indicator solution (1:1 reagent A (1 % (w/v) sulphanilamide in 37 % HCl) and reagent B (0.1 % (w/v) N-[1-naphthylene] ethylenediamide in water) was added to 900 µl of filtrate and after 5 min incubation at room temperature, absorbance was recorded at 543 nm using the Nanodrop spectrophotometer (ND-1000, Thermo Scientific, Northumberland, UK). Each experiment of net nitrate / nitrite transport was carried out at least using four different concentrations of an inhibitor and repeated at least three times. The concentration of nitrate / nitrite in the filtrate was determined from the standard curves, and net transport was determined by decrease in absorbance in filtrate from 0 to 20 min samples. Rate of net nitrate / nitrite transport was calculated as nmol/mg DW/h.

2.5.4 Determination of kinetic parameters
Values of Michaelis constant of the substrate (Kₘ) and maximum velocity of uptake (Vₘₐₓ) were calculated by linear regression of Hofstee analysis (plotting v against v/s, where ‘v’ is net transport and ‘s’ is substrate concentration) and Lineweaver-Burk double reciprocal analysis (1/v plotted against 1/s). The values for Kᵢ (inhibition constant) were determined by plotting rate of net transport (nitrate / nitrite) versus the log of inhibitor concentration and then calculating the concentration of inhibitor that reduced the rate of transport to 50 %.
2.6 Cysteine-scanning mutagenesis and thiol cross-linking studies in NrtA

Single cysteine replacement mutants in NrtA transporter protein of *A. nidulans* were generated by site-directed mutagenesis. Double cysteine mutants were constructed by cloning the restriction fragments containing corresponding single cysteine mutations.

For bacterial growth, LB broth (Sigma, Dorset, UK) was used and prepared following the manufacturer’s instructions and 1.2 % agar was added for solid LB medium. Ampicillin (Melford, Suffolk, UK) was added to the medium to a final concentration of 0.1 mg / ml as selection marker. Plasmids were transformed in *E. coli* strain DH5α and strain stocks maintained in LB containing 20 % glycerol at -80 °C for long term preservation.

2.6.1 Plasmid preparation

A single isolated bacterial colony was used to inoculate the 5 ml LB broth medium supplemented with ampicillin (0.1 mg / ml) and incubated at 37 °C and 250 rpm for 12 - 16 h. Minipreps of plasmids were made by using QIAprep Spin Miniprep Kit (Qiagen, West Sussex, UK). The concentration of the plasmid DNA (µg / µl) was recorded by Nanodrop spectrophotometer.

2.6.2 Preparation of *E. coli* competent cells

The competent cells of *E. coli* were prepared following the method of Cohen *et al.* (1972). *E. coli* strain DH5α was streaked on solid LB and incubated overnight at 37 °C. On the following day, 5 ml LB medium was inoculated with a isolated bacterial colony and incubated overnight at 37 °C orbital shaking at 250 rpm. In a 1 L Erlenmeyer flask, 1 ml of this starter culture was used to inoculate the 100 ml of SOB medium (2 % (w/v) trypton, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO₄, 10 mM MgCl₂) and incubated at 18 °C and 250 rpm until the OD₆₀₀ reached to 0.4 - 0.8. After incubating on ice for 10 min, cells were collected by centrifugation at 2500 rpm and 4 °C in SS-34 rotor (Sorvall Evolution RC). The cell pellet was suspended in 40 ml of sterilised transformation buffer (10 mM PIPES (C₅H₁₈N₂O₆S₂), 15 mM CaCl₂, 2H₂O, 250 mM KCl, 55 mM MnCl₂, 4H₂O, pH 6.7). After incubation on ice for 10 min, a second centrifugation step was performed as before. Transformation buffer was removed and cell pellet was suspended in 4 ml of transformation buffer. To the suspended cells 300 µl of DMSO (dimethyl sulfoxide) was added. Such suspended cells were incubated on ice for further 10 min, aliquoted to pre-cooled Eppendorf tubes and stored at -80°C.

2.6.3 Transformation of plasmids in *E. coli* strain DH5α

An aliquot of 0.5-1.0 µl of plasmid (depending on the concentration) was mixing gently to 50 µl of *E. coli* strain DH5α competent cells and incubated on ice for 20 min. A brief heat shock was carried out
at 42 °C for 90 sec. 10 µl of the transformed cells were plated out on LB medium supplemented with 0.1 mg / ml ampicillin and incubated overnight at 37 °C.

2.6.4 Preparation of single cysteine mutants

The amino acid residues in transmembrane domains (Tm) 2 and 8 of NrtA protein were replaced individually with cysteine. Such single cysteine mutants were prepared using the template plasmid DNA, pNRTAV5CL, which has the coding region for cysteine-less NrtA with C-terminal V5 epitope tag and flanked by EcoRI restriction sites (Unkles et al., 2005). For single cysteine mutants, residues were replaced with cysteine by site-directed mutagenesis by the PCR overlap extension method as described by Warrens et al. (1997). Plasmid DNA was amplified in two PCR reactions, first with non-mutagenic forward primer and mutagenic reverse primer, and second with complementary mutagenic forward and non-mutagenic reverse primers (One cycle of 98 °C for 90 sec followed by 30 cycles each with denaturation at 98 °C for 10 sec, annealing at 60 °C for 20 sec and elongation at 72 °C for 10 sec) in the G-Storm Thermocycler (Essex, UK). The PCR products were purified using the Marligen Biosciences PCR Purification Kit (Ijamsville, USA).

A third and final PCR reaction was carried out with both PCR products as the DNA template in 1:1 ratio that amplified the DNA fragment containing the required replacement of amino acid residue with cysteine. This PCR product was purified from the agarose gel using the DNA Isolation Kit (Qiagen, West Sussex, UK). Digestion of this mutation containing DNA fragment and parent plasmid DNA (cysteine-less) was carried out by restriction enzymes with the appropriate buffers. After isolation from the agarose gel, digested DNA fragments were ligated.

The ligation reaction was carried out using the Rapid DNA Ligation Kit (Fermentas, Yorkshire, UK). After ligation, plasmids were transformed into E. coli strain DH5α. 1 ml of SOC (20 mM glucose added to SOB) was added to transformation mixture and incubated at 37 °C for 1 h at 250 rpm. Cells collected by centrifugation were plated out on LB medium supplemented with ampicillin and plates were incubated overnight at 37 °C.

Several colonies of transformants were cracked by mixing the colony with a sterile tooth-pick in 20 µl water and 20 µl cracking buffer (200 mM NaOH, 0.5 % (w/v) SDS, 20 % (w/v) sucrose and a few crystals of bromocresol green dye). Cracked colonies were run on 1 % agarose gel (with ethidium bromide) along with the control plasmid of similar molecular size. Colonies with the correct molecular size compared to the control plasmid and DNA size marker were selected for plasmid isolation.
Plasmid DNAs were isolated and sequenced for the verification of desired mutation without PCR induced mutation, either from Macrogen Sequencing Service (Seoul, South Korea) or The Dundee Sequencing Service (University of Dundee, Scotland, UK). DNA sequence results were analysed by Sequencher software (Gene Codes Corporation, Ann Arbor, USA).

2.6.5 Construction of double cysteine mutants
Plasmid construct pGPDAATXα, generated by engineering two tandem recognition sequences (IEGR) for factor Xa protease digestion into the central loop between Tm 6 and 7 in the cysteine-less construct of NrtA was used to generate the double cysteine mutants. Three double cysteine mutants, R87C (Tm 2) paired with R368C (Tm 8), R87C (Tm 2) paired with N459C (Tm 11), R368C (Tm 8) paired with N168C (Tm 5) were constructed by two step replacement of the restriction fragments containing single cysteine mutation into the plasmid construct pGPDAATXα.

For the creation of the R87C or N168C replacements, plasmid DNA was digested with FseI and BsrGI restriction enzymes and for R368C or N459C mutations double digestion was carried out by the restriction enzymes AatII and Clai using appropriate buffers. Digested DNA fragments were isolated from the agarose gel by DNA Isolation Kit (Qiagen) and ligated to the respective digested fragment to obtain the final construct that has factor Xa protease site between the two cysteine residues. After verification by DNA sequencing, plasmid constructs were transformed in E. coli strain DH5α and stored in glycerol at -80 °C for future use.

2.6.6 Transformation of mutant plasmids in A. nidulans
Single and double cysteine mutants were transformed in A. nidulans strain JRK 1060 (nrtA747, nrtB110, argB2) using the method described by Tilburn et al. (1983); Yelton et al. (1984); Ballance and Turner (1985) and reviewed by Riach and Kinghorn (1995) based on arginine selection marker.

2.6.6.1 Mycelia preparation
A. nidulans strain JRK1060 was grown from a single point inoculum at 37 °C on complete agar supplemented with 10 mM arginine until full plate growth followed by incubation for 2-3 weeks at room temperature for maturation of conidia. Harvested conidia from such plates were suspended in saline Tween 80 by vortexing and used to inoculate a 1 L flask containing 400 ml of liquid minimal medium supplemented with 5 mM urea and 10 mM arginine. Flask was incubated at 4 °C for 6 h without shaking, then for 10 h at 25 °C and 250 rpm, finally at 37 °C for 3-4 h (until conidia started to germinate) with orbital shaking at 250 rpm. Such germlings were harvested on the ethanol sterilised Miracloth and incubated on ice after washing with cold sterile 0.6 M MgSO₄.
2.6.6.2 Protoplast preparation
The harvested cells were suspended in 5 ml of filter sterilised osmotic solution, OSMO (1.2 M MgSO₄, 10 mM Na₂HPO₄, pH adjusted to 5.8 with 0.2 M Na₂HPO₄). 1 ml of 50 mg glucanex / ml OSMO was mixed to the cell suspension, a further 1 ml of OSMO was added to the cells and incubated on ice for 5 min. 250 µl of 12 mg / ml BSA in OSMO was added to cell suspension, a further 1 ml of OSMO was added to the cells and incubated on gentle orbital shaking (60-70 rpm) at 28 ºC for approximately 2 h. The digested suspension was layered by equal volume of trapping buffer (0.6 M sorbitol, 100 mM Tris, pH 7) carefully avoiding mixing of the layers and then centrifuged at 4 ºC at 3500 rpm for 20 min. The white protoplast layer at the interface was pipette off into a fresh cold tube. For washing the protoplasts, 15-20 ml of sterilised ice-cold STC (1.2 M sorbitol, 10 mM Tris pH 7.5, 10 mM CaCl₂) was added to the protoplast tube and centrifuged at 3500 rpm at 4 ºC for 5 min. The protoplast pellet was suspended in cold STC at the rate of 90 µl per transformation. All steps of protoplast preparation were performed on ice.

2.6.6.3 Genetic transformation
To 90 µl of protoplast suspension, 10 µl of plasmid DNA (~ 1 µg / µl) and 25 µl of 60 % PEG 6000 was added in a 15 ml centrifuge tube. After mixing gently, these tubes were incubated on ice for 20 min. Then 1 ml of 60 % PEG was added to each tube and left for further 20 min at room temperature. After suspending in 5 ml of cold STC, tubes were centrifuged at 2500 x g for 5 min at room temperature. The protoplast pellet was suspended in 65 µl of cold STC and all of this mixture was spread on minimal agar medium supplemented with 1.2 M sorbitol and 5 mM ammonium tartrate, and incubated at 37 ºC for 2-3 days. Transformants were selected on the basis of their arginine prototrophy and screened for their ability to grow on minimal agar medium with nitrate as a sole nitrogen source.

2.6.7 Southern blotting
Southern blotting of the genomic DNA was carried out to identify the single copy gene integrated transformants. The DNA from the transformants was digested with BamHI restriction enzyme and hybridised by radiolabelled XbaI DNA fragment (1 kb) as probe.

2.6.7.1 Fungal DNA isolation
The Nucleon II Kit (Scotlab, Strathclyde, UK) was used for genomic DNA isolation from the transformants. Fungal spores harvested in saline Tween 80 were taken to inoculate 100 ml of liquid minimal medium supplemented with 5 mM urea as nitrogen source. Cells were grown overnight at room temperature in an orbital shaker at 200 rpm, harvested by ethanol sterilised Miracloth and
washed with cold sterilised distilled water. The mycelia cells were squeezed between the folds of paper towels to remove excessive water and after recording their weight stored in liquid nitrogen.

About 300 mg of these frozen cells were ground to fine powder in liquid nitrogen in a sterile pester and mortar. The powdered cells were incubated at 37 °C for 30 min in 2 ml Nucleon Reagent B (400 mM Tris pH 8, 120 mM EDTA, 150 mM NaCl and 1 % (w/v) SDS) with 0.5 µl of 10 mg / ml RNase A in a sterile 5 ml polypropylene tube. 0.5 ml of 5 M sodium perchlorate was added and mixed thoroughly by inverting the tube several times. An aliquot of 2 ml of chloroform at -20 °C was added and mixed vigorously. The tube was then centrifuged at 1800 x g for 3 min (MSE Mistral 1000). The supernatant was carefully transferred to new sterile tube and 2 ml of ice cold 96 % ethaol was added. Precipitated DNA was collected with the help blocked end of a glass Pasteur pipette and washed with 70 % ethanol. Air dried DNA was suspended in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8) and incubated at 65 °C for 15 min to inhibit potential DNase activity.

2.6.7.2 Blot preparation
Depending on the concentration, 5-10 µl of DNA was digested by BamHI at 37 °C overnight and run on 1 % agarose gel at 30 volts. The gel was viewed on a transilluminator, intensity of bands was noted. Surplus gel from all sides including the comb was removed, a cut was made on the top corner for orientation and for record a photograph was taken (Herolab EASY Photographic Suite, Scotlab). The gel was depurinated on gentle shaking in 0.25 M HCl for 20 min then rinsed with distilled water and denaturated for 40 min in denaturating solution (1.5 M NaCl, 0.5 M NaOH). As before, the gel was first washed with distilled water and then neutralised in neutralising buffer (1.5 M NaCl, 1 M Tris EDTA, pH 7.4) for 40 min followed by a final washing with distilled water.

The Southern blotting procedure was as described basically for northern blot (section 2.3.6) with the exception that 20 x SSC was used. The procedure for DNA hybridisation and autoradiography was that discussed for northern blot except the membranes were incubated at 65 °C (instead of 42 °C) in DNA hybridisation buffer (5 x SSPE (750 mM NaCl, 50 mM NaH₂PO₄, 5 mM EDTA, pH 7.4), 9 % (w/v) PEG, 0.5 % (w/v) powdered milk, 0.1 % (w/v) SDS, 5 % (w/v) Na₄P₂O₇, 0.2 mg / ml herring sperm DNA) with the radioactive argB gene probe and all washings with SSC of membranes after hybridisation were also carried out at 65 °C.

2.6.7.3 Genomic DNA extraction from single copy transformants
For sequencing purpose, DNA from fungal cells was isolated by cell disruption. 1 ml of minimal medium supplemented with 0.5 % (w/v) yeast extract and 5 mM urea was inoculated with a wire loopful of spores of single copy transformant identified by DNA labelling. The spores were grown as
static culture at 37 °C for 16-20 h. The mycelial mat was collected and excessive medium was removed by squeezing the cells between layers of paper towels. Such cells were transferred to a screw capped tube containing 300 µl of silica beads (BioSpec) and 700 µl of ice cold DNA breaking buffer (2 % (v/v) Triton X-100, 1 % (w/v) SDS, 100 mM NaCl, 10 mM Tris pH 8, 1 mM EDTA, pH 8). The cells were homogenised in MP FastPrep 24 (MP Biomedicals, Cheshire, UK) at 4.5 m/sec for 20 sec and incubated on ice for 1 min. This step of homogenisation was repeated three times. The supernatant was transferred to a sterilised Eppendorf tube after centrifugation at 14,000 rpm for 3 min in bench top Eppendorf centrifuge. 20 µl of proteinase (20 mg / ml) were added to the supernatant, incubated at 37 °C for 1 h, 1 ml chloroform:isoamylalcohol (24:1) was mixed thoroughly at room temperature and centrifuged at 14,000 rpm for 20 min. The supernatant was transferred to fresh Eppendorf tube and after adding 1 ml of chloroform:isoamylalcohol:phenol (24:1:25) centrifuged as before. To the supernatant, 500-600 µl of ice-cold ethanol was added and centrifuged for a further 10 min at 14,000 rpm at room temperature. The DNA pellet was washed with 70 % ethanol, air dried and suspended in 50 µl of TE buffer. Finally isolated DNA was incubated with 0.5 µl of RNase (10 mg / ml) at 37 °C for 30 min.

2.6.7.4 Polymerase chain reaction (PCR)
The coding region gene containing the mutational sites were amplified using the genomic DNA as template (isolated as described above) in the G-storm thermocycler (Essex UK). Phusion polymerase (Finnzymes, Espoo, Finland) with appropriate buffer was used for amplification in a 50 µl PCR reaction mixture. (1 µl of DNA, 0.2 mM dNTPs, 0.4 µM of each primer, PGPD forward (5΄- CAT CGC AGC TTG ACT AAC AG -3΄) and MUT reverse primer (5΄- CGG TAT CGA TAA GCT TGA TAT C -3΄) and 0.5 u of Phusion polymerase). The PCR reaction was carried out according to the following programme; one cycle at 98 °C for 90 sec followed by 30 cycles each of denaturation at 98 °C for 10 sec, annealing at 60 °C for 20 sec and elongation at 72 °C for 10 sec. The PCR product was purified and fully sequenced.

2.6.8 Western blotting and protein expression
The crude membrane proteins isolated from the transformant strains, containing the appropriate mutation, confirmed by DNA sequencing, were western blotted and protein was probed with V5 antibodies.

2.6.8.1 Crude plasma membrane preparation
The mutant strains were grown on solid complete solid medium and conidia harvested in saline Tween 80 were used to inoculate the 100 ml of minimal liquid medium with 5 mM urea as the
nitrogen source. The cells were grown at 37 °C and 250 rpm for 6.5 h and induced by 10 m M NaNO₃ for 100 min. Cells were harvested on sterile Miracloth, washed with cold sterile water, pressed in paper towels to remove excessive moisture, weighed and stored in liquid nitrogen. Approximately 50 mg of fungal mass was transferred to a tube containing 300 µl of glass beads (BioSpec) and 500 µl of cold extraction buffer (10 mM Na₃PO₄, 200 mM NaCl, 10 % (v/v) glycerol, 0.1 mM PMSF, 1 mM benzamidine, pH 7). Fungal cells were broken in MP FastPrep at 4.5 m / sec for 20 sec and then incubated on ice for 1 min. This step of cell disruption was repeated three times. The centrifugation step was carried out at 10,000 rpm for 1 min at room temperature. The supernatant was transferred to fresh and ice-cold tube and centrifuged in a SORVAL SS-34 rotor at 18,000 rpm for 45 min at 4 °C to collect the membranes. The membrane pellet was suspended in 50 µl of cold extraction buffer. The concentration of protein was determined by Protein Quantification BCA Kit (Pierce) with bovine serum album as standard and following the manufacturer’s instructions.

2.6.8.2 SDS-PAGE

Protein samples were run on 10 % SDS- PAGE gels (separating gel: 0.37 mM Tris pH 8.8, 0.1 % SDS (w/v), 10 % (v/v) acrylamide, 0.05 % (w/v) (NH₄)₂S₂O₈, 0.05 % (v/v) TEMED; stacking gel: 125 mM Tris pH 6.8, 0.125 % (w/v) SDS, 5 % acrylamide, 0.5 % (NH₄)₂S₂O₈, 0.5 % (v/v) TEMED). Protein samples were thawed on ice and 0.5 µl of 1 % (w/v) n-dodecyl β-maltoside (DDM) was mixed with the 10 µl protein sample, 1 µl of this soluble protein was mixed with 8 µl of distilled water and 1 x running buffer loading dye (60 mM Tris pH 6.8, 25 % (v/v) glycerol, 5 % (w/v) SDS, 1 % (v/v) saturated bromophenol blue). After incubating for 30 min at room temperature, protein samples along with positive and negative controls were run in 1 x SDS running buffer (250 mM Tris pH 8.3, 500 mM glycine, 1 % (w/v) SDS) at 200 volts with a pre-stained protein size marker, PageRuler Plus (Fermentas, Yorkshire, UK) until the dye was 1 - 2 mm from the end of the gel. Each sample was run on two gels, one for Coomassie Blue staining and other gel for western blotting. Therefore one gel was stained in a Coomassie Blue stain solution (0.1 % (w/v) Coomassie brilliant blue, 45 % (v/v) methanol and 10 % (v/v) acetic acid) for 20-30 min and then washed with PAGE-destain (10 % (v/v) acetic acid, 45 % (v/v) ethanol) for several times to visualise protein bands.

2.6.8.3 Western transfer

From the protein gels to be western blotted, the stacking gel was removed with a scalpel and the top right corner of the gel was nicked for orientation. Before transfer, the gel was equilibrated in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 20 % (v/v) methanol, 0.1 % (w/v) SDS) for 20 min by gentle shaking. A piece of Hybond-P membrane (GE Healthcare, Amersham) of the gel size was cut, activated by wetting in methanol and then soaked in transfer buffer.
The membrane and gel with one piece of 3 MM Whatman paper (wetted with transfer buffer) on either side was assembled avoiding trapping of air bubbles. Assembled frame was placed in cold transfer buffer in the transfer tank (Bio-Rad) in ice bath and transfer at 100 volts for 50 min. After transfer of protein, the membrane was washed twice for 15 min each in TBST (0.9 % (w/v) NaCl, 10 mM Tris pH 7.4, 0.1 % (v/v) Tween 20) at room temperature and incubated in the 5 % membrane block (dry milk, Marvel) in TBST overnight at 4 °C. The following day membrane was washed twice for 10 min in TBST at room temperature and incubated in TBST containing 0.05 % (w/v) block and 1/5000 anti - V5HRP antibody (Invitrogen, Paisley, UK.) for 4 h at room temperature. The membrane was washed twice with TBST each for 5 min then twice for 20 min per wash. After removing the excessive TBST, ECL Plus detection solution (GE Healthcare, Buckinghamshire, UK) was transferred onto the membrane with the protein side up and incubated for 5 min at room temperature. Finally the membrane was placed between two layers of plastic sheets with protein side up in an autoradiography cassette and exposed to ECL Hyper-film (GE Healthcare).

2.6.9 Thiol cross-linking of double cysteine mutants followed by Factor Xa digestion

Commercially available, homobifunctional thiol cross-linkers with different spacer arm lengths; o-phenylenedimaleimide (o-PDM) (6 Å), bis (maleimido) ethane (BMOE) (8 Å) copper phenanthroline. A complex of CuSO₄ and 1, 10 phenanthroline (Cu-Phen) (8 Å), p-phenylenedimaleimide (p-PDM) (10 Å), bis (maleimido) hexane (BMH) (13 Å), 1, 8 bis (maleimido) diethylene glycol (BM (PEG)₂) (14.7 Å) and 1,4-di-(3’-[2’-pyridyldithio]propionamido) butane (DPDPB) (19.9 Å) were used in this study. The chemical cross-linkers o-PDM, p-PDM and 1, 10 phenanthroline were purchased from Sigma, Aldrich while BMOE, BMH, BM (PEG)₂ and DPDPB were acquired from Pierce.

For cross-linking experiments, the membrane pellet prepared in extraction buffer (section 2.6.8.1) was suspended in PBS (10 mM Na₂PO₄, 150 mM NaCl, pH 7.4) with 0.5 mM EDTA at a concentration 1 mg / ml. Total protein in the crude membrane samples was quantified by Protein Quantification Kit (Pierce), following the instructions provided by the manufacturer. All cross-linking experiments were carried out on 25 µg of total protein (1 µg / µl) at 25 °C. Stock solutions of all cross-linkers were made in fresh before use in DMSO with exception of the Cu-Phen cross-linker. The working solution of Cu-Phen was made by mixing the stock solutions of CuSO₄ (in water) and 1, 10 phenanthroline (in 95 % ethanol) in 3:1 ratio (Hamdan et al., 2002). The concentrations used for o-PDM and p-PDM for cross-linking was 500 µM with an incubation time of 30 min; 2.5 mM for BMH, BM (PEG)₂ and DPDPB, incubation period of 1 h; and 1 mM Cu-Phen (concentration of Cu-Phen actually shows the concentration of 1, 10 phenanthroline) the time of incubation for cross-linking was 15 min.
After cross-linking, centrifugation was performed at 18,000 rpm and 4 °C to remove the cross-linker and PBS buffer in SS-34 rotor. Membrane pellet was washed twice with Factor Xa buffer (20 mM Tris pH 8.5, 100 mM NaCl, 2 mM CaCl₂) by suspension followed by centrifugation at 18000 rpm, 4 °C for 10 min. The cross-linker treated membrane pellet was suspended in the Factor Xa buffer at the concentration 1 mg / ml and incubated with / without Factor Xa protease (New England Biolabs, Hitchin, Herts, UK) for overnight digestion at 4 °C. Equal amount of protein samples was run on a 12 % SDS-PAGE. The resulting protein was western blotted and probed with V5 antibody (as described in section 2.6.8.3).

2.7 Crystallography attempts of prokaryotic nitrate transporters

2.7.1 Construction of fusion proteins

The genes encoding nitrate transporter proteins from Escherichia coli, NarU; Bacillus cereus, Nark; Pseudomonas aeruginosa, Nark1 and Nark2; and Thermus thermophilus, NarK1 and NarK2 were cloned into expression vector pWaldo-GFPd (Waldo et al., 1999). pWaldo-GFPd is derived from the standard pET28 (a⁺) plasmid with a GFP reporter and a kanamycin resistance gene (Km'), T7 promoter, and a 8 x His tag. Between the transporter and GFP genes, a TEV protease cleavage site was inserted for the removal of GFP - 8 x His part of the fusion protein by affinity chromatography (Figure 2.1).

Figure 2.1: A schematic representation of pWaldo-GFPd.

(a) pWaldo-GFP vector that contained multicloning sites (MCS) for the insertion of nitrate transporter. (b) Insertion of nitrate transporter coding sequence in NdeI/BamHI restriction sites in pWaldo-GFP. (Please note that this diagram is not to the scale).
Table 2.6: Bacterial proteins examined in this present study.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Target protein</th>
<th>MW (kDa)</th>
<th>No. of amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>NarU</td>
<td>50.0</td>
<td>463</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>NarK</td>
<td>42.6</td>
<td>389</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>NarK1</td>
<td>47.2</td>
<td>431</td>
</tr>
<tr>
<td></td>
<td>NarK2</td>
<td>50.6</td>
<td>468</td>
</tr>
<tr>
<td><em>Thermus thermophilus</em></td>
<td>NarK1</td>
<td>49.6</td>
<td>448</td>
</tr>
<tr>
<td></td>
<td>NarK2</td>
<td>49.0</td>
<td>443</td>
</tr>
</tbody>
</table>

The protein sequences, number of amino acids and molecular weight of the above mentioned prokaryotic nitrate transporters were obtained from European Bioinformatics Institute website (www.ebi.ac.uk).

2.7.2 Optimisation of GFP-fused protein expression
Plasmid vectors encoding Waldo-GFP *E. coli* NarU, or Waldo-GFP *T. thermophilus* NarK1 were transformed into *E. coli* strains (i) BL21(DE3) (fhuA2 [lon] ompT gal (DE3) [dcm] ΔhsdS) and (ii) Lemo 21(DE3) (fhuA2 [lon] ompT gal (DE3) [dcm] ΔhsdS/ pLemo(CamR)) competent cells by the same procedure used for the DH5α strain (section 2.6.3). For the selection of transformants, 50 µg /ml kanamycin was added to LB medium and for Lemo 21 (DE3), in addition to kanamycin, 30 µg /ml chloramphenicol was added.

2.7.3 Protein over-expression trails for *E. coli* NarU and *T. thermophilus* NarK1

2.7.3.1 Culture media and antibiotics
Two different media, LB or auto-induction (Studier, 2005) were used for over-expression trials of proteins. In case of auto-induction medium, the inoculum culture was prepared in ZYP-0.8G medium which is a rich growth medium without inducer. The composition of ZYP-0.8G was 9.3 % (v/v) ZY (1 % (w/v) N-Z-amine, 0.5 % (w/v) yeast extract), 0.1 mM MgSO₄, 0.08 % (w/v) glucose, 1 x NPS (20 x NPS: 0.5 M (NH₄)₂SO₄, 1 M KH₂PO₄, 1 M Na₂HPO₄, pH 6.75. For protein expression, auto induction medium ZYP-5052, was used which was 92.8 % (v/v) ZY, 1 mM MgSO₄, 1 x 5052 (50 x 5052: 2.5 % (v/v) glycerol, 0.25 % (w/v) glucose, 1 % (w/v) α-lactose), 1 x NPS. As adequate aeration was required for better performances of these media therefore only 20 % of the total volume of the flask (or bottle) was filled with media.
2.7.3.2 Induction of protein expression

a. LB medium

2 ml of LB medium supplemented with appropriate antibiotics was inoculated with a single freshly transformed E. coli colony and incubated for 8 h at 37 ºC and 250 rpm orbital shaking to produce a starter / inoculum culture. 5 ml of LB supplemented with antibiotic(s) were inoculated by 200 µl of starter culture and incubated at 37 ºC at 250 rpm orbital shaking. After 2 h of incubation (when the OD$_{600}$ of the bacterial culture was ~ 0.4-0.5) protein expression was induced by adding different concentrations of Isopropyl-β-D-thio-galactopyranoside (IPTG) (Melford, Suffolk, UK) and the cultures transferred to incubators set for temperatures 37, 30, 25 and 20 ºC and grown at 250 rpm.

b. Auto-induction medium

For auto-induction medium, to produce an inoculum culture, 2 ml of ZYP-0.8G medium containing the antibiotic(s) was inoculated with a freshly transformed bacterial colony and incubated at 37 ºC for 6-8 h. 5 ml of ZYP-5052 medium with appropriate antibiotic(s) were inoculated with the 25 µl of inoculum culture, first incubated at 37 ºC for 2 h and then transferred to incubators set at 37, 30, 25 or 20 ºC temperature with orbital shaking at 250 rpm.

2.7.3.3 Recording protein expression levels

At different times between 0 to 24 h after induction in case of LB medium and inoculation in case of ZYP-5052 medium, an aliquot of 1 ml bacterial culture was taken and pelleted to observe green fluorescence on UV transilluminator. At the same time, the level of expression was also recorded as relative fluorimetric units (RFU). To record RFU values, 1 ml of the culture was centrifuged at 13,000 rpm at room temperature. The bacterial cell pellet was washed by suspending in 500 µl of PBS and centrifuged again at 13000 rpm to remove any PBS. The cell pellet was dissolved in 150 µl of lysis buffer (250 mM Tris pH 7.5, 0.2 % Triton X-100, 4 mg / ml Na-deoxycholate), incubated at room temperature for 5 min, centrifuged as before and the supernatant transferred to cuvette of VersaFluor Fluoremeter (Bio-Rad) to record RFU values.

2.7.4 Crude membrane preparation from E. coli

E. coli crude membranes were extracted by Water Lysis method (Ward et al., 2000). The cells were grown under the conditions showing the optimum expression of GFP. The cells were harvested by centrifugation, suspended in 10 ml of 0.2 M Tris HCl pH 8.0, by gentle shaking at room temperature for 20 min. For the lysis of cells, 4.85 ml of sucrose buffer (1 M sucrose, 0.2 M Tris pH 8, 1 mM EDTA) were added to the suspended cells and after 90 sec continuous shaking by hand, 65 µl of freshly prepared 10 mg / ml lysozyme (Fluka, Dorset, UK) in sucrose buffer were added and mixed
well for another 30 sec and finally 9.6 ml of sterile distilled water was added. This mixture was stirred for 20 min at room temperature following by centrifugation in SS-34 at 18,000 rpm at 4 °C for 20 min. The periplasmic fraction was removed by decanting. The pellet was resuspended by homogenisation in 15 ml of distilled water followed by incubation of 30 min at room temperature. A further centrifugation step was carried out as before, to remove the cytoplasmic fraction. Finally the pellet was homogenised in 30 ml of phosphate buffer (0.1 M NaH₂PO₄, 1 mM β-mercaptoethanol) and the membranes collected by centrifugation. The pellet of membranes was suspended in 500 µl of phosphate buffer and stored at -20 °C until further use.

The protein concentration was determined by BCA Protein Assay Kit (Pierce, Thermo Scientific). 15 µg of total protein was run on a SDS-NuPAGE precast gel (Invitrogen, Paisley, UK) along with the molecular marker (PrecisionPlus Protein Standards, Bio-Rad). After fixing in gel fixative solution (45 % ethanol, 10 % acetic acid, 45 % distilled water), the gel was stained by His-tag stain (Invitrogen, Paisley, UK) to confirm the presence of His-tagged nitrate transporter protein. Coomassie Blue staining of SDS gel was also carried out to estimate expression levels. The band of the protein was cut from the Coomassie stained gel and its identity was confirmed by mass spectroscopy.

2.7.5 Pre-crystallisation screening of detergent

Fluorescence detection size-exclusion chromatography (FSEC) was used to screen the stability of GFP-fused nitrate transporter proteins in detergent following the method described by Kawate and Gouaux, (2006). Five detergents of different micelle sizes, n-dodecyl β-maltoside (DDM), n-decyl β-D-maltopyranoside (DM), n-octyl β-D-glucopyranoside (OG), n-octyl β-D-thioglucopyranoside (OTG) and n-dodecyl-N-N-dimethylamine-N-oxide (LDAO) were examined for proteins solubility and stability. All detergents (except DDM which was obtained from Glycon Biochemistry, GmbH Biotechnology, Germany) were purchased from Affymetrix, Anatrace (Woodburn Green, UK). 10 ml of the bacterial cultures were grown under the same conditions as optimised for the over-expression of GFP-fused proteins from a freshly transformed colony. The cells were collected by centrifugation at 10,000 rpm in a SS-34 rotor at 4 °C for 15 min. The cell pellet was suspended in 500 µl of sonication buffer (50 mM Tris HCl pH 8, 190 mM NaCl, 10 mM KCl, 15 mM EDTA, 10 µg / ml lysozyme from chicken egg white, (Fluka, Dorset, UK) and a tablet of complete protease inhibitor cocktail (Roche, Sussex, UK) per 100 ml of buffer). The suspended cells were lysed by sonication (Soniprep 150, MSE) three times in accord with the programme: sonication time 10 sec at 15 amplitude microns using a small probe and incubation on ice for 1 min to prevent the over-heating. The cell debri was removed by centrifugation at 8000 rpm using Eppendorf rotor F45-30-11 (Eppendorf centrifuge) at 4 °C for 20 min. Membranes were collected by ultra-centrifugation of the
supernatant in TLA 110 rotor (Beckman Coulter Optima Max-XP Ultra centrifuge) at 60,000 rpm at 4 °C for 20 min. The membrane pellet was solubilised in solubilisation buffer (50 mM Tris-HCl pH 8, 190 mM NaCl, 10 mM KCl, 15 mM EDTA, complete protease inhibitor and 40 mM of detergent tested) overnight at 4 °C on gentle mixing followed by centrifugation at 60,000 rpm in TLA 100 rotor for 20 min. 200 µl of the supernatant (sample) was run through a Superose 6 10 / 300 column (Superdex, GE Healthcare, Amersham) pre-equilibrated with running buffer (20 mM Tris-HCl pH 8.0, 190 mM NaCl, 10 mM KCl and 1 critical micelle concentration (CMC) of detergent to be tested).

Table 2.7: Concentration of detergents (1 CMC) used in solubilisation buffer.

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Mol. wt (g/mol)</th>
<th>1 CMC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDM</td>
<td>510.6</td>
<td>1</td>
</tr>
<tr>
<td>DM</td>
<td>482.6</td>
<td>1.8</td>
</tr>
<tr>
<td>OG</td>
<td>292.4</td>
<td>20</td>
</tr>
<tr>
<td>OTG</td>
<td>308.4</td>
<td>10</td>
</tr>
<tr>
<td>LDAO</td>
<td>229.4</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The values of 1 CMC for each detergent are according to the information provided by the supplier.

GFP fluorescence of eluent was detected by a fluorescence detector (Jasco, Intelligent Florescence Detector, Essex UK) at the excitation wavelength 395 nm and emission wavelength 509 nm attached to the ÄKTA Design Purifier using the Unicorn software (GE Healthcare, Amersham Biosciences, Uppsala, Sweden). Fractions were collected in 96-well microtiter plates in a fraction collector, Frac-950 (GE Healthcare).

2.7.6 Western blots
Plasmids were transformed in E. coli strains BL21(DE3) and cells were grown in LB (without induction) and auto-induction medium to study protein expression by western blotting. The cells were collected by centrifugation and the cell pellet was suspended in 500 µl sonication buffer. Such cells were disrupted by sonication at 4 °C using the sonicator (as described in section 2.7.5). Lysed cells were directly run on a NuPAGE Gel using the 35 min programme; 200 volts, 120 mA and 25 W mixed with 1 x NuPAGE LDS sample buffer in 1 x NuPAGE MES SDS running buffer (50 mM MES, 50 mM Tris Base, 0.1 % SDS, 1 mM EDTA, pH 7.3) with pre-stained protein size marker (PrecisionPlus Protein Standards, Bio-Rad). A PVDF membrane (Invitrolon), pore size 0.45 µm and activated by methanol was used to blot the protein bands with the semi dry transfer apparatus (Trans-Blot SD, Semi dry transfer cell, BioRad). The blot was assembled by placing two 3 MM Whatman papers of gel sized strips soaked in anode buffer 1, A1 (300 mM Tris, 20 % (v/v) methanol). Atop of this, a 3 MM paper wetted with anode buffer 2, A2 (25 mM Tris, 20 % (v/v) methanol) then the
PVDF membrane, pre-equilibrated with A2 buffer and over the membrane, gel pre-equilibrated with A2 buffer was placed. Finally three 3 MM Whatman papers wetted with cathode buffer, K (5.2 % (w/v) Aminocaproic acid, 20 % (v/v) methanol, 0.01 % (w/v) SDS) were placed on the very top of this set up. Transfer of protein was performed for 1 h at 25 volts, 160 mA and 17 W. The membrane was incubated for 1 h in 5 % membrane block (dry milk) at room temperature. After blocking, the membrane was incubated with anti-His antibodies (Invitrogen) in PBS at room temperature for 1 h. The membrane was washed with PBS for 3 periods of 10 min. Detection of protein was carried out by Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) and the membrane was exposed Fujifilm Intelligent Dark box and analysed by its programme Fujifilm LAS-1000.

2.7.7 Large scale bacterial protein extraction

2.7.7.1 Bacterial grow-ups
On the basis of observed FSEC results, scale-up preparation of proteins was carried out to purify the protein for crystallisation attempts. 6 L of ZYP-5052 medium (1 L medium / 5 L baffled culture conical flask) supplemented with 50 µg / ml kanamycin, was inoculated by 500 µl of starter culture produced in ZYP-0.8G medium (as described in section 2.7.3.2b). Flasks were incubated at 37 ºC for 1 h followed by the temperature reduced to 23 ºC. Such cultures were left to grow for 38-40 h. The OD₆₀₀ of the culture was recorded and cells were collected in a Thermo Scientific PTi F9S-4x1000Y rotor (Sorval RC-6 Plus) at 10,000 rpm, 4 ºC for 15 min. Finally the weight of the cells was recorded.

2.7.7.2 Membrane preparation
The bacterial cells were suspended in 500 ml of PBS, containing protease inhibitor and 10 µl of 30 mg / ml DNase I (Sigma) and incubated on a magnetic stirrer at 4 ºC. The suspended cells were lysed by passing through the cell disrupter (Constant Systems, Ltd) twice both at a pressure 30 Kpsi. Disrupted cells were centrifuged at 8000 rpm in a SS-34 rotor at 4 ºC for 30 min. The supernatant was transferred to new clean tube and the membranes were collected by centrifugation of supernatant at 40,000 rpm, 4 ºC for 1 h in 50.2 Ti rotor (Beckman L-60 Ultra-centrifuge). The membrane pellet was suspended in 100 ml of extraction buffer (50 mM Tris-HCl pH 8, 190 mM NaCl, 10 mM KCl and 1 CMC of detergent to be tested (see Table 2.7 for 1 CMC values of detergents) and incubated overnight at 4 ºC with magnetic stirring. Un-dissolved fractions were removed by ultra-centrifugation at 40,000 rpm 4 ºC for 1 h in 50.2 Ti rotor and precipitated protein removed. The supernatant was filtered through Millipore filters.
2.7.7.3 Programmed multistep protein purification

Using the ÄKTA purifier and Unicorn software (GE Healthcare, Amersham Biosciences, Uppsala, Sweden), filtered solubilised protein samples in extraction buffer were run through the nickel affinity or His-trap column (HisTrap FF 5 ml, GE Healthcare) pre-equilibrated with Buffer A (20 mM Tris-HCl pH 8.0, 190 mM NaCl, 10 mM KCl, 0.08 % (w/v) DDM / DM or 0.3 % (w/v) OTG) from one column volume of 150 ml Superloop (GE Healthcare) at the flow rate of 2 ml / min. Total protein was monitored by reading the absorbance at 280 nm and GFP-fused protein by detecting florescence by fluorescence detector. His-tagged protein that was trapped by the His-trap column was eluted and collected by the increasing concentration of imidazole in buffer A in a 96-well microtiter in fraction collector. This was done by mixing buffer A with buffer B (1 M imidazole added to buffer A).

2.7.7.4 Manual purification of protein

Purification of recombinant proteins containing poly histidine tags was also performed using the nickel beads (agarose beads coated with nickel) by ion metal affinity. Protein samples were incubated with nickel beads on slow revolving overnight at 4 ºC. The beads were collected using a syringe followed by washes with buffer A. Starting with the 25 mM imidazole in buffer A, protein was eluted with increasing concentration of imidazole in buffer A (40 mM, 100 mM, 300 mM and finally by 1 M imidazole).

2.7.7.5 TEV cleavage and second nickel column

To remove residual imidazole, protein fractions were concentrated to 5 ml in the Viva spin ultrafiltration tube (Sartorius Stedium, Goettingen, Germany) at 4500 rpm at 4 ºC. Buffer A was added to final volume 100 ml to the concentrated protein and incubated with an aliquot of 5 mg of TEV protease for overnight digestion at 4 ºC. The cleaved protein was purified by incubating the digest with nickel beads. The His-tagged-GFP section of the protein attached to the nickel beads and nitrate transporter was purified. Purified protein samples were run on a NuPAGE SDS gel with protein size marker and its identification was verified by mass spectroscopy.
In vivo pathogenicity studies

3.1 Introduction

3.1.1 Health risks associated with Aspergillus species

The aspergilli are ubiquitous in nature and have been involved in human affairs mainly due to their industrial applications and pathogenicity. This is exemplified by the following. (i) Aspergillus sojae and A. oryzae, the so-called the koji molds, have a longest history over many centuries in making miso sake and soya sauce. A. niger is another industrially important species and involved in the production of citric acid and certain commercial enzymes such as, pectinases, amylases and proteinases. These three fungi, A. sojae, A. oryzae and A. niger have been given the status of ‘generally regarded as safe’ (GRAS) by USA Food and Drug Administration. (ii) Whilst other Aspergillus species such as A. fumigatus, A. flavus and A. terreus are not specialised, such that they infect animals or humans, they can act instead as opportunistic organisms causing infection in immuno-suppressed individuals (Raper and Fennell, 1965; Bennett, 2009 and references therein). (iii) A. flavus and A. parasiticus are prominent in producing highly toxic secondary metabolites, including aflatoxins (Payne and Brown, 1998; Yu et al., 2004; Keller et al., 2005).

With regard to human health risk, A. fumigatus arguably is the most notorious species of the genus Aspergillus (Bennett, 2009 and references therein) and causes Aspergillosis, a condition of three main clinical subgroups; allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis (Latge, 1999; Latge, 2001). A. flavus too causes invasive aspergillosis (Hedayati et al., 2007). In this regard studies on the healthy and immuno-deficient mice demonstrated that A. flavus pathogenicity is approximately 100 times higher than A. fumigatus (Mosquera et al., 2001). Pathogenicity of A. terreus is also well established (Chang and King, 1986; Tiwari et al., 1995; Iwen et al., 1998; Schett et al., 1998). A. niger is the low potential pathogen and reported infection by A. niger include otomycosis, an chronic ear infection (Araiza et al., 2006), cutaneous infections and pneumonia (Person et al., 2010).

Aspergillus nidulans was selected by Pontecorvo as a potential genetic laboratory tool in the 1950’s to explore eukaryotic genetics. This organism seemed to be harmless but later unusual pathogenic features were observed (Kim et al., 1997; Bennett, 2009). Similar to other Aspergillus species, the infections by A. nidulans have been reported mostly in patients with chronic granulomatous disease (CGD). CGD is a rare disorder in which phagocytes are defective in generating reactive antimicrobial
oxidants and hence patient is immuno-deficient for microbial infections (Lucas et al., 1999). However there are a few reports of diseases by A. nidulans, for example brain abscess, in the individuals without immuno-suppression have also been reported (Chakrabarti et al., 2006). Primary cutaneous infection (Lucas et al., 1999), cerebral aspergillosis and extensive spinal cord infection are some reported cases by A. nidulans.

3.1.2 Galleria mellonella, a model host to study fungal pathogenesis

The use of mammalian infection laboratory models, such as mice, rats and guinea pigs added to our knowledge of mammalian immune systems, pathogenicity features of microbes, drug testing and resistance to drugs (Kavanagh and Fallon, 2010 and references therein). However, the use of mammalian models has practical problems relating to space required, expensive, legal and ethical constrains. All of these considerations discourage their use. Therefore for in vivo testing of microbial pathogenicity in alternate hosts, free of these constrains and provide reliable data, would of great benefit (Ratcliffe, 1985; Mylonakis, 2008).

Given the high degree of structural and functional homology between the insect and mammalian innate immune system (Ratcliffe, 1985; Hoffman, 1995; Salzet, 2001; Kimbrell and Beutler, 2001), the use of insects as an alternate mammalian host in studying microbial infection could provide similar information (Vilmos and Kurucz, 1998; Salzet, 2001; Kavanagh and Fallon, 2010). The function of haemocytes present in the haemolymph of insects is comparable to phagocytes of mammals. Phagocytosis in insects is a sequential process that includes the recognition of foreign particle by the receptors present on the surface of haemocytes, that produce antimicrobial peptides and enzymes which finally engulf and destroy the foreign body (Baggiolini and Wymann, 1990; Vilmos and Kurucz, 1998).

The greater wax moth, G. mellonella has emerged as a promising invertebrate host to study fungal pathogenesis (Mylonakis, 2008; Fuchs et al., 2010a; Fuchs et al., 2010b; Kavanagh and Fallon, 2010). The reasons that have made this particular insect larva a desirable model to study pathogenesis as well as host defence mechanism since larvae are, (i) inexpensive, (ii) readily available from local suppliers, (iii) of a reasonable size, (iv) maintained in standard petri dishes (or plastic containers) without the need for special equipment in a temperature range 25 to 37 °C, (v) inoculated with a known quantity of fungal inoculum injected directly into the haemocoel with the minimum of training, (vi) that no UK Government Home Licence is required, (vii) monitored simply on the basis of their mortality and finally (viii) that post-infection changes in the larvae can also be studied by
examining the infected internal structures directly under the microscope (Mylonakis, 2008; Fuchs et al., 2010a).

The correlation between the level of pathogenicity and type of host has been used to compare the response of *G. mellonella* larvae or mammalian host, mice. Strong agreement of results in both types of hosts was found when inoculated with lipopolysaccharide-deficient mutants of *Pseudomonas aeruginosa* (Jander et al., 2000), strains of *Bacillus thuringiensis* and *B. cereus* (Salamitou et al., 2000) and the dimorphic yeast *Candida albicans* (Brennan et al., 2002). In all these experiments positive correlation between pathogenicity profiles of these pathogens in *G. mellonella* larvae and mice demonstrated that comparable results may be obtained by using simple, ethically acceptable and cost effective insect larvae.

Although *G. mellonella* has many advantages over mammalian hosts, these insects cannot be used to study certain disease stages that are specific to mammalian tissues. Another problem associated with the use of the larvae is the unavailability of its genome sequence. Finally, unlike the fly and nematode, the moth larva does not have an adaptive immune system. Therefore pathogenicity might be significantly higher or lower in larvae than the mammalian hosts (Mylonakis et al., 2005).

Fungal infections are important and commonly found in immuno-suppressed patients (Raper and Fennell, 1965; Bennett, 2009). One example of such opportunistic infection is aspergillosis, mostly caused by *A. fumigatus* and *A. flavus* (Latge, 1999; Latge, 2001; Daly and Kavanagh, 2001; Bennett, 2009). *G. mellonella* larvae have been successfully used to investigate the pathogenesis of fungal strains including *Aspergillus fumigatus*, *A. flavus*, *A. niger* and *A. nidulans* (St. Leger et al., 2000; Reeves et al., 2004; Mylonakis et al., 2005; Renwick et al., 2006; Fuchs et al., 2010a; Fuchs et al., 2010b).

3.1.3 Studies of fungal pathogenicity using *Galleria mellonella* larvae

St. Leger et al. (2000) investigated the ability of the filamentous fungi, *A. flavus*, *A. fumigatus* and *A. nidulans* to infect *G. mellonella* larvae. They studied the pathogenicity level of eight different clinical isolates of *A. flavus*, four isolates of *A. fumigatus* (two human, one rat and one from cockatoo) and a *A. nidulans* mutant strain with the veA1 affecting conidiation. The results demonstrated that all strains of these three aspergilli could not invade the larva when applied on the cuticle. However, in contrast, spore injection of all *A. flavus* human derived strains resulted in 100 % larval kill within 48 h post-inoculation. St. Leger and colleagues also observed that spores of *A. fumigatus* and *A. nidulans* did
not kill a single living larva 5 days post-injection, however they were able to colonise the freeze-killed larvae.

*G. mellonella* larvae had also been used to identify a role of the fungal metabolite, gliotoxin (an immuno-suppressive), produced by *A. fumigatus* clinical isolates (Reeves *et al.*, 2004). In another study, using the *G. mellonella* larvae it was established by Reeves and colleagues that the germination stage of conidia greatly influenced *A. fumigatus* pathogenicity. In this respect it was observed that the haemocytes could not phagocytose outgrowing conidia, resulting in significant killing with fewer numbers of spores as compared to the non-germinating or in early germination stage conidia (Renwick *et al.*, 2006).

Larvae of *G. mellonella* have been used to study the pathogenicity of certain human yeast pathogens, for example, *Candida albicans* (Dunphy *et al.*, 2003; Fuchs *et al.*, 2010a; Fuchs *et al.*, 2010 b), *Cryptococcus neoformans* (Mylonakis *et al.*, 2005; Fuchs *et al.*, 2010a). The results indicated that the rate of mortality depends on inoculum size and the type of strain studied.

### 3.2 Objective

The objective of the work presented in this chapter is to ascertain if *A. nidulans*, first identified and exploited by Pontecorvo half a century or more ago, as an experimental eukaryotic organism and to be used throughout this current study, is indeed safe to handle by laboratory workers. Previous work by St. Leger and colleagues (2000; and see introduction) showed that both *A. nidulans* and *A. fumigatus* were non-pathogenic. However, they used a *A. nidulans* strain containing a conidial mutation. In addition, there are more recent but contradictory reports (see introduction) of *A. fumigatus* causing high-level larval deaths. Consequently, we decided to revisit potential pathogenicity exhibited by *A. nidulans*, the organism used to study nitrate metabolism in this thesis and extended the study to examine further aspergilli.
3.3 Results

3.3.1 Determination of viable spore number in the fungal inoculum

Each larva was injected with $10^5$ spores (10 µl of a spore suspension at a concentration of $1 \times 10^7$ spores / ml) of individual *Aspergillus* species studied (for detail of species see Materials and Methods, Chapter 2, Table 2.1). The number of viable spores or colony forming units (CFU) present in the spore suspension injected to the larvae was recorded and presented in Table 3.1.

Figure 3.1: Selection and inoculation of larvae.

(a) Healthy larvae, without any grey or black markings on the body, of approximate equal size were selected for present study. (b) The pro-legs of larva were exposed for inoculation. (c) Fungal inoculation procedure was injection directly into the haemocoel through the left pro-leg using a 100 µl Hamilton syringe.
Table 3.1: Viable conidial spore number in fungal inocula.

<table>
<thead>
<tr>
<th>Species / treatment</th>
<th>No of viable spores per ml inoculum</th>
<th>Approximate number of viable spores injected into each larva</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. fumigatus (MMG)</td>
<td>$1.3 \times 10^6$</td>
<td>13,000</td>
</tr>
<tr>
<td>A. fumigatus (MMG - heated)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. nidulans (STA171)</td>
<td>$2.2 \times 10^6$</td>
<td>22,000</td>
</tr>
<tr>
<td>A. nidulans (C51)</td>
<td>$2.2 \times 10^6$</td>
<td>22,000</td>
</tr>
<tr>
<td>A. nidulans (C49)</td>
<td>$4.8 \times 10^6$</td>
<td>48,000</td>
</tr>
<tr>
<td>A. flavus (MMG)</td>
<td>$2.9 \times 10^6$</td>
<td>29,000</td>
</tr>
<tr>
<td>A. flavus (MMG - heated)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. oryzae (IMI283863)</td>
<td>$1.7 \times 10^6$</td>
<td>17,000</td>
</tr>
<tr>
<td>A. oryzae (IMI283863 - heated)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. oryzae (IMI283874)</td>
<td>$1.1 \times 10^6$</td>
<td>11,000</td>
</tr>
<tr>
<td>A. oryzae (IMI283874 - heated)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. sojae (RIB1045)</td>
<td>$2.1 \times 10^6$</td>
<td>21,000</td>
</tr>
<tr>
<td>A. sojae (RIB1045 - heated)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. terreus (MMG)</td>
<td>$4.2 \times 10^6$</td>
<td>42,000</td>
</tr>
<tr>
<td>A. terreus (MMG - heated)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. niger (IMI60286)</td>
<td>$3.9 \times 10^6$</td>
<td>39,000</td>
</tr>
<tr>
<td>A. niger (Gb)</td>
<td>$1.1 \times 10^6$</td>
<td>11,000</td>
</tr>
</tbody>
</table>

Serial dilutions of spore suspensions for each Aspergillus species were carried out to calculate the number of viable spores that was actually used to inject larvae in this survey. Aliquots of dilutions were plated out on complete medium. The number of colonies (from viable germinating spores) was determined after 2 days incubation at 37 ºC. Strains denoted as ‘heated’ refers to spores subjected to heat treatment at 70 ºC for 45 min. Values shown in this table are the mean of three independent viable counts for each strain. The number of viable spores present in a 10 µl aliquot of fungal inoculum was calculated and presented in the table. Strains designated as follows; MMG, Department of Medical Mycology, University of Glasgow, UK; IMI, International Mycological Institute, UK; RIB, Research Institute of Brewing, Tokyo, Japan; Gb, (Gist-brocades, Dutch Industrial company); C, Croft, University of Birmingham; STA, a prototrophic wild type recombinant with green spores, University of St. Andrews.

3.3.2 A trial experiment

Initially to test experimental operational feasibility and to become familiar with the procedures, ten larvae were used for the controls as well as the test system. For the test experiment, spores of A. fumigatus were used since recent publications reported of its pathogenicity (Reeves et al., 2004; Renwick et al., 2006). The data presented in Figure 3.3 shows that the experiments were being
performed satisfactory. However as 10 larvae were on the low side for statistical analyses, the number of larvae was increased to 30 in all further experiments.

With respect to the trial experiment, 10 µl of an *A. fumigatus* spore suspension containing approximately 13,000 living spores, was injected to each larva to assess pathogenicity. Two control groups were run in parallel, one group injected with equal number of heat-killed spores and other one with spore suspension solvent (saline Tween 80). These insect cultures were incubated at 37 ºC. The appearance of inoculated larvae was monitored every 24 h up to 192 h post-injection for their survival. The results demonstrated that no death of larvae infected with the viable spores of *A. fumigatus* was recorded until 72 h post-inoculation and after 192 h of inoculation 48 % larvae survived. The killing of larvae by *A. fumigatus* living spores was significant (*P* = 0.00039) compared to the heat-treated spores and with those injected with saline Tween 80 (control). Extensive melanisation in the body of dead larvae was observed (Figure 3.2). It was assumed from this experiment, our system was working satisfactory and we preceded to exam other aspergilli.

Figure 3.2: Infection of *G. mellonella* larvae by *A. fumigatus*.

(a) Living larvae after 192 h inoculation with saline Tween 80 only. (b) Larvae injected with *A. fumigatus* living spores that have subsequently died. The blackening of the dead body (cadaver) is an indicative of melanisation.
Figure 3.3: Kaplan-Meier survival probability plot of *G. mellonella* larvae by *A. fumigatus*.

Living spores of *A. fumigatus* showed significant killing (*P* < 0.05) of *G. mellonella* larvae compared to heat-killed spores and control group (injected with saline Tween 80) of larvae.

3.3.3 *A. nidulans*

*A. nidulans* strains STA171, C51 and C49 were analysed for pathogenicity by determining the survival of larvae injected with living spores. Each larva was injected with approximately 22,000 viable spores (calculated on from the plates after 2 days growth, as for *A. fumigatus*) for both STA171 and C51, and 48,000 living spores for the C49 strain were injected to each larva. The results showed that 91% larvae injected with *A. nidulans* strain STA171 and 86% injected with C51 survived 168 h post-inoculation. Larval death due to *A. nidulans* spores showed non-significant difference (*P* = 0.246 and 0.0737 for STA171 and C51 respectively) from control group (saline Tween 80) (Figure 3.4). Moreover larvae treated with strains STA171 and C51 did not show any larval morphological differences from the control group.

After 168 h of larvae incubation with spores of strain C49, 80% larvae survived and the mortality was significant (*P* = 0.006) as compared to the control group of larvae. Some melanisation on the surface of infected larvae was observed.
3.3.4 *A. flavus*

Each larva was infected with approximately 29,000 viable spores of *A. flavus*. The data presented in Figure 3.5, shows that injection of *A. flavus* living spores resulted in rapid killing of larvae and 90% larvae died within 24 h of post-inoculation. Compared with the control group of larvae (injected with saline Tween 80), significantly high mortality by the viable spores of *A. flavus* occurred suggested its role as a pathogen. After 24 h of death, a white mycelial mass was observed penetrating from the inside to the outside of dead larval cuticle. On the following day, the entire larval body became covered with green spores that resulted from most likely asexual sporulation (Figure 3.10).

To confirm that these larvae died from fungal infection, a similar number of heat-treated (i.e. non-viable) *A. flavus* spores (Table 3.1) were injected and larvae were monitored for survival. Infection with the heat-killed spores was significantly less than the living spores. However, compared to the control group, heat-killed spores injection resulted in observable larval killing ($P = 0.000531$) with 70% larvae surviving the 168 h treatment (Figure 3.5).
Figure 3.5: Kaplan-Meier survival probability plot of *G. mellonella* larvae by *A. flavus*.

Non-significant larval death (*P* > 0.05) by heat killed spores as compared to viable spores was recorded.

3.3.5 *A. oryzae*

Spore injection of either *A. oryzae* strain IMI283863 (17,000 viable spores injected per larva) or IMI283874 (11,000 viable spores injected per larva) resulted in a 100 % kill after 24 h incubation (Figure 3.6). The insect cadavers were observed to be black probably due to melanisation (Figure 3.10).

On the other hand, injection of heat-treated spores of *A. oryzae* strain IMI283863 failed to kill larvae even after 168 h incubation (*P* = 0.285). Infection with strain IMI283874 heat-treated spores resulted in a kill of just 14 % larvae. However the mortality rate by heat-killed IMI283874 spores was not significantly different from the saline Tween 80 larval control group (*P* = 0.0662).
Infecting larvae with the viable spores of both strains of *A. oryzae* caused significant killing of larvae but heat-killed spores of both strains were non-infectious.

### 3.3.6 A. sojae

Injection of approximately 21,000 living spores of *A. sojae* (RIB1045) resulted in a high larval mortality rate. More than 80 % larvae died during the first 24 h of inoculation and the remaining expired over the following 24 h. When heat-treated spores were examined, 96 % of the larvae survived after 168 h incubation and this figure was not significantly different (*P* = 0.924) from the saline Tween 80 control group (Figure 3.7).

### 3.3.7 A. terreus

The *A. terreus* strain used for this present study, proved pathogenic for *G. mellonella* larvae. After 96 h of inoculation with the 42,000 living spores, 70 % of the larvae survived. The number of surviving larvae dropped to 30 % in 120 h post-infection and to 20 % after 168 h of spore injection. Darkening on the bodies of dead larvae, the indication of melanisation was observed. Injecting larvae with the heat-killed spores of *A. terreus* resulted in significantly prolonged survival (*P* = 0.072) and 86 % larvae survived after 168 h of injection (Figure 3.8).
Living spores of *A. sojae* were significantly pathogenic while heat killed spores were absolutely non-pathogenic for *G. mellonella* larvae.

Living spores of *A. terreus* were significantly pathogenic for *G. mellonella* larvae than the heat-killed spores.
3.3.8 *A. niger*

The strains *A. niger* (Gb) and *A. niger* (IMI60286) were tested for their pathogenicity. The inoculum size injected was approximately 11,000 viable spores for *A. niger* (Gb) and around 39,000 living spores for (IMI60286). After 72 h of infection, no deaths were recorded for either strain. However by the end of the 168 h incubation period, the strains exhibited different results with respect to pathogenicity (Figure 3.9). *A. niger* (Gb) was clearly non-pathogenic ($P = 0.513$) with 93 % surviving larvae after 168 h of inoculation. Although 80 % larvae survived by the end of experiment but statistical analyses showed larval mortality by *A. niger* strain IMI60286 was significant ($P = 0.00659$).

Figure 3.9: Kaplan-Meier survival probability plot of *G. mellonella* larvae by *A. niger* strains.

Statistical analysis revealed that the spores of *A. niger* (Gb) caused non-significant killing of larvae but spores of *A. niger* strain IMI60286 resulted in significant mortality.
Figure 3.10: Pathogenesis of *Aspergillus* species in *G. mellonella* larvae.

The figure shows a phenotypic comparison of larvae killed by pathogenic fungi in this present study. The control group was injected with saline Tween 80. The body of *A. flavus* infected dead larvae was covered with green spores. As a result of defence response, melanisation could be observed on the bodies of other dead larvae (as they turned black). MMG is for, Department of Medical Mycology, University of Glasgow, UK; IMI, International Mycological Institute, UK; RIB, Research Institute of Brewing, Tokyo, Japan and Gb, Gist-brocades, Industrial company, Netherlands.

### 3.4 Discussion

The main object of this section of the work was to determine if *Aspergillus nidulans* is safe to use, at least as determined by insect larval bioassays. The results presented here show that all the three strains of *A. nidulans* have extremely low or zero larval morbidity. This included, a prototrophic derivative (STA171) of the original strain developed by Pontecorvo (University of Glasgow) and two strains (C49 and C51) isolated by Croft (University of Birmingham) from the natural environment. The studies on host specialisation of *Aspergillus* species by St. Leger *et al.* (2000) identified *A. nidulans* as non-pathogenic fungus for *G. mellonella* larvae. However they used just 3000 spores per larva and spore numbers were deduced from microscopic counts only and therefore many spores may not have been viable. In the present study, spores of *A. nidulans* laboratory strain (STA171) were completely avirulent even although (i) the inoculum size used was much larger (22,000 per larva) than that used by St. Leger and colleagues (3000 spores per larva) (ii) all spores were shown to be viable and (iii) a genetically wild type strain was used in this study. Moreover, number of viable spores (48,000) for the Croft strain C49 did not bring about large fatalities (Table 3.1). It has been reported in *A. fumigatus* (Renwick *et al.*, 2006) and also in human pathogen *Cryptococcus neoformans* (Mylonakis *et al.*, 2005) that the killing correlates to the inoculum size. The number of viable spores of strain C49 that injected to each larva was 48,000, more than the double counted for...
the other two strains, STA171 and C51 (Table 3.1). Finally, it is noteworthy that they found that the A. nidulans strain St. Leger and co-workers used, did not have the ability to resist the digestion by the haemocytes of larvae, an experiment which we did not repeat.

The strain from the industrial enzyme Dutch company, Gist-brocades (Gb) was mostly likely found to be safe and therefore deserving of the status ‘generally regarded as safe’ (GRAS). The results of the present study recommended A. niger (Gb) as a non-pathogenic strain. However A. niger strain IMI60286 caused significant larval death. This difference in the pathogenicity of two strains of the same species may be due to differences in the capacity of mycotoxins production. Revees and colleagues (2004) identified an environmental isolate of A. niger for the production of gliotoxin, a fungal metabolite known as an immuno-suppressive agent which might have caused death of the G. mellonella larvae. Another reason which could explain the strain difference in pathogenicity could be the number of viable spores injected (Table 3.1). Each larvae infected with A. niger (Gb) received 11,000 viable spore while for the strain IMI60286 received 39,000 viable spores, almost four times higher than the inoculum size injected than A. niger (Gb). However, strain Gb experiments should be repeated with different spore inocula sizes to investigate such strain differences. In contrast, A. flavus (MMG), A. oryzae (IMI283863 and IMI283874), A. terreus (MMG) and A. sojae (RIB1045) strains were found to have the ability to kill moth larvae.

Our finding of A. flavus pathogenesis was similar with that reported by St. Leger and colleagues (2000) who observed high larval death rates. From this present study, it is clear that A. flavus caused lethal larval infection and not only killed the larvae at a rapid rate but also grew on the dead larval bodies as a saprophyte and sporulated on the surface of body in order to disseminate the species.

To my knowledge, three species of Aspergillus included in this survey, A. terreus, A. oryzae and A. sojae have not been studied previously for pathogenicity at least using G. mellonella. As the strain of A. terreus used for this present study was a clinical isolate, it is perhaps not unsurprising that it exhibited pathogenic characteristics in our present experiment. However a surprising finding was the significant pathogenicity exhibited by A. oryzae (strains IMI283863 and IMI283874) as well as A. sojae (RIB1045) (Figure 3.6, Figure 3.7). Pathogenicity of A. oryzae and A. sojae could be explained by their ability to produce mycotoxin molecules. Both A. oryzae and A. sojae belong to the taxonomic group Genus Aspergillus section Flavi, a group of closely related Aspergillus species and some of them such as A. flavus and A. parasiticus are aflatoxin producing. Both A. oryzae and A. sojae are not only morphologically similar to the aflatoxin producing species, A. flavus, but share such a high DNA sequence similarity such that both these species could be given taxonomic status of ‘variety’ of A.
flavus. The homologues of several aflatoxin producing genes are also present in A. oryzae and A. sojae (Takahashi et al., 2002). These genes were found to be non-functional as they apparently do not produce a transcript (Kurtzman et al., 1987; Chang et al., 1995; Klich et al., 1995; Kusumoto et al., 1998). It is possible that under conditions provided in our present experiment, such genes were actually expressed and thus resulting in toxin production. A second possibility is that as the larvae do not have an adaptive immune system, these fungal strains are pathogenic in larvae but non-pathogenic to mammals and nematodes (Mylonakis et al., 2005). As both A. oryzae strains and A. sojae were procured from fungal culture collection centres (IMI and RIB) and were identified many decades before on the basis of conventional microscopic characterization, a third possibility could be one of mis-identification due to close morphological resemblance with A. flavus.

3.5 Conclusion
The main results presented here are (i) a wild type prototrophic laboratory strain of A. nidulans (as well as genetic derivatives and transformants) used extensively in this research work on nitrate assimilation and presented in this thesis, is found to be safe to handle in the laboratory when pathogenicity is tested using the G. mellonella larvae model, (ii) A. fumigatus and A. flavus are pathogenic as reported before in certain published research papers and (iii) previously unreported aspergilli; A. terreus, A oryzae and A. sojae have been observed to have quite nasty consequences for moth larvae and most likely constitute a health risk for humans.

Acknowledgement
I wish to thank Dr. Peter Coote (University of St. Andrews) for help and assistance during performing this section of my research work.
Chapter Four

Characterisation of newly discovered genes associated with nitrate metabolism

4.1 Introduction

4.1.1 Background

Aspergillus nidulans has been used for more than 50 years as a model eukaryotic organism to study nitrate metabolism, cofactor biosynthesis and the regulation of gene expression (Cove and Pateman, 1963; Pateman et al., 1964; Pateman et al., 1967; Pateman and Cove, 1967; Johnson et al., 1980; Brownlee and Arst, 1983; Unkles et al., 1997; Millar et al., 2001; Unkles et al., 2001; Heck et al., 2002; Wang et al., 2008a; Takaya 2009; Schinko et al., 2010 and see Chapter 1, General Introduction).

The fully sequenced genome of A. nidulans, consisting of 8 chromosomes, with an assembly size of 30,068,514 base pairs (bp), has been made available (Galagan et al., 2005). Genome sequencing information not only provides evolutionary relationships between different groups of organisms, but may also unearth novel genes involved in a specific physiological or metabolic process. The availability of the fully sequenced genome of A. nidulans is a technical landmark in many respects not least for determining hidden aspects of nitrate metabolism in this eukaryotic model organism.

4.1.2 Discovery of four putative genes for nitrate metabolism

A preliminary BLASTP (Basic Local Alignment Search Tool) search, using the A. nidulans assimilatory nitrate reductase, NiaD protein (see Chapter 1, General Introduction), as the query sequence was carried out by S E Unkles. The BLASTP results identified two further nitrate reductases, which were designated as NiaB and NiaC. The NiaC protein, encoded by the niaC gene located on chromosome V, was annotated in the Aspergillus Genome Database (AspGD) as an assimilatory nitrate reductase. High sequence homology was observed between amino acid sequences of the NiaC and assimilatory nitrate reductase of A. nidulans, NiaD. On the other hand, the NiaB protein, encoded by the niaB gene positioned on chromosome VII, was annotated as a dissimilatory nitrate reductase. Surprisingly when S E Unkles explored surrounding DNA to niaB, a contiguous gene was predicted to be involved in molybdenum cofactor (MoCo) biosynthesis and consequently designated cnxK (for background information of known cnx genes see Chapter 1, General Introduction). The BLASTP results with the protein CnxK as the query revealed that CnxK was clearly related to MoeA which is a domain of the CnxE protein. The MoeA domain is involved in the conversion of molybdopterin (MPT) to the molybdenum cofactor required for assimilatory nitrate reductase (NiaD) (Chapter 1, General Introduction). A further gene contiguous to cnxK, encoded a putative protein which showed
high similarity with *E. coli* MobA, a protein involved in molybdopterin guanine dinucleotide (MGD) biosynthesis from the MoCo (see Chapter 1, General Introduction). This protein was designated CnxL (S E Unkles, Unpublished Data). The discovery of these four genes was somewhat surprising given that they had lain hidden for more than half a century of nitrate assimilation research. The new genes identified by computational analyses raised very interesting questions regarding their potential function and expression in nitrate metabolism in this model eukaryotic organism.

### 4.2 Objective

The aim of this line of research was to characterise the function and expression of the *niaB*, *niaC*, *cnxK* and *cnxL* genes by bioinformatics approaches, northern blotting and knock-out gene technology to gain clues as to their potential functions in nitrate metabolism.

### 4.3 Results

4.3.1 *In silico* analysis

4.3.1.1 Gene structure and arrangement

Accession numbers in AspGD for the *cnxL*, *cnxK*, *niaB* and *niaC* genes are AN12130, AN9038, AN9037 and AN8449, respectively. The genes *cnxL*, *cnxK* and *niaB* were found to be contiguous and located on chromosome VII in the gene order *cnxL*-*cnxK*-*niaB*. In this gene cluster, between *niaB* and *cnxK* is an intergenic region of around 200 bp (Figure 4.1). This could be a promoter region with the genes being divergently transcribed. Upstream of the *cnxK* gene is *cnxL* with only 6 bp between the putative stop codons of *cnxK* and *cnxL* (Figure 4.1). The *niaC* gene is present on a different chromosome, chromosome V. Studying gene annotations in AspGD and carrying out BLASTP searches, there do not appear to be any other potential nitrate assimilation genes in the near vicinity of *niaC*. A summary of the general characteristics of *cnxL*, *cnxK*, *niaB* and *niaC* genes is presented in Table 4.1.
Table 4.1: Summary of the general characteristics of newly identified cnx and nia genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Located on chromosome</th>
<th>Accession no.</th>
<th>No. of nucleotides</th>
<th>No. of putative introns</th>
<th>No. of amino acid residues in putative protein</th>
<th>Annotated function</th>
</tr>
</thead>
<tbody>
<tr>
<td>cnxL</td>
<td>VII</td>
<td>AN12130</td>
<td>690</td>
<td>0</td>
<td>225</td>
<td>MGD synthesis</td>
</tr>
<tr>
<td>cnxK</td>
<td>VII</td>
<td>AN9038</td>
<td>1329</td>
<td>0</td>
<td>442</td>
<td>MoCo biosynthesis</td>
</tr>
<tr>
<td>niaB</td>
<td>VII</td>
<td>AN9037</td>
<td>3193</td>
<td>3</td>
<td>1009</td>
<td>Dissimilatory nitrate reductase</td>
</tr>
<tr>
<td>niaC</td>
<td>V</td>
<td>AN8449</td>
<td>3297</td>
<td>5</td>
<td>1016</td>
<td>Assimilatory nitrate reductase</td>
</tr>
</tbody>
</table>

Proteins characteristics were searched on AspGD, the Aspergillus Genome Database (www.aspergillusgenome.org). MGD, molybdopterin guanine dinucleotide, MoCo, molybdenum cofactor.

Figure 4.1: Genomic region of A. nidulans containing the gene cluster cnxL-cnxFK-niaB on chromosome VII.

Genes are shown with coloured arrows at their relative position in the gene cluster cnxL-cnxFK-niaB in chromosome VII of A. nidulans. The top line scale shows the chromosome coordinate numbering of this region of the chromosome according to AspGD. The chromosome coordinates for the intergenic regions are indicated below the relevant region. If functional the cnxFK and cnxL genes would transcribe convergently as indicated by the direction of arrows and have an intergenic region of 6 bp (from 65977 to 65983). The niaB gene is downstream of the cnxFK with an intergenic region from 67313 to 67512 (i.e 200) bp. The black horizontal bars on the coloured arrows (genes) represent the positions of putative introns.

4.3.1.2 Presence of cnxL, cnxFK and niaB genes in other aspergilli

Genes homologous to cnxL, cnxFK and niaB were revealed using the Orthologous Clusters Tool in AspGD. NCBI (National Centre for Biotechnology Information) BLAST searches were carried out using the amino acid sequences of the proteins translated from the cnxL, cnxFK and niaB genes as queries and the percentage homologies obtained were recorded in Table 4.2.
Table 4.2: Identity of CnxL, CnxK and NiaB proteins with their homologues in *Aspergillus* spp.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Orthologues found</th>
<th>Gene ID</th>
<th>No. of amino acid residues in putative protein</th>
<th>% amino acid identity</th>
<th>Annotated function</th>
</tr>
</thead>
<tbody>
<tr>
<td>cnxL</td>
<td><em>A. fumigatus</em></td>
<td>Afu3g15160</td>
<td>163</td>
<td>58</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td><em>A. flavus</em></td>
<td>AFLA_023580</td>
<td>154</td>
<td>57</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td><em>A. fischeri</em></td>
<td>NFIA_061920</td>
<td>230</td>
<td>54</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td><em>A. oryzae</em></td>
<td>AOR_1_2612154</td>
<td>229</td>
<td>60</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td>cnxK</td>
<td><em>A. fumigatus</em></td>
<td>Afu3g15170</td>
<td>448</td>
<td>50</td>
<td>MoCo biosynthesis</td>
</tr>
<tr>
<td></td>
<td><em>A. flavus</em></td>
<td>AFLA_023570</td>
<td>451</td>
<td>49</td>
<td>MoCo biosynthesis</td>
</tr>
<tr>
<td></td>
<td><em>A. fischeri</em></td>
<td>NFIA_061910</td>
<td>451</td>
<td>50</td>
<td>MPT biosynthesis</td>
</tr>
<tr>
<td></td>
<td><em>A. oryzae</em></td>
<td>AOR_1_2948154</td>
<td>450</td>
<td>51</td>
<td>MPT biosynthesis</td>
</tr>
<tr>
<td>niaB</td>
<td><em>A. fumigatus</em></td>
<td>Afu3g15190</td>
<td>910</td>
<td>71</td>
<td>Periplasmic nitrate reductase</td>
</tr>
<tr>
<td></td>
<td><em>A. flavus</em></td>
<td>AFLA_023560</td>
<td>981</td>
<td>71</td>
<td>Periplasmic nitrate reductase</td>
</tr>
<tr>
<td></td>
<td><em>A. fischeri</em></td>
<td>NFIA_061900</td>
<td>972</td>
<td>71</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td></td>
<td><em>A. oryzae</em></td>
<td>AOR_1_2616154</td>
<td>990</td>
<td>72</td>
<td>Periplasmic nitrate reductase</td>
</tr>
</tbody>
</table>

Amino acid sequences of proteins encoded by *cnxL*, *cnxK* and *niaB* genes were obtained from the AspGD using their accession numbers (gene ID). The online Orthologous Clusters Tool identified the orthologous proteins in other species of the genus *Aspergillus*. The % identity of amino acid sequence of such identified genes with that of corresponding homologue in *A. nidulans* is presented in the table above. MoCo, molybdenum cofactor; MPT, molybdopterin.

4.3.1.3 Synteny of the gene cluster, *cnxL*-*cnxK*-*niaB*, in the aspergilli

An interesting observation from this present study was the clustering of homologous *cnxL*, *cnxK* and *niaB* genes in *A. fumigatus*, *A. fischeri*, *A. flavus* and *A. oryzae* (Table 4.2). Using AspGD, orthologues of *cnxL*, *cnxK* and *niaB* genes were identified in *A. fumigatus*, with accession numbers Afu3g15160, Afu3g15170 and Afu3g15190, respectively. These were present contiguously on chromosome III of *A. fumigatus* in the same order that found in *A. nidulans*. The directions of transcription, in which these genes may be transcribed, were also observed to be identical. The *A. oryzae*, *A. fischeri* and *A. flavus* homologues of *cnxL*-*cnxK*-*niaB* genes were also found in similarly arranged gene clusters (Figure 4.2, Table 4.2, Table 4.3).

4.3.1.4 Analysis of putative introns in *cnxL*-*cnxK*-*niaB* homologues

The number, position and length of putative introns in *cnxL*-*cnxK*-*niaB* homologues were studied and information collected by AspGD is summarised in Table 4.3. The comparison of introns in homologous genes indicated that the gene *cnxK* and homologues of *cnxK* found in other aspergilli were devoid of introns. However for other two genes, *cnxL* and *niaB*, the homologous genes were different from each other with respect to the number, position or size of their introns (Figure 4.2). Please note that information provided in Table 4.3 is based on computer predictions.
Figure 4.2: Comparison of *A. nidulans* cnxL-cnxK-niaB gene cluster with homologue clusters in aspergilli.

The gene *cnxL* and homologues are shown in green, *cnxK* and homologues in blue and *niaB* and homologues in red coloured arrows. The direction of arrow indicates the putative direction of transcription. Black vertical bars on the genes represent the position and number of introns. The values in ‘bp’ indicate the intergenic distance between two contiguous genes. Note that this figure is not to scale and shows approximate sizes and distances only. Due to incomplete information of *A. oryzae* homologue gene cluster cnxL-cnxK-niaB on AspGD, this cluster could not be included in above figure.
Table 4.3: Comparative analysis of putative introns in cnxL, cnxK and niaB genes in the aspergilli.

<table>
<thead>
<tr>
<th>Aspergillus sp.</th>
<th>Gene ID</th>
<th>Gene name / homologue</th>
<th>No. of nucleotides</th>
<th>No. of putative intron(s)</th>
<th>Position of putative intron(s) (bp)*</th>
<th>Intron length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. nidulans</em></td>
<td>AN12130</td>
<td>cnxL</td>
<td>690</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>AN9038</td>
<td>cnxK</td>
<td>1329</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>AN9037</td>
<td>niaB</td>
<td>3193</td>
<td>3</td>
<td>147-190, 302-358, 2398-2454</td>
<td>43, 56, 56</td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>Afu3g15160</td>
<td>cnxL</td>
<td>571</td>
<td>1</td>
<td>479-560</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Afu3g15170</td>
<td>cnxK</td>
<td>1344</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Afu3g15190</td>
<td>niaB</td>
<td>2792</td>
<td>1</td>
<td>225-286</td>
<td>61</td>
</tr>
<tr>
<td><em>A. fischeri</em></td>
<td>NFIA_061920</td>
<td>cnxL</td>
<td>690</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NFIA_061910</td>
<td>cnxK</td>
<td>1353</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NFIA_061900</td>
<td>niaB</td>
<td>2916</td>
<td>5</td>
<td>705-762, 1568-1593, 2421-2482, 2655-2701, 2766-2817</td>
<td>57, 25, 61, 46, 51</td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td>AFLA_023580</td>
<td>cnxL</td>
<td>465</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>AFLA_023570</td>
<td>cnxK</td>
<td>1353</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>AFLA_023560</td>
<td>niaB</td>
<td>2973</td>
<td>3</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td><em>A. oryzae</em></td>
<td>AOR_1_2612154</td>
<td>cnxL</td>
<td>690</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>AOR_1_2948154</td>
<td>cnxK</td>
<td>1353</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>AOR_1_2616154</td>
<td>niaB</td>
<td>2973</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
</tbody>
</table>

* The positions of the introns are numbered relative to the adenosine (1) of the start codon. For further detail of these homologous genes see Table 4.2. ND; not determined.

4.3.1.5 Conservation of the gene cluster, cnxL-cnxK-niaB in fungi other than the aspergilli

During NCBI BLAST searches it was observed that homologues genes (or domains) of all three were present and clustered (in the same *A. nidulans* gene order, cnxL-cnxK-niaB) in *Fusarium oxysporum*. Other common features of the *F. oxysporum* gene cluster are that cnxK (FOXB_12270) is intron-less and cnxL (FOXB_12269) and niaB (FOXB_12271) are transcribed in the same direction but opposite to cnxK (FOXB_12270) (Figure 4.3).

In the human pathogen *Talaromyces stipitatus*, homologues of all three genes, cnxL, cnxK and niaB (TSTA_118160, TSTA_118170 and TSTA_118190, respectively) are located contiguously with similar gene transcription direction as before. The only difference is the presence of an uncharacterised putative protein coding region TSTA_118180 between TSTA_118170 and TSTA_118190 (Figure 4.3). In the opportunistic human pathogen *Penicillium marneffei*, cnxL and cnxK homologues (PMAA_083230 and PMAA_083240, respectively) are present as neighbours but the niaB (PMAA_083320) is not the cnxL-cnxK cluster. In the biotechnologically important *Penicillium chrysogenum*, cnxK and cnxL are present as a two gene cluster (Pc20g03380 and Pc20g03360, respectively), with an uncharacterised protein (Pc20g03370) separating them. In *Chaetomium*
**globosum** only the homologues of *cnxK* and *niaB* (CHGG_09578 and CHGG_09579, respectively) are present and as a two gene cluster.

Figure 4.3: Comparison of *cnxL-cnxK-niaB* homologous gene clusters in fungi.

Homologues of *cnxL* are shown in green, *cnxK* in blue and *niaB* in red coloured arrows. The grey coloured arrows represent genes that are not homologues to *cnxL*, *cnxK* or *niaB* but present in between them. The direction of arrow shows the putative direction of gene transcription. Genes are annotated with their names (ID). Fungal strains to which these gene clusters belong are provided on the left hand-side. Vertical black bars on the genes represent the position and number of introns. The values in ‘bp’ indicate the intergenic distances between genes. Note that this figure is not to the scale and shows approximate sizes and distances.
Table 4.4: An overview of non-aspergilli fungal cnxL-cnxA-niaB homologues gene cluster.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Gene ID</th>
<th>Homologue of</th>
<th>% amino acid identity with homologue</th>
<th>No of amino acid residues in putative protein</th>
<th>No of putative introns</th>
<th>Putative intron length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FOXB_12269</td>
<td>cnxL</td>
<td>34</td>
<td>795</td>
<td>2</td>
<td>62, 457</td>
</tr>
<tr>
<td>F. oxysporum</td>
<td>FOXB_12270</td>
<td>cnxK</td>
<td>37</td>
<td>259</td>
<td>1</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>FOXB_12271</td>
<td>niaB</td>
<td>60</td>
<td>1739</td>
<td>6</td>
<td>54, 55, 54, 47, 316, 608</td>
</tr>
<tr>
<td>T. stipitatus</td>
<td>TSTA_118170</td>
<td>cnxL</td>
<td>34</td>
<td>785</td>
<td>2</td>
<td>80, 56</td>
</tr>
<tr>
<td></td>
<td>TSTA_118160</td>
<td>cnxK</td>
<td>39</td>
<td>942</td>
<td>6</td>
<td>114, 64, 51, 49, 51, 137</td>
</tr>
<tr>
<td></td>
<td>TSTA_118190</td>
<td>niaB</td>
<td>68</td>
<td>744</td>
<td>6</td>
<td>56, 73, 40, 52, 52, 53</td>
</tr>
<tr>
<td>P. chrysogenum</td>
<td>PC20G03380</td>
<td>cnxK</td>
<td>41</td>
<td>402</td>
<td>1</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>PC20G03360</td>
<td>niaB</td>
<td>70</td>
<td>935</td>
<td>3</td>
<td>59, 59, 59</td>
</tr>
<tr>
<td>P. marneffei</td>
<td>PMAA_083230</td>
<td>cnxL</td>
<td>39</td>
<td>163</td>
<td>1</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td>PMAA_083240</td>
<td>cnxK</td>
<td>39</td>
<td>916</td>
<td>7</td>
<td>73, 202, 57, 369, 62, 65, 97</td>
</tr>
<tr>
<td>C. globosum</td>
<td>CHGG_09578</td>
<td>cnxK</td>
<td>42</td>
<td>414</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CHGG_09579</td>
<td>niaB</td>
<td>59</td>
<td>1801</td>
<td>6</td>
<td>58, 53, 55, 201, 214, 164</td>
</tr>
</tbody>
</table>

In case of multi-domain proteins, the percentage homology mentioned in above table is over the domain.

4.3.1.6 Non-clustered homologous genes of cnxL, cnxK, and niaB in fungi, bacteria and plants

Detail of non-clustered homologues of cnxL, cnxK, and niaB in fungi, bacteria and plants were searched by NCBI BLAST. All three genes identified the homologues in bacteria and fungi. However in plants, only homologues of CnxK were found but these are most likely to be homologues of the already characterised two-domain CnxE. The description of representative homologues has been summarised in Table 4.5, Table 4.6 and Table 4.7.
Table 4.5: Examples from non-clustered fungal homologues of \textit{cnxL}, \textit{cnxK} and \textit{niaB} genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fungal species</th>
<th>Gene ID</th>
<th>% amino acid identity with homologue</th>
<th>Annotated function</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{cnxL}</td>
<td>\textit{Colletotrichum graminicola} **</td>
<td>GLRG_06992</td>
<td>35</td>
<td>MoCo synthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{Rhodotorula glutinis}</td>
<td>RTG_01962</td>
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</tr>
<tr>
<td></td>
<td>\textit{Ajellomyces dermatitidis} *</td>
<td>EEQ84860</td>
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</tr>
<tr>
<td></td>
<td>\textit{Magnaporthe oryzae} **</td>
<td>EHA50395</td>
<td>40</td>
<td>MoCo synthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{Debaryomyces hansenii} *</td>
<td>XP_456625</td>
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</tr>
<tr>
<td></td>
<td>\textit{Trichophyton verrucosum} *</td>
<td>EFE42324</td>
<td>30</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td>\textit{Magnaporthe oryzae} **</td>
<td>MGG_03258</td>
<td>35</td>
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</tr>
<tr>
<td></td>
<td>\textit{Neurospora crassa}</td>
<td>NCU09417</td>
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</tr>
<tr>
<td></td>
<td>\textit{Neurospora tetrasperma}</td>
<td>NEUTE1DRAFT_102683</td>
<td>35</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td>\textit{Sordaria macrospora}</td>
<td>SMAC_05676</td>
<td>33</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td>\textit{Ajellomyces dermatitidis} *</td>
<td>EGE84671</td>
<td>48</td>
<td>MoCo synthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{Thielavia terrestris}</td>
<td>THITE_2089085</td>
<td>39</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td>\textit{Verticillium dahlia} **</td>
<td>EGY16895</td>
<td>34</td>
<td>MPT biosynthesis</td>
</tr>
<tr>
<td>\textit{cnxK}</td>
<td>\textit{Uncinocarpus reesi}</td>
<td>UREG_04837</td>
<td>63</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td>\textit{Ajellomyces dermatitidis} *</td>
<td>BDBG_04405</td>
<td>53</td>
<td>Periplasmic nitrate reductase</td>
</tr>
<tr>
<td></td>
<td>\textit{Metarrhizium acridum}</td>
<td>MAC_01227</td>
<td>50</td>
<td>Periplasmic nitrate reductase</td>
</tr>
<tr>
<td></td>
<td>\textit{Magnaporthe oryzae} **</td>
<td>MGG_03242</td>
<td>57</td>
<td>Uncharacterised</td>
</tr>
</tbody>
</table>

MoCo, molybdenum cofactor; MPT, molybdopterin. The fungal strains marked with an asterisk (*) are human pathogens and (**) are plant pathogens.

Table 4.6: Examples from non-clustered bacterial homologues of \textit{cnxL}, \textit{cnxK} and \textit{niaB} genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Bacteria</th>
<th>Gene ID</th>
<th>% amino acid identity with homologue</th>
<th>Annotated function</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{cnxL}</td>
<td>\textit{Chlorobium tepidum}</td>
<td>MobBA</td>
<td>37</td>
<td>MGD biosynthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{Zobellia galactanivorans}</td>
<td>MobA</td>
<td>37</td>
<td>MGD biosynthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{Chrysoobacterium gleum}</td>
<td>MoaC3</td>
<td>36</td>
<td>MGD biosynthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{Glucobacter oxydans}</td>
<td>GOX0444</td>
<td>36</td>
<td>MGD biosynthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{Thermosynechococcus elongates}</td>
<td>MoaC</td>
<td>36</td>
<td>MGD biosynthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{Lentisphaera araneosa}</td>
<td>LNTAR_03284</td>
<td>32</td>
<td>MGD biosynthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{Cellulophaga lytica}</td>
<td>MobA</td>
<td>32</td>
<td>MGD biosynthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{Glucanacetobacter sp.}</td>
<td>SXCC_03515</td>
<td>32</td>
<td>MGD biosynthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{Microscilla marina}</td>
<td>M23134_07659</td>
<td>31</td>
<td>MoCo biosynthesis</td>
</tr>
<tr>
<td>\textit{cnxK}</td>
<td>\textit{Xanthomonas oryzae}</td>
<td>XO02952</td>
<td>36</td>
<td>MPT biosynthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{X. axonopodis}</td>
<td>MoeA</td>
<td>35</td>
<td>MPT biosynthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{X. gardneri}</td>
<td>XGA_0385</td>
<td>35</td>
<td>MoCo biosynthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{X. fuscans}</td>
<td>MoeA</td>
<td>34</td>
<td>MPT biosynthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{X. campestris}</td>
<td>XCR_2280</td>
<td>34</td>
<td>MPT biosynthesis</td>
</tr>
<tr>
<td></td>
<td>\textit{Burkholderia thailandensis}</td>
<td>BTH_11704</td>
<td>32</td>
<td>MPT biosynthesis</td>
</tr>
<tr>
<td>\textit{niaB}</td>
<td>\textit{Spirosoma linguale}</td>
<td>Slin_0131</td>
<td>57</td>
<td>MPT oxidoreductase</td>
</tr>
<tr>
<td></td>
<td>\textit{Saccharomonospora viridis}</td>
<td>Svir_19050</td>
<td>56</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td>\textit{Arthrobtactor phenanthreivorans}</td>
<td>Asphe3_00380</td>
<td>54</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td>\textit{Nocardiosis dassonvillei}</td>
<td>Ndas_4662</td>
<td>54</td>
<td>MPT oxidoreductase</td>
</tr>
<tr>
<td></td>
<td>\textit{Streptomyces ghanaensis}</td>
<td>SSFG_00811</td>
<td>53</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td></td>
<td>\textit{Mycobacterium colombiense}</td>
<td>MCOL_08348</td>
<td>53</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td></td>
<td>\textit{Methylbacterium sp.}</td>
<td>M446_6438</td>
<td>49</td>
<td>MPT oxidoreductase</td>
</tr>
</tbody>
</table>

MGD, molybdopterin guanine dinucleotide; MPT, molybdopterin.
4.3.1.7 CnxL homology and conserved residues with MobA proteins

The amino acid sequence of CnxL was aligned with *E. coli* MobA and 21.7 % identity and 35.4 % similarity was recorded. Multiple alignments of MobA proteins from different bacteria and CnxL (Figure 4.4) revealed that the CnxL protein shares a highly conserved sequence motif, LAGG, characteristic of MobA proteins. Other highly conserved residues G15, R19, K25, G78, G82, D101, N180 and N182 of *E. coli* MobA (also conserved in other bacteria) that are involved in GTP binding or MGD formation (Guse et al., 2003; Neumann et al., 2010), were also found conserved in the CnxL protein (Figure 4.4).

**Table 4.7: Examples from homologues of the cnxK gene in protists and plants.**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Gene ID</th>
<th>% amino acid identity</th>
<th>Annotated function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>EDP07827</td>
<td>33</td>
<td>MPT biosynthesis</td>
</tr>
<tr>
<td><em>Selaginella moellendorffii</em></td>
<td>SELMORDRAFT_150666</td>
<td>32</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td><em>Micromonas</em> sp.</td>
<td>AC06159</td>
<td>30</td>
<td>MoCo biosynthesis</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>CNX1_ARATH</td>
<td>30</td>
<td>MoCo biosynthesis</td>
</tr>
<tr>
<td><em>Hordeum vulgare</em></td>
<td>AAF73075</td>
<td>30</td>
<td>MoCo biosynthesis</td>
</tr>
</tbody>
</table>

4.3.1.7 CnxL homology and conserved residues with MobA proteins

The amino acid sequence of CnxL was aligned with *E. coli* MobA and 21.7 % identity and 35.4 % similarity was recorded. Multiple alignments of MobA proteins from different bacteria and CnxL (Figure 4.4) revealed that the CnxL protein shares a highly conserved sequence motif, LAGG, characteristic of MobA proteins. Other highly conserved residues G15, R19, K25, G78, G82, D101, N180 and N182 of *E. coli* MobA (also conserved in other bacteria) that are involved in GTP binding or MGD formation (Guse et al., 2003; Neumann et al., 2010), were also found conserved in the CnxL protein (Figure 4.4).

**Figure 4.4: Alignment of bacterial MobA proteins with CnxL.**

The abbreviation used for bacterial strains are: Ec, *E. coli*; Rs, *Rhodobacter sphaeroides*; Pp, *Pseudomonas putida*. Bs, *Bacillus subtilis*. Strictly conserved residues (as described in text) are highlighted in blue. The numbers on the right hand side represent the residue positions in respective protein. The alignment was generated using ClustalW2 Multiple alignment tool (www.ebi.ac.uk).
4.3.1.8 CnxK homology and residues conserved with MoeA and MoeA like domains

Using the EMBOSS (European Molecular Biology Open Software Suite) Water, protein alignment tool, from the European Bioinformatics Institute (EBI) (www.ebi.ac.uk), amino acid sequences were compared. The sequence of CnxK was aligned against the MoeA domains of *A. nidulans* CnxE, *Arabidopsis thaliana* Cnx1, *Drosophila melanogaster* (Cinnamon), *Rattus norvegicus* (Gephyrin) and *E. coli* MoeA. The results of amino acid sequence homology of CnxK are summarised in Table 4.8.

Figure 4.5: Sequence alignment of CnxK with MoeA or MoeA like domains.

<table>
<thead>
<tr>
<th>MoeA</th>
<th>CnxK</th>
<th>Cinnamon</th>
<th>Gephyrin</th>
<th>CnxE</th>
<th>CnxK</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFLDVFTDFNLHMEQRVRADIAS---------GQLPFWAGKSFAQGQYKQGEMFAG--------</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>---</td>
<td>----</td>
<td>---</td>
<td>-----</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cnx1</td>
<td>Cinnamon</td>
<td>Gephyrin</td>
<td>CnxE</td>
<td>CnxK</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>CnxK</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Amino acid sequence alignment of CnxK with homologues in *E. coli* (MoeA), *Arabidopsis thaliana* (Cnx1), *Drosophila melanogaster* (Cinnamon) *Rattus norvegicus* (Gephyrin) and *A. nidulans* (CnxE).

Strictly conserved residues characteristic of MoeA protein family are highlighted in blue and conserved residues among the proteins aligned are shown in yellow. The numbers on the right hand side refer to the residue number in the respective protein. The alignment was generated using ClustalW2 (www.ebi.ac.uk).
The multiple CnxK amino acid sequence alignment with homologous proteins (Figure 4.5) indicated that CnxK protein have essential residues critical for structure or function of MoeA protein and also conserved in all homologues in eukaryotic and prokaryotic organisms (Figure 4.5). For example in *E. coli* MoeA amino acid residues P51 D59, A62, T100, P112, D142, E188, N205, D228, F286 and L297 are considered structurally or functionally essential (Xiang *et al.*, 2001; Heck *et al.*, 2002). All these residues are also conserved in the CnxK protein. Moreover certain strictly conserved residues in all members of MoeA protein family were also found conserved in the CnxK protein.

Table 4.8: *cnxK* homology with biochemically characterised proteins.

<table>
<thead>
<tr>
<th>Homology of CnxK</th>
<th>CnxL</th>
<th>CnxE</th>
<th>Cnx1</th>
<th>Cinnamon</th>
<th>Gephyrin</th>
<th>MogA</th>
<th>MoeA</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Identity</td>
<td>22.0</td>
<td>26.8</td>
<td>27.4</td>
<td>27.3</td>
<td>27.1</td>
<td>21.3</td>
<td>31.5</td>
</tr>
<tr>
<td>% Similarity</td>
<td>31.7</td>
<td>41.5</td>
<td>40.9</td>
<td>45.9</td>
<td>45.7</td>
<td>37.6</td>
<td>46.8</td>
</tr>
</tbody>
</table>

Identity refers to conservation of amino acid residues while similarity is based on the common characteristics of residues. Note that the for CnxE, Cnx1, cinnamon and gephyrin, the percentage homology is over the MoeA domain of the protein.

4.3.1.9 Analysis of the NiaB protein

The automated annotation for the NiaB protein provided by AspGD, suggested that NiaB is a periplasmic nitrate reductase and its putative function is in dissimilatory nitrate metabolism. Also the amino acid sequence of NiaB was denoted as a NapA-like nitrate reductase (respiratory nitrate reductase) by the NCBI Database. The AspGD as well as NCBI Conserved Domain search tool revealed that the NiaB protein has amino acid sequence domains for predicted molybdopterin binding (polysulfide binding sites).

An alignment of NiaB with the respiratory nitrate reductase NapA, from *E. coli* was carried out. These results showed that NiaB has 21.2 % identity and 31.9 % similarity with the *E. coli* NapA. In NapA proteins four cysteine residues are conserved which bind to the 4Fe-4S cluster and molybdenum cofactor. It was observed that in NiaB, one of the essential cysteine residues is not conserved and instead of cysteine a glycine residue is present in the respective position (Figure 4.6).
Figure 4.6: Amino acid sequence alignment of NiaB with NapA of *E. coli*.

<table>
<thead>
<tr>
<th>NiaB</th>
<th>KEYVYATVFRTYNHQSHLYPFNLQMVSNHPRLTHLPFFRNHIDDRDIARLHGYEQVRHKSEPT</th>
<th>59</th>
</tr>
</thead>
<tbody>
<tr>
<td>NapA</td>
<td>KLLRERFEMANVAAAAAAGSAGVAVAVQPEAIFPKDKARHIFTLVVPLLTVLVTYK</td>
<td>60</td>
</tr>
<tr>
<td>NiaB</td>
<td>KEYVYATVFRTYNHQSHLYPFNLQMVSNHPRLTHLPFFRNHIDDRDIARLHGYEQVRHKSEPT</td>
<td>59</td>
</tr>
<tr>
<td>NapA</td>
<td>KLLRERFEMANVAAAAAAGSAGVAVAVQPEAIFPKDKARHIFTLVVPLLTVLVTYK</td>
<td>60</td>
</tr>
<tr>
<td>NiaB</td>
<td>KEYVYATVFRTYNHQSHLYPFNLQMVSNHPRLTHLPFFRNHIDDRDIARLHGYEQVRHKSEPT</td>
<td>59</td>
</tr>
<tr>
<td>NapA</td>
<td>KLLRERFEMANVAAAAAAGSAGVAVAVQPEAIFPKDKARHIFTLVVPLLTVLVTYK</td>
<td>60</td>
</tr>
<tr>
<td>NiaB</td>
<td>KEYVYATVFRTYNHQSHLYPFNLQMVSNHPRLTHLPFFRNHIDDRDIARLHGYEQVRHKSEPT</td>
<td>59</td>
</tr>
<tr>
<td>NapA</td>
<td>KLLRERFEMANVAAAAAAGSAGVAVAVQPEAIFPKDKARHIFTLVVPLLTVLVTYK</td>
<td>60</td>
</tr>
<tr>
<td>NiaB</td>
<td>KEYVYATVFRTYNHQSHLYPFNLQMVSNHPRLTHLPFFRNHIDDRDIARLHGYEQVRHKSEPT</td>
<td>59</td>
</tr>
<tr>
<td>NapA</td>
<td>KLLRERFEMANVAAAAAAGSAGVAVAVQPEAIFPKDKARHIFTLVVPLLTVLVTYK</td>
<td>60</td>
</tr>
</tbody>
</table>

This amino acid sequence alignment has been generated by ClustalW2 (www.ebi.ac.uk). Conserved residues are highlighted in yellow. Essential cysteine residues conserved in both NiaB and NapA are highlighted in blue. The non-conserved cysteine residue is shown with black background.
4.3.1.10 Homology studies on the NiaC protein

Homologues of the NiaC protein were identified in other *Aspergillus* species, fungi other than aspergilli as well as in plants. Interestingly, some of the homologues of NiaC found in fungi are human pathogens (Table 4.10).

### Table 4.9: The NiaC homologues in genus *Aspergillus*.

<table>
<thead>
<tr>
<th>Aspergillus sp.</th>
<th>Gene ID</th>
<th>No of amino acid residues in putative protein</th>
<th>% amino acid identity</th>
<th>Annotated function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. fumigatus</em></td>
<td>Afu5g10420</td>
<td>1026</td>
<td>64</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td><em>A. fischeri</em></td>
<td>NFIA_076810</td>
<td>1033</td>
<td>64</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td><em>A. terreus</em></td>
<td>ATEG_07595</td>
<td>1000</td>
<td>64</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td><em>A. clavatus</em></td>
<td>ACLA_013480</td>
<td>1037</td>
<td>62</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td>AFL2G_01616</td>
<td>823</td>
<td>57</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>Aa07g08920</td>
<td>1048</td>
<td>48</td>
<td>Nitrate reductase</td>
</tr>
</tbody>
</table>

Amino acid sequences of proteins were obtained from the AspGD using the accession numbers shown. Using the Orthologous Clusters Tool, orthologues in other species of the genus *Aspergillus* were identified. Percentage identities of homologous proteins were recorded.

### Table 4.10: Examples of the NiaC protein homologues from fungi and plants.

<table>
<thead>
<tr>
<th>Group</th>
<th>Organism</th>
<th>Gene ID</th>
<th>% amino acid identity</th>
<th>Annotated function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fungi</strong></td>
<td><strong>Trichophyton rubrum</strong></td>
<td>TERG_07501</td>
<td>57</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td></td>
<td><em>Arthroderma otae</em></td>
<td>MCYG_04628</td>
<td>55</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td></td>
<td><em>Ajellomyces dermatitidis</em></td>
<td>BDCG_06319</td>
<td>54</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td></td>
<td><em>Ajellomyces capsulata</em></td>
<td>HCBG_04374</td>
<td>53</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td></td>
<td><em>Coccidioides posadasi</em></td>
<td>CPSG_07496</td>
<td>52</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td></td>
<td><em>Talaromyces stipitatus</em></td>
<td>TSTA_079850</td>
<td>52</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td></td>
<td><em>Fusarium oxysporum</em></td>
<td>FOXB_14074</td>
<td>51</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td><em>Paracoccidioides brasiliensis</em></td>
<td>PABG_00814</td>
<td>50</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td></td>
<td><em>Uncinocarpus reessii</em></td>
<td>UREG_04837</td>
<td>50</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td><em>Phaeosphaeria nodorum</em></td>
<td>SNOG_02336</td>
<td>50</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td><em>Metarhizium robertsii</em></td>
<td>MAA_07505</td>
<td>48</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td><em>Verticillium albo-atrum</em></td>
<td>VDBG_06582</td>
<td>47</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td></td>
<td><em>Sordaria macrospora</em></td>
<td>SMAC_07620</td>
<td>46</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td><strong>Plants</strong></td>
<td><strong>Beta vulgaris</strong></td>
<td>NR</td>
<td>38</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td></td>
<td><em>Oryza sativa</em></td>
<td>NIA1</td>
<td>37</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td></td>
<td><em>Hordeum vulgare</em></td>
<td>-</td>
<td>37</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td></td>
<td><em>Medicago truncatula</em></td>
<td>NR2</td>
<td>36</td>
<td>Nitrate reductase</td>
</tr>
</tbody>
</table>

Fungal and plant homologues were identified by NCBI BLAST search using the amino acid sequence of NiaC as query. Fungal strains marks with an asterisk are human pathogens.
4.3.1.11 NiaC and characterised assimilatory nitrate reductases

The amino acid sequence alignment of NiaB and NiaC proteins revealed that these are only 15% identical and 24.1% similar with each other. The NCBI BLAST results indicated that the homologues of NiaC were annotated as involved in assimilation of nitrate although few have been characterised biochemically. Basic information provided for NiaC in AspGD and NCBI Conserved Domain Search, indicated that NiaC has all three essential assimilatory nitrate reductase domains i.e. predicted electron transport activity (FAD), heme binding and molybdenum cofactor (MoCo) binding domains. The amino acid sequence of NiaC was compared with the well-characterised assimilatory nitrate reductase in *A. nidulans* NiaD (Garde *et al.*, 1995) and *A. thaliana*, NIA1 (Cheng *et al.*, 1988).

The essential residues in all these domains in NiaD are also conserved in the NiaC protein along with a number of other highly conserved residues, C150, H547, W618, K626, H654, Y693 and G721 (Figure 4.7).

**Table 4.11: Homology of NiaB and NiaC with the hitherto characterised nitrate reductase proteins.**

<table>
<thead>
<tr>
<th></th>
<th>NiaD % Identity</th>
<th>NiaD % Similarity</th>
<th>NIA1 % Identity</th>
<th>NIA1 % Similarity</th>
<th>NapA % Identity</th>
<th>NapA % Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NiaB</td>
<td>11.6</td>
<td>18.7</td>
<td>15.2</td>
<td>27.0</td>
<td>21.2</td>
<td>31.9</td>
</tr>
<tr>
<td>NiaC</td>
<td>29.3</td>
<td>43.9</td>
<td>31.9</td>
<td>46.9</td>
<td>13.5</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Percentage identity and similarity has been determined by pairwise alignment using the EMBOSS Needle Alignment Programme. The amino acid residues of NiaB or NiaC, putative nitrate reductases were compared with the characterised assimilatory nitrate reductase of *A. nidulans* (NiaD), the assimilatory nitrate reductase of *A. thaliana*, NIA1, and the dissimilatory nitrate reductase of *E. coli*, NapA.
Figure 4.7: Alignment of NiaC with characterised nitrate reductases.

A multiple amino acid alignment generated by ClustalW2 (www.ebi.ac.uk). Conserved residues are highlighted with yellow and strictly conserved amino acid residues in all assamplified nitrite reductases (residue positions C150, H547, W618, K626, H654, Y693 and G721 in NiaD) are highlighted in blue.
Northern blot analysis (vis-à-vis DNA-RNA hybridisation), using DNA radiolabelled probes of individual \( cnxL \), \( cnxK \), \( niaB \) or \( niaC \) genes was carried out to attempt to identify mRNA profiles related to gene expression. The wild type, as well as mutants affecting the regulation of nitrate metabolism, were included in the survey. Strains were grown under various aerobic and anaerobic conditions, for 5 h (germination of conidia) and 24 h (mature mycelia), and expression of these genes was studied from total RNA preparations. No transcript signal was detected in RNA from the wild type strain or any of the mutants for any of the newly identified genes. This included RNA isolated from cells grown under aerobic and anaerobic conditions on different carbon sources i.e. glucose, ethanol or acetate. Total RNA was extracted from the wild type strain (with respect to the nitrate metabolism) and from mutants strains of \( A. \) nidulans defective in nitrate metabolism, \( niaD17, niaD\Delta506, cnxE16, nirA1, areA300 \) and \( xprD1 \) (see Material and methods, Chapter 2 for strain details). Cells were grown on 5 mM urea for 5 h or 24 h as discussed above and then transferred to one of the three different carbon sources, 1 % glucose, 3 % acetate or 3 % ethanol with nitrate or ammonium as the nitrogen source. Extracted RNA was blotted on to nylon membranes and hybridised with the radiolabelled gene probes as discussed in Chapter 1). No mRNA signals were detected for either gene probe, under any condition of growth (Figure 4.8).
Figure 4.8: Northern blot analysis and hybridization of total RNA with radiolabelled DNA probes.

Legends are on the following page.
Total RNA was extracted from *A. nidulans* strains defective in nitrate metabolism (Chapter 2, Materials and methods, Table 2.2). Strains were grown in liquid minimal medium supplemented with 10 mM NaNO₃ and one of the three different carbon sources, 1 % glucose (G), 3 % acetate (A) and 3 % ethanol (E). Gel electrophoresis of total RNA stained with ethidium bromide (a), blotted on nylon membrane and hybridized with radiolabelled *cnxK* (b) and *actA* (actin) (c) gene probes. Membranes were exposed to phosphor screens for two weeks in the case of *cnxK* and 2-3 h for *actA*, and developed by a Personal Molecular Imager FX (BioRad) using the programme Quantity One. No mRNA signals were detected for *cnxK* gene. The high pixilation of the image in panel (b) is due to very long exposure. The two bands in autoradiograph (b) are rRNA subunits (18S and 23S) due to non-specific binding of *cnxK* gene probe. The blot was washed with 0.1 % SDS and re-probed (c) using a 0.83 kb fragment of the *A. nidulans* actA gene as positive control. Size markers (kb) are shown on the left of the image.

4.3.3 Generation and characterisation of knock-out mutants

Knock-out mutants of the entire gene cluster (*cnxL-cnxxK-niaB*) as well as individual genes were generated by S E Unkles (Chapter 2; Materials and methods, Section 2.4.7). Solid minimal medium used for phenotypic testing contained glucose, ethanol or acetate as the sole carbon source with nitrate or ammonium as the sole nitrogen source (Table 4.12). Knock-out mutants were grown under aerobic and semi-anaerobic conditions in attempts to determine the function of individual genes and/or the whole gene cluster. The putative knock-out mutants grew well under the conditions tested and could not be distinguished phenotypically from each other or indeed from the wild type strain. These results suggested that these genes are non-functional at least under the conditions employed.

**Table 4.12: Growth response of knock-out mutants grown under semi-anaerobic conditions on various carbon and nitrogen sources.**

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Nitrogen source</th>
<th>Wild type</th>
<th>ΔniaB (T12320-T12328, T12380-T12388)</th>
<th>ΔcnxL (T12360-T12367)</th>
<th>ΔcnxK (T12400-T12407)</th>
<th>ΔcnxL-cnxxK-niaB (T12340-T12348)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 % glucose</td>
<td>100 mM NO₃⁻</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>1 % glucose</td>
<td>10 mM NO₃⁻</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>1 % glucose</td>
<td>1 mM NO₃⁻</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>1 % glucose</td>
<td>1 mM NH₄⁺</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.5 % glucose</td>
<td>1 mM NH₄⁺</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3 % ethanol</td>
<td>2 mM NO₃⁻</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3 % acetate</td>
<td>2 mM NO₃⁻</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Phenotypic analysis of putative knock-out mutants was carried out with individual sole carbon and nitrogen sources added to solid minimal medium, pH 6.5. Transformant numbers are included and all were assayed for their growth response. Percentages of carbon source (1 % glucose, 3 % ethanol or 3 % acetate) were selected to provide the similar levels of carbon. In one of the growth condition 0.5 % glucose was used to create a carbon source limitation and therefore stress. Growth responses are shown on a scale where ‘+++’ represents the wild type growth, ‘++’ is for growth less than the wild type (moderate) and ‘+’ denotes poor but still tangible growth.
4.4 Discussion

In the present study we have identified four new putative genes designated cnxL, cnxK, niaB and niaC that may have a role in *Aspergillus nidulans* nitrate metabolism. Three of the four genes, cnxL, cnxK and niaB genes are clustered and locate on chromosome VII. The presence of contiguous genes (cnxL-cnxK-niaB) to form a short gene cluster suggests the possibility of functionality and by extension, in this case involvement in nitrate metabolism. Functionally related clustered genes had been observed previously in *A. nidulans* and in the field of nitrate assimilation. For example, and of particular relevance to the work carried out in this thesis, the gene cluster nrtA-niiA-niaD- on chromosome VIII, is required for nitrate assimilation in which nitrate is reduced to ammonium (Johnstone *et al.*, 1990 and references therein and reviewed in the General Introduction, Chapter 1). By analogy, it is tempting to suggest that cnxL-cnxK-niaB is functional and is involved in nitrate metabolism.

Several other gene clusters have been found in *A. nidulans* that are functional and therefore support the above prediction. First, four genes prnA, prnB, prnC and prnD, related to proline metabolism cluster on chromosome VII (Cubero and Scazzocchio, 1994; Reyes-Dominguez *et al.*, 2008 and references therein). Second, five genes designated alcM, alcS, alcO, alcP and alcU cluster and participate in the ethanol breakdown pathway (Fillinger and Felenbok, 1996 and references therein). Further, a number of gene clusters have been reported, that play a role in secondary metabolism. In this regard three examples of characterised secondary metabolic gene clusters in *A. nidulans* are the sterigmatocystin (an insecticidal mycotoxin) metabolism (Brown *et al.*, 1996), penicillin (an antibiotic) biosynthesis (Buades and Moya, 1996 and references therein) and for terraquinone A (an antitumor agent) metabolism gene cluster.

Homology studies may play an important role in the prediction of protein function if biochemical analyses are available for that protein in another organism. *In silico* analysis of *A nidulans* CnxL revealed that CnxL shares high amino acid similarity with the *E. coli* MobA protein. In this respect, MobA has been shown to be involved in the catalytic conversion of MoCo to molybdopterin guanine dinucleotide (MGD) cofactor (Schrag *et al.*, 2001; Neumann *et al.*, 2010). CnxL protein alignment with MobA from a variety of bacteria (including *E. coli*), indicated that CnxL shares the highly characteristic amino acid sequence, LAGG, of the MobA protein family. Moreover, highly conserved residues G15, R19, K25, G78, G82, D101, N180 and N182 in *E. coli* MobA which are either structurally important or essential for MGD formation. These residues are perfectly conserved in the bacterial MobA proteins, and interestingly in *A. nidulans* CnxL (Figure 4.4). Therefore, conservation
of MobA essential amino acid residues in CnxL is strong evidence that CnxL is functional and could be involved in the conversion of MoCo to MGD (Figure 4.4).

Kinghorn and colleagues (Millar et al., 2001) reported that the MoeA section of the A. nidulans CnxE has high similarity with E. coli MoeA, D. melanogaster Cinnamon, A. thaliana Cnx1 and rat gephyrin. All these proteins perform a single crucial function, that of insertion of molybdenum into molybdopterin to produce the molybdenum cofactor. E. coli MoeA, amino acid residues P51 D59, A62, T100, P112, D142, E188, N205, D228, F286 and L297, are highly conserved in bacteria. Such conserved residues in MoeA proteins from a variety of organisms and also observed in A. nidulans CnxK is suggestive evidence that CnxK has indeed a functional role and this of course may be conversion of molybdopterin into the molybdenum cofactor (Figure 4.5, Figure 4.9).

Figure 4.9: Comparison of molybdenum cofactor biosynthesis pathway of E. coli and A. nidulans.

This figure, adapted from www.stonybrook.edu/biochem/BIOCHEM/facultypages/schindelin/, originally describes the E. coli molybdenum cofactor biosynthesis pathway and has been adapted and annotated to explain molybdenum cofactor biosynthesis pathway in A. nidulans. Each step in the pathway shows the protein involved. ‘Mo’ denotes E. coli proteins and Cnx A. nidulans. The newly identified proteins, CnxL and CnxK are speculatively indicated carrying out reaction steps in red.
No NiaB homologues in plants were observed. Plants are strictly aerobic and probably do not require enzymes related to anaerobic metabolism (Takasaki et al., 2004a). Two putative NirA (the positive regulator protein mediating nitrate induction of the of A. nidulans niaD gene) nucleotide binding sites, CCGCGGAG (Punt et al., 1995) were observed upstream of niaB. It is unlikely that niaB is a duplication product of niaD as the proteins encoded by both of these genes have very low amino acid identity. Instead, NiaB has higher similarity with dissimilatory nitrate reductases, such as NapA, a periplasmic E. coli nitrate reductase (Figure 4.6). One major difference between NapA and NiaB proteins is that out of four cysteine residues that bind to 4Fe-4S and to MGD, only three are conserved in NiaB (Figure 4.6). NiaC amino acid sequence alignment indicated that the protein is very similar to the well-characterised assimilatory nitrate reductases, NiaD or NIA1. Four putative NirA binding sites were observed upstream niaC. With regard to protein structure, NiaC has all three functional domains associated with assimilatory nitrate reductase, i.e. FAD, heme binding (FeS) and MoCo domains. Furthermore, conserved residues in the three domains of NiaD are also conserved in the NiaC protein. In common with a number of other highly conserved residues, C150 present in the MoCo domain is predicted to form a ligand with the molybdenum atom (Barber and Neame, 1990), H547, in the heme domain is thought to be involved in binding to heme iron. All these observations suggest that NiaC functions as an assimilatory nitrate reductase.

It is interesting that Takasaki et al. (2004b) identified a further role for the assimilatory niaD and niiA gene products (nitrate reductase and nitrite reductase activities respectively) in fungal ammonium fermentation. Using niaD and niiA mutants, Takasaki and colleagues provided evidence that NiaD and NiiA activities were essential for ammonium fermentation in A. nidulans. Following a similar experimental growth strategy to Takasaki and co-workers (2000b), we examined a variety of A. nidulans strains, mutant in a number of genes involved in nitrate metabolism. These genes were niaD (nitrate reductase), cnxE (molybdenum cofactor biosynthesis), nirA (regulatory gene mediating nitrate induction) and the areA (control gene for ammonium or glutamine repression). The mutants were grown aerobically and transferred to anaerobic conditions with ethanol or acetate as the sole carbon source and 10 mM nitrate as the sole nitrogen source. Analysis of northern blots containing RNA from these mutants did not reveal any hint of mRNA transcript, from any of the four novel gene probes (Figure 4.8). There are three possibilities regarding failure to observe a message. First, the expression level of the four novel genes is too low to be detected by DNA-RNA hybridisation. Second, the correct growth conditions required for their expression were not utilised. For example, a different nitrogen sources other than the nitrate, may be the inducer of gene expression levels of the four novel genes. Finally all four genes could be pseudogenes that have lost their function due to mutations.
arising during the course of genome evolution. Pseudogenes are generally of three types, (i) processed pseudogenes formed due to the re-integration of mature mRNA (without promoter sequence) into the genome, (ii) duplicated pseudogenes that arise due to mutations in a copy (duplicate) of a functional gene (iii) disabled genes in which a single gene harbours mutations resulting in loss-of-function (Vanin, 1985; Spieth and Lawson, 2006).

On the basis of overall protein architecture, the predicted functional role of NiaB is that of dissimilation or respiration of nitrate. There is circumstantial evidence of involvement of the linked cnxL and cnxK genes in the synthesis of molybdenum cofactor and/or molybdopterin guanine dinucleotide required for dissimilatory nitrate reductase activity. Knock-out of the individual gene or the whole gene cluster (cnxL-cnxK-niaB) did not affect the growth of mutants on a variety of carbon sources with nitrate as the sole nitrogen source pointed towards cnxL-cnxK-niaB being non-functional. This gene cluster is conserved in species of Aspergillus as well as several other fungi including human as well as plant pathogens. Therefore the possibility that they are duplicated pseudogenes is perhaps less likely. As cnxL and niaB genes possess putative introns so these genes might not be the processed pseudogenes because usually processed pseudogenes have their introns spliced out (Spieth and Lawson, 2006). The number, position and nucleotide length of introns vary in homologues genes. One possible explanation non-functional NiaB is the mutation in the native cysteine that has been replaced with a glycine residue resulting in loss-of-function of NiaB. If niaC is indeed a pseudogene, that has high similarity with niaD, it is possible that duplication of niaD occurred from chromosome VIII to V and this event was followed by mutations in niaC resulting in the non-functional gene product (Zhou and Wang, 2008).

4.5 Conclusion

On the basis of gene homology, localisation and conservation of essential amino acid residues, on the one hand, we may predict that (i) the CnxL protein is involved in the formation of molybdopterin guanine dinucleotide from the molybdenum cofactor (ii) CnxK is involved in the synthesis of molybdenum cofactor from molybdopterin (iii) NiaB is a dissimilatory nitrate reductase and that CnxL and CnxK is needed for its activity and (iv) NiaC is an assimilatory nitrate reductase. However, no expression of any of the four genes at the transcript level could be detected, at least under the conditions employed. Furthermore, mutant knock-outs of the gene cluster cnxL-cnxK-niaB did not yield a phenotype which would have most likely identified the functional role of these genes or protein products. On the other hand the four genes are simply pseudogenes. Unfortunately as the research stands, it is not possible to conclude that any of the four genes are non-functional genes or functional,
at least under certain growth or environmental conditions employed in these experiments. A further investigation using more mutants and growth conditions such as different combinations of carbon and nitrogen sources, incubation time and temperature might facilitate the determination of their metabolic role (if any). Some of the homologues of these genes are present in human pathogens, therefore discovery of the function of these genes could be of particular interest in the field of pharmacology by targeting these genes for drugs.

Acknowledgement

I wish to thank Mr. Ryan Huddleston and Ms. Lucy Finnegan for help and collaboration with this section of work.
Chapter Five

Studies of residue proximity in the NrtA transporter by thiol cross-linking technique

5.1 Introduction

5.1.1 General characteristics of NrtA protein
The *Aspergillus nidulans* nitrate transporter protein NrtA, encoded by *nrtA* gene, belongs to the nitrate / nitrite porter family (NNP) of MFS (Unkles *et al.*, 1991; Trueman *et al.*, 1996; Forde, 2000; Unkles *et al.*, 2001 and see Chapter 1, General Introduction). NrtA, a high affinity nitrate transporter protein, has a number of conserved amino acid residues and motifs characteristic of MFS and NNP are conserved. The characteristic amino acids motif of NNP AAGXGNXGGG is called the nitrate signature and in NrtA two copies of nitrate signatures are present. The first copy of nitrate signature is in Tm 5 spanning from amino acid residue 163 to 172 while the second copy is in Tm 11 (Trueman *et al.*, 1996; Forde, 2000; Kinghorn *et al.*, 2005). Mutational analysis of NrtA Tm 5 and Tm 11, harbouring the nitrate signature (NS) sequence, suggested that nitrate signature motifs might directly participate in substrate binding and transport (S E Unkles, Unpublished Data). A number of glycine residues are also conserved in both copies of the nitrate signature. Glycines, due to their small side chain, allow not only helix flexibility but also close packing of protein helices (Ulmschneider and Sansom, 2001). Alanine replacement of conserved glycine residues G165, G167 and G170, within the nitrate signature motif resulted in complete growth arrest on nitrate as sole source of nitrogen indicating that these glycine residues are essential for nitrate transport. Among the conserved asparagine residues N168 (Tm 5, NS1) and N459 (Tm 11, NS2), within the nitrate signature sequence, N168 has been reported to be irreplaceable and N459 can be replaced with glutamine only (Unkles *et al.*, 2004a; S E Unkles, Unpublished Data).

Similar to all other prokaryotic and eukaryotic high affinity nitrate transporters, the *A. nidulans* NrtA protein has positively charged arginine residues, R87 (Tm 2) and R368 (Tm 8) and these are also conserved (Unkles *et al.*, 2004a; Kinghorn *et al.*, 2005). Mutational analysis of the NrtA protein revealed that R87 and R368 are involved in substrate binding and probably present in the close proximity to N459 (Tm 11). It was also suggested that both asparagine residues, N459 (Tm 11) and N168 (Tm 5) most likely work together with conserved arginine residues to form a translocation pore for nitrate transport (Unkles *et al.*, 2004a). Such a pair of conserved arginine residues, vital for substrate transport activity, has been observed in other characterised MFS transporters. R87 (Tm 2) and R303 (Tm 8) from *E. coli* nitrate transporter NarU (Jia *et al.*, 2009), R45 (Tm 1) and R 269 (Tm 7)
in GlpT (Law et al., 2008), LacY R144 (Tm 5) and R302 (Tm 9) (Wolin and Kaback, 2000; Zhang et al., 2002) and these residues were found to be essential in their respective substrate translocation.

5.1.2 Thiol cross-linking of cysteine residues

Crystal structures of several prokaryotic MFS transporter proteins are available. These include E. coli LacY (Abramson et al., 2003; Chaptal et al., 2011), GlpT of E. coli (Huang et al., 2003), oxalate-formate transporter, OxlT of Oxalobacter formigenes (Hirai et al., 2002), the multidrug transporter protein, EmrD (Yin et al., 2006), E. coli FucP, the fucose transporter (Dang et al., 2010) and PepTso, the peptide transporter protein from bacterium, Shewanella oneidensis (Newstead et al., 2011).

Although both LacY and GlpT belong to entirely different sub-groups of MFS transporters, both have very low homology and also LacY is a symporter and GlpT is an antiporter but their crystal structures revealed that they share the same architecture (Law et al., 2008). With the available crystal structures of proteins, the position and orientation of amino acid residues can be accurately determined but for functional studies of a transporter, biochemical information is essential.

The homobifunctional thiol cross-linking methodology has been used to characterise a number of prokaryotic as well as eukaryotic proteins by determining the residue proximity. This technique also provides insights into the helix packing of the proteins. Cross-linking reagents provide the information on the distances between two cross-linked amino acids thereby helping to elucidate protein structure. Mapping the distance between the residues by such methods is invaluable where the crystal structure of protein is not available (Kwaw et al., 2000). Thiol cross-linking of pair-wise cysteine residues introduced into a cysteine-less background has been employed successfully for a number of membrane proteins, for example the lactose permease (LacY) of E. coli (Wolin and Kaback, 2000; Kwaw et al., 2000; Zhang et al., 2002; Zhou et al., 2008), oxalate transporter of Oxalobacter formigenes, OxlT (Yang et al., 2005), E. coli twin arginine translocase TatB (Lee et al., 2006), human glucose transporter, GLUT1 (Alisio and Mueckler, 2004) and human reduced folate carrier, RFC (Hou et al., 2010).

Information on helix packing of membranes, identification of critical residues in transport activity and the special relationship of these critical residues provides the insights into substrate transport mechanisms (Guan et al., 2001). In E. coli LacY, thiol cross-linking has been studied either using the split permease (two separate functional C-terminal and N-terminal halves each containing one cysteine) (Kwaw et al., 2000; Guan et al., 2001; Zhang et al., 2002) or intact LacY permease with engineered the factor Xa cleavage site (Wu et al., 1996; Wolin and Kaback, 2000; Zhou et al., 2008). Furthermore
double cysteine mutants of LacY (Wu et al., 1996) and also of the glucose transporter, GLUT1, have the functional activity comparable to cysteine-less mutant.

On the basis of homology and crystal structure of the MFS anion transporter, glycerol-3-phosphate transporter (GlpT), a model for NrtA has been created (Figure 5.1). The homology of these two transporters is only 20% but the basic structural framework is conserved throughout the MFS family. According to our model, the distance between two arginine residues at position 87 (Tm 2) and 368 (Tm 8) is approximately 18 Å, R87 and N459 are approximately 6 Å apart from each other and N168 and R368 are relatively closer to each other and are only 4 Å apart (Figure 5.1b). The diameter of nitrate molecule is 2.6 Å that also suggest that the R87 (Tm 2) and R368 (Tm 8) are closer than that illustrated by the existing model of NrtA.

Figure 5.1: Model of A. nidulans NrtA protein.

(a) Tms are shown as ribbons, coloured from 1 in blue to 12 in red. Predicted locations of residues selected for thiol cross-linking are shown as sticks in their respective Tms. (b) Residues used in this present study are shown at their relative positions as in the model. Distances between paired residues have been estimated by model. These figures have been generated using Pymol software (www.pymol.org).

For this present study, three double cysteine mutants R87C/R368C, R87C/N459C and N168C/R368C were generated in an otherwise cysteine-less construct. In the large central loop between Tm 6 and Tm 7 of the double cysteine NrtA mutant protein, two tandem sequences (IEGR) were introduced for site specific cleavage by factor Xa protease site (Figure 5.2). Site specific cleavage by factor Xa protease combined with the thiol cross-linking with the commercially available homobifunctional thiol cross-linkers (of known distance between thiol reactive groups) was employed to determine the proximity of cysteine residues in double cysteine mutants. Cysteine-less mutant, T22 (containing factor Xa protease
cleavage site) as well as two single cysteine mutants, R368C and N459C (having factor Xa protease cleavage site) were used as control strains.

Figure 5.2: Schematic representation of thiol cross-linking in NrtA double cysteine mutants.

The NrtA protein is shown in two halves, (C- and N-terminal, each consisting of six Tms). Both C- and N-terminal halves are joined by large central loop between Tm 6 and Tm 7. This central loop contains two tandem sequences, IEGR for factor Xa protease recognition. If the two cysteines, one from each half of the protein, cross-link, the protein band in western blot would be a full length wild type NrtA protein size. However if there is no cross-linking, only the C-terminal half of the protein (harbouring the His-tag) would be observed by western blot analysis. Tms are represented as cylinders and cross-linking agent as an orange coloured bar. C denotes to cysteine residue.
If cross-linking occurs between the cysteine pair of a double cysteine mutant, after treating with the factor Xa protease, the protein band should appear on the corresponding intact full length NrtA protein band size in western blots. In other words, the protein with cross-linked cysteine residues, either proteolyised with factor Xa or not, should be present at the same position (corresponding wild type NrtA protein band size, ~ 48 kDa). If cross-linking with the thiol reactive reagent does not occur, only the C-terminal half of the protein will react with the His-antibody (Figure 5.2).

5.2 Objectives

The objectives of this line of study are two-fold (i) to determine the distance of amino acid residues R368 (Tm 8) from R87 (Tm 2) and N168 (Tm 5); and the distance of asparagine residue at position 459 (Tm 11) from R87, (ii) to investigate the conformational changes of these highly conserved arginine (87 and 368) and asparagine (168 and 459) residues in the presence of substrate.

5.3 Results

5.3.1 Construction of cysteine mutants in the NrtA protein

Mutant construct plasmids each containing a single alteration to cysteine were constructed (Figure 5.3; Table 5.1) by site-directed mutagenesis. The double cysteine mutants were generated by ligation of restriction fragments containing the appropriate single cysteine mutation as described in Chapter Two (Materials and methods). Factor Xa protease site was also engineered by ligation of restriction fragment containing two tandem recognition sequences, IEGR. All mutations were verified by DNA sequencing.

Table 5.1: Generation of single cysteine mutants in a cysteine-less NrtA mutant plasmid.

<table>
<thead>
<tr>
<th>Residue position</th>
<th>Tm location</th>
<th>Residue replaced with cysteine (C)</th>
<th>Primer used (5’ – 3’)</th>
<th>Codon altered</th>
</tr>
</thead>
<tbody>
<tr>
<td>87</td>
<td>2</td>
<td>Arginine (R)</td>
<td>CTA CTA GTT TGC CTT ATC GCC</td>
<td>CGA - TGC</td>
</tr>
<tr>
<td>168</td>
<td>5</td>
<td>Asparagine (N)</td>
<td>GTC TAG GTT GCG CTG GTG G</td>
<td>AAC - TGC</td>
</tr>
<tr>
<td>368</td>
<td>8</td>
<td>Arginine (R)</td>
<td>ATT GTC TCA TGC CCG GCA GG</td>
<td>CGT - TGC</td>
</tr>
<tr>
<td>459</td>
<td>11</td>
<td>Asparagine (N)</td>
<td>ATT CGG GTG CCT CGG CGG T</td>
<td>AAC - TGC</td>
</tr>
</tbody>
</table>

Single cysteine mutants were generated using site-directed mutagenesis by the PCR overlap extension method. (For detail procedures see Chapter 2, Section 2.6.4).
Figure 5.3: 2D structure model of A. nidulans NrtA showing factor Xa protease cleavage sites.

Tms are numbered and presented as cylinders inside the membrane with both C- and N-termini in the cytoplasm. Two tandem sequences (IEGR) were introduced into the large central loop of NrtA (Tm 6 / Tm 7) for site specific proteolysis. The amino acid residues selected for this present study are shown in their respective Tms. This figure is based on the NrtA model proposed by Kinghorn et al. (2005).

5.3.2 DNA:DNA hybridisation and identification of single copy nrtA mutants

Single cysteine mutational plasmid constructs, R368C and N459C (controls for thiol cross-linking experiments) and double cysteine mutants, R87C/R368C, R87C/N459C and N168C/R368C constructs (all constructs possessing the factor Xa cleavage site) were transformed in A. nidulans strain JRK1060 and targeted to the argB chromosomal locus (Chapter 2, Section 2.6.6). After Southern blotting, DNA labelling, DNA:DNA hybridisation with radioactive $^{32}$P probe, transformants with single copy integration patterns were selected and mutation(s) was verified by DNA sequencing. A representative autoradiograph after DNA hybridisation is presented in Figure 5.4.
Figure 5.4: Southern blot analysis and DNA hybridisation with $^{32}$P of mutant N168C/R368C.

DNA from the *A. nidulans* transformants was digested with the *Bam*HI restriction enzyme along with standard single copy (SC) and multiple copy (MC) controls, Southern blotted and cross-linked to the Hybond-N nylon membrane. Hybridisation using a $^{32}$P labelled *Xba*I DNA fragment of the *argB* gene was carried out and mutants with a single copy integration of gene were selected for further study. Values in kb are indicated for standard DNA molecular markers. Single copy transformants exhibited two bands of molecular sizes of 11.7 and 6.6 kb while multiple copy transformants showed an additional band of 9.5 kb. In this presented autoradiograph T10611 and T10615 are single copy transformants. This figure is a representative of all Southern blot analysis results carried out in this study.

5.3.3 Growth testing of transformed strains

Single and double cysteine mutant transformants (all possessing the factor Xa cleavage site) were grown on solid minimal medium with 100 mM nitrate as the sole nitrogen source. No growth was recorded in any of the mutants indicating that the NrtA protein was non-functional in both single and double cysteine mutants. This was not unexpected since the residues under study are essential.

5.3.4 Protein expression levels in cysteine mutants

Once the desired mutation (in single copy transformants) was confirmed by DNA sequencing, crude membranes were extracted from young mycelium and 5 µg of total protein (determined using a BCA protein quantification kit with bovine serum album as standard) was run on a 10 % SDS-PAGE gel. Western blot analysis confirmed the presence of NrtA protein in crude membranes from all single and double cysteine mutants created for this survey. A band of approximately 48 kDa was observed for the wild type strain T454 (positive control), cysteine-less strain T22, both single cysteine mutants R368C or N459C, and all three double cysteine mutant strains (R87C/R368C, R87C/N459C and N168C/R368C). When the level of NrtA protein expression in the mutant strains was compared with
the wild type strain (T454), the expression of protein from the single cysteine mutants, R368C and N459C was higher than that of double cysteine mutants (R87C/R368C, R87C/N459C and N168C/R368C) and the cysteine-less strain T22, but lower than the wild type strain. Amongst the double cysteine mutants, the least protein expression level was observed in mutant N168C/R368C (Figure 5.5). As the double mutants express NrtA protein, we continued with this line of experimentation.

Figure 5.5: NrtA protein expression in mutant strains containing the factor Xa protease site.

![Protein expression of double and single cysteine mutants as well as transformant T22 (cysteine-less NrtA) strain was determined. All strains contained the factor Xa protease recognition sequence. The wild type strain (i.e. with no NrtA amino acid alterations) T454, was used as a positive control. For protein expression, mutants were grown on 5 mM urea for 5 h followed by 100 min induction with 10 mM NaNO₃. Crude membranes were extracted, 5 µg of total protein was run on a 10 % SDS-PAGE gel, western blotted and detected on gels by anti-V5 antibodies. Figure shown here is the representative of three independent experiments.]

5.3.5 Studies of cysteine residues proximity by thiol cross-linking
Disulphide cross-linking (S-S) by homobifunctional thiol reagents combined with factor Xa protease cleavage was used as a molecular ruler to measure the proximity of cysteine residues in a double cysteine knock-in mutant. All cross-linking experiments were carried out using the crude membrane preparations and at 25 °C. Ligand induced conformational changes in these residues were studied by performing the cross-linking in the presence of 10 mM nitrate.

5.3.5.1 Time course study of factor Xa protease digestion
Western blot analysis of single and double cysteine mutants (all possessed the factor Xa protease recognition sequence) displayed one or two protein bands, of less than 30 kDa in size, in addition to the expected full length wild type protein band (~ 48 kDa). Of course such additional small sized bands resulted by proteolysis the reason of which is not known.
To distinguish the protein band actually resulted by factor Xa proteolysis from such degraded protein bands by unknown proteolysis, time course digestion of factor Xa protease was carried out using the strain T22, a cysteine-less mutant NrtA protein with two tandem factor Xa protease recognition sequences.

Factor Xa proteolysis of NrtA protein resulted in an approximately ~ 20 kDa protein band. This protein band continued to undergo further unknown degradation / proteolysis and finally disappeared (Figure 5.6).

![Figure 5.6: Time course of factor Xa proteolysis in the NrtA cysteine-less strain, T22.](image)

Crude membranes were extracted and suspended in factor Xa buffer at a concentration of 1 mg of total protein per ml of factor Xa buffer. An aliquot of 5 µl (5 µg protein) was taken before adding the factor Xa protease (Lane 1). After adding factor Xa protease, the membranes were incubated at 25 ºC. At each time-point (as indicated in the figure), an aliquot of 5 µl was withdrawn and mixed by pipetting with 0.5 mM PMSF (protease inhibitor) to stop further proteolysis and the material stored at -20 ºC. Samples were run on a 12 % SDS PAGE gel and protein was detected using V5 antibody.

5.3.5.2 Optimisation of thiol cross-linking and factor Xa digestion using the cysteine-less strain

The specificity of thiol cross-linkers for cysteine residues was assessed by performing the cross-linking experiments on cysteine-less NrtA strain T22. Results of thiol cross-linker treatment followed by factor Xa digestion, in the presence or absence of 10 mM nitrate suggested that no non-specific cross-linking had occurred by any of the cross-linker tested (Figure 5.7).
Figure 5.7: Specificity of thiol cross-linkers in the cysteine-less strain, T22.
Western blots of membrane proteins from the cysteine-less strain, T22 that underwent thiol cross-linking followed by factor Xa digestion. Western blot presented here is the representative of experiments performed in triplicate.

5.3.5.3 Optimisation of thiol cross-linking and factor Xa digestion using the single cysteine mutants

As cross-linking experiments were carried out on crude membranes, to investigate the specificity of thiol cross-linking between the cysteine pair of a double cysteine mutant, cross-linking experiments were also carried out on single cysteine mutants (with factor Xa protease cleavage site) of its corresponding double cysteine mutants.

Single cysteine mutant R368C (containing the factor Xa protease recognition site) was the control for double cysteine mutants, R87C/R368C and N168C/R368C. N459C (also possesses the factor Xa protease site) was the control for mutant R87C/N459C. Both of these single cysteine mutant controls were treated with the homobifunctional thiol cross-linking reagents by the same method used later in this present study, followed by factor Xa proteolysis.

No cross-linking was observed with any of the cross-linkers in either single cysteine mutants (Figure 5.8). Therefore it was obvious from the preliminary cross-linking experimental trials with Xa protease containing single cysteine mutants, R368C and N459C, that there was no non-specific cross-linking taking place.
Figure 5.8: Optimisation of thiol cross-linking and factor Xa digestion.

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<tr>
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</table>

Immunoblots showing cross-linking followed by factor Xa digestion of single cysteine mutants. Left hand panel shows N459C blots while right hand panel R368C (as annotated under each lane). Crude membranes were incubated in the presence or absence of cross-linker, subjected to factor Xa cleavage and ran on a 12 % SDS-PAGE gel.
5.3.5.4 Proximity of cysteines in mutant R87C/R368C

Cross-linking reagents of varying arm lengths, ranging from 6 to 19.9 Å were used as molecular rulers to measure distances involved in disulphide bond formation between the cysteine pair present in mutant R87C/R368C. Western blot results of cross-linking experiments using homobifunctional thiol cross-linkers between the cysteine residues at position 87 (Tm 2) and 368 (Tm 8) in the presence or absence of nitrate are presented in Figure 5.9. Cysteine pair 87/368 exhibited weak cross-linking of approximately similar strength in the presence or absence of 10 mM nitrate with the rigid cross-linker α-PDM (6 Å). Weak cross-linking was observed between the cysteine residues at position 87 and 368 with Cu-Phen (8 Å) that is also a rigid cross-linker, in the absence of nitrate but the same double cysteine mutant in the presence of 10 mM nitrate did not exhibit the cross-linking suggesting they moved apart in the presence of the substrate. Although BMOE (flexible cross-linker) that also has the same arm length as Cu-Phen (8 Å), moderate level of cross-linking of similar strength in the presence or absence of nitrate, was observed. Significantly stronger disulphide bond formation was achieved with the cross-linking reagents p-PDM (10 Å), BMH (13 Å) and BM(PEG)2 (14.7 Å). Lastly with the longest spacer arm length cross-linker, DPDPB (19.9 Å) very minimal cross-linking was detected in the presence of 10 mM nitrate but no cross-linking recorded in the absence of nitrate.

Another prominent observation on the western blots was the recovery of the cross-linked membrane protein in two bands. The top band was similar to the full length NrtA protein band size (~ 48 kDa) while the other band was around 40 kDa. The ratio of the top band to the smaller (by unknown proteolysis) was estimated and presented in Table 5.2.

From the above thiol cross-linking results, it was concluded that the two cysteines at the position 87 and 368 are approximately 10 Å apart from each other and both of these residues or at least one of them might have moved away slightly from the other residue in the presence or during transportation of nitrate. The semi-quantitative analysis of R87C/R368C (Table 5.2) showed that the best or complete cross-linking was achieved with the 14.7 Å crosslinker, BM(PEG)2. The side chain of arginine residue is approximately 8.9 Å in length and that of cysteine is ~ 4.1 Å. Therefore if both arginine residues were replaced with cysteine the underestimated distance between R87 and R368 by thiol cross-linking is 2 x 4.8 Å. Considering this difference in side chain of cysteine and arginine residues it was concluded that both arginine residues are in close proximity (0.4 Å) if they directly face each other (Figure 5.13, Figure 5.14). However this is the minimum estimated distance between R87 and R368 and depending upon the orientation of the arginine side chain, this distance could be significantly greater.
Figure 5.9: Cross-linking between cysteine pair R87C/R368C.

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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>Xa protease</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
All cross-linking experiments were carried out with crude membranes and at 25 ºC in the presence (+) or absence (−) of 10 mM NaNO₃. Following cross-linking, membranes were incubated overnight with factor Xa protease at 4 ºC. The control, without treatment with cross-linker (0 cross-linker) was compared with the cross-linker treated membranes. All experiments were repeated at least three times and results of one representative experiment shown here.

Table 5.2: Semi-quantitative analyses of R87C/R368C cross-linking results.

<table>
<thead>
<tr>
<th>Cross-linking reagent</th>
<th>Arm length (Å)</th>
<th>Ratio of cut protein band to top protein band*</th>
<th>Content fraction of cut bands compared to control without enzyme**</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-PDM</td>
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<td>20/80</td>
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</tr>
<tr>
<td>Cu-Phen</td>
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<td>30/70</td>
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<td>0.5</td>
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<td>DPDPB</td>
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<td>-</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* The ratio (out of 100) of two bands (for same cross-linked protein) in the factor Xa added lane in the presence of cross-linker. ** refers to the ratio (out of 1) of the protein in the lane with Xa protease versus the lane without Xa protease in the presence of cross-linker.

5.3.5.5 Proximity of cysteines in mutant R87C/N459C

With the mutant R87C/N459C, the pattern of cross-linking by each cross-linking reagent tested was carried out in the presence or absence of 10 mM NO₃. No cross-linking was observed with o-PDM, the shortest length cross-linker. Moderate level of cross-linking was achieved with Cu-Phen (8 Å), a rigid cross-linker while with the BMOE, a cross-linker of also 8 Å in length but flexible, considerable cross-linking was detected. Significant cross-linking was observed with p-PDM a rigid homobifunctional cross-linker in which the bis-maleimide groups are seperated by 10 Å. Maximum cross-linking was
achieved with BMH and BM(PEG)$_2$, flexible cross-linkers of 13 Å and 14.7 Å in length respectively that exhibited the intense band of protein at the position of full length NrtA protein (Figure 5.10; table 5.3). In contrast to mutant R87C/R368C, the cysteine pair at positions 87 and 459 cross-linked significantly with the longest spacer arm length cross-linker DPDPB (19.9 Å) used.

Similarly to the mutant R87C/R368C, the cross-linked proteins of mutant R87/N459 usually appeared as two bands on western blots, a top band of the full length NrtA protein band size ~ 48 kDa and a smaller band of around 40 kDa. The ratio of both these bands is given in Table 5.3.

Results suggested that the inter-thiol distance between position 87 and 459 is approximately 10 Å and the substrate did not alter the conformation or relative position of both residues. The semi-quantitative analysis of R87C/N459 (Table 5.3) cross-linking results indicated that 100 % cross-linking of cysteine residues at position 87 and 459 was accomplished with BMH (13 Å). The side chain of asparagine residue is approximately 3 Å longer than cysteine. Therefore by considering the side chains of arginine (87) and asparagines (459) the distance between R87 and N459 is {10 - (3+4.8)} 2.2 Å which is the minimum distance between these two residues when their side chains directly face each other. However, depending on the placement or angle of the residue side chains, the distance could be significantly longer.

Figure 5.10: Cross-linking between cysteine pair R87C/N459C.
Crude membranes at the concentration 1 mg/ml PBS/0.5 mM EDTA were incubated with cross-linkers at 25 ºC in the presence (+) or absence (–) of 10 mM NaNO₃. After cross-linking membranes were incubated with factor Xa protease. Immunoblots shown are the representative of at least three independent thiol cross-linking experiment.
Table 5.3: Semi-quantitative analyses of R87C/N459C cross-linking results.

<table>
<thead>
<tr>
<th>Cross-linking reagent</th>
<th>Arm length (Å)</th>
<th>Ratio of cut protein band to top protein band*</th>
<th>Content fraction of cut band compared to control without enzyme**</th>
</tr>
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<tbody>
<tr>
<td>o-PDM</td>
<td>6</td>
<td>-</td>
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<td>DPDPB</td>
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<td>0.8</td>
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* The ratio of two bands (for same cross-linked protein) in the factor Xa added lane in the presence of cross-linker. ** refers to the ratio of the protein in the lane with Xa protease versus the lane without Xa in the presence of cross-linker.

5.3.5.6 Proximity of cysteines in mutant N168C/R368C

Similar to the other two double cysteine mutants, R87C/R368C and R87C/N459C results reported above, the distance between two cysteine residues at positions 168 and 368 was also determined by using homobifunctional thiol cross-linker of various lengths coupled with western blotting. No detectable cross-linking of cysteine residues at position 168 and 368 occurred with the two short and rigid cross-linkers, o-PDM (6 Å) and Cu-Phen (8 Å). With the BMOE, a flexible having 8 Å distance between the functional thiol groups, very weak cross-linking was detected that was comparatively better in the absence of nitrate than in the presence of nitrate. A moderate level of cross-linking was achieved with the rigid molecule p-PDM (10 Å) and flexible molecule BMH (13 Å) between the cysteine residues at position 168 and 368. With mutant N168C/R368C, the efficiency of cross-linking was maximum with the flexible cross-linker BM(PEG)_2 (14.7 Å) as most of the protein after factor Xa cleavage migrated to the position of the full length wild type NrtA protein band position. However with the cross-linker DPDPB of 19.9 Å distance between the reactive thiol groups, negligible amount of protein migrated at the position of wild type NrtA on the western blot, indicating very minimal cross-linking in the absence of substrate (nitrate) (Figure 5.11).

In case of cross-linking occurred between cysteine pair of mutant N168C/R368C, two proteins bands; one full length (48 kDa) and other one smaller/proteolysed NrtA protein band (~ 40 kDa) were observed. The semi-quantitative analysis of these top and smaller bands is given in Table 5.4. The estimated distance between the thiols at position 168 and 368 as determined by the cross-linking results was approximately 14 Å. Therefore by considering the side chain lengths of amino acids, the minimum distance between these two residues is {14 - (3+4.8)} 6.2 Å.
Figure 5.11: Cross-linking between cysteine pair N168C/R368C.
The crude membranes from the *A. nidulans* mutant expressing NrtA double cysteine mutant N168C/R368C were extracted. The effect of substrate was studied by conducting cross-linking experiments in the presence (+) or absence (−) of 10 mM NaNO₃. After treating with cross-linker, the membranes were incubated with factor Xa protease at 4 ºC overnight. Equal amount of protein samples were run on a 12 % SDS-PAGE gel and probed using polyclonal V5 antibody. Immunoblots shown here is a representative result from at least three independent experiments.

### Table 5.4: Semi-quantitative analyses of N168C/R368C cross-linking results.

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<th>Cross-linking reagent</th>
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<th>Content fraction of cut bands compared to control without enzyme**</th>
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<td>DPDPB</td>
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* The ratio of two bands (for same cross-linked protein) in the factor Xa added lane in the presence of cross-linker. ** refers to the ratio of the protein in the lane with Xa protease versus the lane without Xa in the presence of cross-linker.
Table 5.5: Summary of the thiol cross-linking results.

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<th>R87C/N459C</th>
<th>N168C/R368C</th>
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</tr>
<tr>
<td>DPDPB</td>
<td>19.9</td>
<td>flexible</td>
<td>–</td>
<td>+</td>
<td>++/–</td>
</tr>
</tbody>
</table>

Cross-linkers o-PDM (6 Å), BMOE (8 Å) Cu-Phen (8 Å), p-PDM (10 Å), BMH (13 Å), BM (PEG)₂ (14.7 Å) and DPDPB (19.9 Å) were used in this study. For cross-linking, crude membranes extracted in phosphate buffer were dissolved in PBS (pH 7.4)/0.5 mM EDTA at the concentration 1 mg/ml. Cross-linking experiments were carried out in the presence (+) or absence (−) of 10 mM NaNO₃. Membranes were incubated at 25 ºC with cross-linkers as follows, 500 µM o-PDM or p-PDM for 30 min, 1 mM Cu-Phen for 15 min, 2.5 mM BMOE, BMH, BM(PEG)₂ or DPDPB for 1 h. After cross-linking, centrifugation was carried out at (4 ºC and 18000 rpm) to remove the cross-linker and any PBS. Membranes were washed twice with factor Xa buffer and then incubated overnight at 4 ºC with (+) or without (−) factor Xa protease. Equal amount of protein digest was run on a 12 % SDS-PAGE gel and protein was probed against V5 antibody. For the cross-linking results (−) denoted no cross-linking, (+/−) very minimal, (+) weak, (++) moderate and (++++) strong cross-linking.

5.4 Discussion

Cross-linking between the two cysteine residues, one present in C-terminal half and the other one present in N terminal half of the A. nidulans NrtA protein was carried out to determine the proximity of highly conserved arginine (R87 and R368) and asparagine (N168 and N459) residues. The thiol cross-linking results of double cysteine mutants provide indirect evidence of residues proximity due to the side chain length difference of different amino acids. Therefore to compare the results of the present study that actually represent the distance between two cysteine residues, the native arginine and asparagine residues were replaced with cysteine using the computer programme Pymol (www.pymol.org), and S-S distance of both cysteines were determined. According to the mutated existing model of NrtA, the cysteine residues at position 168 (Tm 5) and 368 (Tm 8) are too close with each other that no distance was detected between their S-S atoms. However results of present study using molecular rulers disagree with the positioning of these residues and provide evidence that both residues (168 and 368) are more distant than suggested by the existing NrtA protein model. On the other hand, residue 87 and 368 are closer (10 Å apart) to each other than suggested by the prevailing model (26 Å). Finally, data from the results of present study suggested a distance of 10 Å between cysteine residues at position 87 (Tm 2) and 459 (Tm 11) which is 13 Å in the NrtA current model...
Figure 5.12: Comparison of residue positions in NrtA existing model and the cross-linking results.

(a) In the homology based NrtA protein structural model, using the Pymol software (www.pymol.org), the native residues at positions 87, 168, 368 and 459 were replaced with cysteine residues. The pair wise distance between cysteine residues as indicated was determined by same computer programme. On the basis of this distance (Å), respective Tms have been placed. (b) The Tms have been rearranged following the present study results of thiol cross-linking. The distances between the paired residues is mentioned. Please note that this is the tentative re-modelling of NrtA protein based on the results of present study combined with personal imagination.

Arginine has a long side chain and it is approximately 4.8 Å longer than the cysteine residue while the side chain of asparagine is approximately 3 Å longer than cysteine (Figure 5.13). While interpreting the results of cysteine cross-linking another factor that should be considered is the volume of an amino acid.

Figure 5.13: Structural comparison of amino acid residues asparagine and arginine with cysteine.

The volume of cysteine (C₅H₉NO₂S) is 108.5 Å³, of asparagine (C₅H₁₀N₂O₃) is 114.1 Å³ and of arginine (C₆H₁₄N₄O₂) is 173.3 Å³. Among these residues, arginine has the longest and cysteine has the shortest side chain.
Therefore taking into account the differences in side chain lengths of these amino acids, from the results obtained in the present study, it is concluded that R87 (Tm 2) and R368 (Tm 8) are very close to each other (Figure 5.15) and lie on the same face of Tm 2 and Tm 8 respectively. The distance between residues R87 and N459 is approximately 2.2 Å, and N168 and R368 is around 6.2 Å.

Figure 5.14: Analysis of thiol cross-linking results.

Thick blue arrows show the backbone and black arrows represent the side chains of amino acid residues.

It is inferred from the results obtained here that R87 and R368 and hence Tm 2 and Tm 8 are in close proximity (Figure 5.12b). Unkles et al. (2004a) using the second-site suppressor studies on these conserved arginine residues, R87 and R368 suggested at least one of these arginine residues is present near to N459. The results from this work also confirm α - helical arrangements of NrtA Tms in the membrane in which these R87, R368 and N459 are present on same face on their respective Tms (Wolin and Kaback, 2002).

It has also been reported that to study residue proximity and transport activity of cross-linked protein, not only length but also the flexibility of thiol cross-linker is an essential factor. Zhou et al. (2008) determined the whole range of S-S distance of thiol cross-linkers. As in present study no (very weak) cross-linking was observed in cysteine pair 87/368 and 168/368 with DPDPB cross-linker while
significant cross-linking was observed by DPDPB in cysteine pair 87/459. The minimum distance of thiol reactive groups in flexible DPDPB cross-linker reported is approximately 9.3 Å (Green et al., 2001). This result suggests that the double mutants N168/R368 should also show good cross-linking with DPDPB. The explanation for no observable cross-linking with DPDPB with mutant N168C/R368C is the orientation of these residues that results in inaccessibility of one or both cysteine residues to DPDPB (Zhang et al., 2002). It is also possible that this region of protein is inflexible to accommodate such a large molecule. The cross-linker BM(PEG)$_2$ has been reported to be sufficiently flexible, with minimum of 3.5 Å distance between thiol reactive groups (Green et al., 2001) and also resulted in highly efficient cross-linking in all the three double cysteine mutants studied in this present study.

One of the problems associated with the thiol cross-linking technique was the weakly cross-linked cysteine residues (Zhang et al., 2002) that made it difficult to decide whether this showed the actual distance between thiol groups of cysteine residues or not. The possible explanation is that at certain residue positions, one of the thiol groups reacts more efficiently than the other one due to its native location in membrane resulting in less protein being recovered at the wild type protein band position (Zhang et al., 2002). Alisio and Mueckler (2004) studying the GLUT1 glucose transporter, suggested that lack of cross-linking is not always due to the relative distance between thiol reactive groups but is could also be due to the inaccessibility of both or one of the cysteine residue to cross-linker.

An additional smaller protein band on our western blots has been observed in a number of NrtA mutants (S E Unkles, Unpublished Data). Obviously this smaller band was due to some proteolysis. A broad-spectrum protease inhibitor cocktail was added to the membrane extraction buffer therefore this proteolysis is unlikely to have occurred during membrane extraction. As the mutants used for present study were non-functional so it might be possible that natural misfolding or degradation of protein occurred.

The presence of arginine residues (R87 and R368) in close proximity (~ 0.4 Å) as indicated by the results obtained in present study raised the question as to how two positively charged residues could be present very close to each other. In case of LacY many of the distances between the residues determined by the thiol cross-linking were consistent with that determined by x-ray crystal structure but still a number of residues were discovered on the cytoplasmic side of LacY where the distances between the amino acid residues were under-estimated probably due to the instability in the conformation of protein (Abramson et al., 2003). It has also been demonstrated that disulphide bond
between cross-linking reagent and cysteine may result in trapping of cysteine molecule and bring them close with each other (Careaga and Falke, 1992). Another problem of this study was all the three double cysteine mutants were non-functional, although the western blot of these non-functional double cysteine mutants exhibited wild type NrtA protein bands but still it is possible that protein is not in natural conformational state. Therefore there is a need to study the cross-linking among the residues around R87 and R368 to come to a conclusion. Examining the thiol cross-linking between the residues present of the same face of Tm 2 and Tm 8 as R87 and R368 (positive control) and on opposite face (negative control) might help to confirm the efficiency of thiol cross-linking technique in this system.

5.5 Conclusion

It is concluded from the present study that R87 and R368 or in a broader sense, Tm 2 and Tm 8 are closer than described by the existing model of NrtA protein. Both arginine residues R87 and R368 move apart to give ~ 2 Å distance from each other to accommodate the nitrate molecule during translocation.

The work presented here is an initial step in collecting biochemical data to study the static or dynamic aspects of NrtA. In the unavailability of crystal structure of NrtA transporter a considerably huge biochemical evidences are required for purposing the structure and functional mechanism of this transporter protein.
Chapter Six

Construction of a library of single cysteine mutants in NrtA transmembrane domains 2 and 8

6.1 Introduction

6.1.1 Background

In the absence of a crystal structure due to difficulties in crystallisation of membrane proteins, mutational analysis combined with the biochemical studies of membrane transporter proteins can provide clues or even identify amino acid residues that play vital roles in substrate binding and translocation (Unkles et al., 2004a; Kinghorn et al., 2005; Guan and Kaback, 2007; Chaptal et al., 2011; S E Unkles, Unpublished Data).

6.1.2 Cysteine-scanning mutagenesis

Cysteine amino acid is of average steric bulk and amenable to specific modifications. In addition, the hydrophobic nature of cysteine is an important feature, which makes cysteine-scanning mutagenesis a powerful technique in structural and functional studies of proteins. Cysteine-scanning mutagenesis may be successfully employed in determining the transmembrane spanning regions of polytopic membranes (Kimura et al., 1996; Frillingos et al., 1998; Kaback et al., 2001; Guan and Kaback, 2007). Initially all native cysteine residues (if present) in a protein are replaced systematically using site-specific mutagenesis to give a cysteine-less, functional protein. In such a cysteine-free protein background, cysteine residues are introduced one by one individually and a crop of single cysteine mutants is generated.

Thiol specific reagents are used for their accessibility to cysteine residue and assist the study of membrane topology (Cao and Matherly, 2004; Punginelli et al., 2007), identifying the amino acid residues in the regions lining the translocation pathway (Kimura et al., 1996; Tang et al., 1999; Karatza et al., 2006; Wang et al., 2008a) as well as the spatial proximity between transmembrane domains (Falke et al., 1997; Kaback et al., 2007).

6.1.3 Cysteine-scanning mutagenesis of E. coli LacY

Escherichia coli LacY is the most extensive studied MFS transporter protein. Cysteine-scanning mutagenesis at almost every position of LacY has been carried out over many years (Frillingos and Kaback, 1996; Frillingos and Kaback, 1997; Venkatesan et al., 2000a; Venkatesan et al., 2000b; Venkatesan et al., 2000c; Kwaw et al., 2001; Ermolova et al., 2003; Zhang et al., 2003; Ermolova et al., 2006). Recently the crystal structure of a single cysteine (at position 122) mutant LacY protein
has been solved (Chaptal et al., 2011) to gain detailed insights into the LacY structure in a different conformational state. The results and observations of cysteine-scanning mutagenesis, for example, the presence of single sugar binding site in the middle of the protein and opening and closing of periplasmic and cytoplasmic cavity alternately during translocation of substrate, are consistent with the information provided by the crystal structures of the LacY (Abramson et al., 2003; Mirza et al., 2006; Guan et al., 2007; Chaptal et al., 2011). Therefore, this powerful technique is quite promising to allow basic architectural study of other membrane proteins including NrtA.

6.1.4 Cysteine-scanning mutagenesis of some other transporter proteins

Cysteine-scanning mutagenesis strategy was successfully employed equally to prokaryotic as well as eukaryotic transporter proteins. TetA, the bacterial metal-tetracycline / H+ antiporter is another MFS membrane protein underwent extensive cysteine-scanning mutagenesis resulting in the mapping of positions and role of most of its amino acid residues. Cysteine-scanning of TetA identified the membrane boundaries of embedded regions and water extruding loops connecting the transmembrane domains (Kimura et al., 1996; Tamura et al., 2001). The results of cysteine-scanning of TetA mutants in Tm 3, Tm 6 and Tm 9 revealed that all these three Tms are completely buried in hydrophobic part of membrane (Kimura et al., 1996; Kimura et al., 1998; Konishi et al., 1999).

In the oxalate transporter, OxlT of Oxalobacter formigenes another member of MFS, the accessibility of the single cysteine mutants spanning Tm 5 to thiol reactive regents not only revealed residues that were present in translocation pathway but also conformational change upon substrate binding (Wang et al., 2008a). Cysteine substitution and sulfhydryl labelling of cysteine residues carried out to probe the topology of TatC component of E. coli twin arginine translocase suggested that TatC is composed of six transmembrane helices instead of four (Punginelli et al., 2007).

Substitutive cysteine-mutagenesis in combination with the sulfhydryl chemistry was used to identify the residues that line the translocation pore of AE1, the chloride / bicarbonate anion antiporter of human erythrocyte (Tang et al., 1999). Using the same method, in another transporter, the human reduced folate carrier (hRFC), the number of Tms (12) was confirmed (Cao and Matherly, 2004).

6.1.5 Cysteine-scanning mutagenesis of A. nidulans NrtA

Cysteine-scanning mutagenesis approach has been effectively employed to study the static and dynamic aspects of structure and function of many prokaryotic as well as eukaryotic (including humans) transporter proteins. Although LacY is a symporter and TetA is an antiporter, the comparative analysis of complete set of cysteine-scanning results of both LacY and TetA identified
interesting similarity in residue positions and helix packing and hence molecular structure of both of these MFS transporters (Tamura et al., 2001).

Therefore cysteine-scanning mutagenesis could be used to determine the molecular structures of other MFS transporters including NrtA symporter. A functional cysteine-less NrtA mutant construct with approximately 40% wild type nitrate transport activity, was generated by Kinghorn and colleagues (Unkles et al., 2005). The basic aim of generating such cysteine-less construct was to alter the individual amino acid to cysteine residue and then study the role of each amino acid residue in transportation of nitrate by thiol chemistry.

6.2 Objective

The main objective of this section of the research was to create a library of single cysteine mutants in the residues spanning Tm 2 and Tm 8 of A. nidulans NrtA and later use this library to determine the local environment (either hydrophobic or hydrophilic) at each residue position and identifying conformational changes upon substrate binding.

6.3 Results

6.3.1 Single cysteine substitutions in Tm 2 and Tm 8 residues in NrtA
The NrtA homology model developed by Kinghorn and colleagues (Kinghorn et al., 2005 and references therein) suggested that Tm 2 or Tm 8 has 21 amino acid residues. According to this model, Tm 2 spans residues N74 to A94 and Tm 8, K355 to A375. Native cysteine residues located at positions 90 and 94 were replaced with alanine (A) and at position 367 with serine (S) in the cysteine-less construct. The residue at each position of Tm 2 and Tm 8 were replaced individually with the cysteine (Figure 6.1) in collaboration with Eugenia Karabika to generate a library of single cysteine mutants encompassing Tm 2 and Tm 8 of NrtA.
Figure 6.1: Simplified provisional secondary structure model of *A. nidulans* NrtA protein.

This model is based on the NrtA model proposed by Kinghorn and colleagues (2005). 12 transmembrane domains (Tms) are presented as numbered cylinders in the membrane. Both Tm 2 and Tm 8 are highlighted. The C- and N-terminal ends of NrtA are towards cytoplasm. The amino acid residues of Tm 2 and Tm 8, which were replaced individually one by one with cysteine, are shown in one-letter amino acid code. The first and last positions of residues in Tm 2 and Tm 8 are numbered. Conserved arginine residues, R87 (Tm 2) and R368 (Tm 8) are highlighted in red and conserved glycine residues, G91 (Tm 2), G371 and G372 (Tm 8) in blue.

6.3.2 Identification of single copy transformants

Mutant plasmid constructs containing single cysteine mutation were transformed into *A. nidulans* strain JRK1060 (*nrtA747, nrtB110, argB2*) by S E Unkles. Single copy integrated transformants were identified by Southern blotting followed by DNA hybridisation with $^{32}$P (Figure 6.2). At least one single copy transformant from each mutant was verified by DNA sequencing. (Table 6.1, Table 6.2).
Figure 6.2: Southern blotting and DNA labelling of mutant A79C transformants.

BamHI digested DNA fragments from approximately 8-10 transformants for each of single cysteine mutant were Southern blotted on the Hybond-N nylon membranes with a standard single copy (SC) and multiple copy (MC) transformants as controls. $^{32}$P hybridisation was carried out using XbaI DNA fragment (argB gene) as the probe. DNA size marker is shown in kb. Single copy transformants were identified by the presence of two bands of molecular sizes 11.7 and 6.6 kb while multiple copy transformants exhibited an additional band of 9.5 kb. This figure is the representative of all Southern blotting and $^{32}$P DNA labelling carried out to identify the single copy transformants. ‘T’ refers to transformant.

6.3.3 Growth tests of single cysteine mutant transformants

Single cysteine mutants encompassing Tm 2 and Tm 8 were grown on minimal medium supplemented with nitrate as the sole source of nitrogen. Among this group of 42 single cysteine mutants, most of the mutants failed to grow on nitrate even provided 100 mM NaNO$_3$ in the medium at 37 ºC. A small percentage of mutants in Tm 2 (I78C, A82C and L84C) and Tm 8 (K355C, A357C, G361C, L363C, P369C, A370C, F373C, L374C) were able to grow on 100 mM NaNO$_3$ but these mutants exhibited a poorer growth relative to the NrtA cysteine-less strain.
Using site-directed mutagenesis by PCR amplification, single cysteine mutants were generated in the cysteine-less NrtA construct. A transformant is represented by ‘T’. Qualitative assessment of protein expression in single cysteine mutants was carried out. ‘+++’ is the wild type level of protein expression, ‘++’ denotes intermediate level while ‘+’ is for the poor expression of protein.

### Table 6.1: The NrtA Tm 2 single cysteine mutants.

<table>
<thead>
<tr>
<th>Residue position</th>
<th>Residue altered with cysteine</th>
<th>Mutant</th>
<th>Single copy transformant(s) confirmed by DNA sequencing</th>
<th>Growth on 100 mM NaNO₃</th>
<th>Level of western protein expressed</th>
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<tr>
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### Table 6.2: The NrtA Tm 8 single cysteine mutants.

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<th>Residue position</th>
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<th>Single copy transformant(s) confirmed by DNA sequencing</th>
<th>Growth on 100 mM NaNO₃</th>
<th>Level of western protein expressed</th>
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Legends are as described for Table 6.1.
6.3.4 Protein expression of NrtA single cysteine mutants

The growth tests results suggested that most of the mutants failed to grow on nitrate as the sole source of nitrogen however the expression of the NrtA protein from all the 42 single cysteine mutants was observed. The level of NrtA protein expression in some of the representative single cysteine mutants is shown in Figure 6.3. Expression level of protein was compared with A. nidulans strain T454 that contained wild type NrtA protein (positive control) and A. nidulans strain T110 lacking the NrtA protein (negative control). Single cysteine mutants possessed a single band of wild type NrtA protein with band size ~ 48 kDa. The majority of single cysteine mutants in both Tm 2 and Tm 8 showed approximately wild type level of protein expression. The level of protein expression from the single cysteine mutants is summarised in Table 6.1 (Tm 2) and Table 6.2 (Tm 8).

Figure 6.3: Protein expression of some representative single cysteine mutants in Tm 8 of NrtA protein.

Single cysteine mutant strains were grown in liquid minimal medium supplemented with 5 mM urea as the sole source of nitrogen for 5 h and then induced with 10 mM NaNO₃ for 100 min. Cells were harvested and crude membrane were prepared. Approximately 5 µg of total protein was run on 10 % SDS-PAGE gel along with positive (strain T454) and negative (strain T110) controls. After western blotting, proteins were probed using anti V5 antibody. Protein size marker is shown in kDa.

6.4 Discussion

Unkles et al. (2005) constructed a cysteine-less NrtA protein to investigate the structurally and functionally important amino acids by cysteine-scanning mutagenesis and thiol chemistry. No change in the Kₘ for nitrate was observed in the cysteine-less NrtA mutant that indicated none of the native cysteine residue was essential or directly involved in nitrate transport activity. In this present study a total of 42 single cysteine mutants in Tm 2 and Tm 8 were generated and thus represents an extension of this particular line of NrtA membrane protein research.
The growth test results indicated that most of the alterations to cysteine in Tm 2 and Tm 8 in the cysteine-less NrtA transporter protein background, were not tolerated and single cysteine mutants did not grow even on higher concentration of nitrate. The possible reasons for this loss-of-function in cysteine substituted strains are (i) side chain constrains of natural amino acids residues i.e some amino acids (eg. methionine) have larger side chains and others have a smaller volume (glycine) than the cysteine residue, (ii) loss of an essential aromatic amino acid (eg. tryptophane, phenylalanine), (iii) loss-of-charge from positively charged (eg. arginine) or negatively charged (eg asparagine) amino acids and finally (iv) the replacement of conserved residues in Tm 2 and Tm 8 that have crucial role in nitrate binding and / or translocation.

In Tm 2, R87 and G91 are highly conserved residues in prokaryotes as well as eukaryotes and the native cysteine residue present at position 90 (that was replaced with alanine in cysteine-less construct) is conserved in eukaryotes. Tm 8 possessed three highly conserved residues R368, G371 and G372 (Kinghorn et al., 2005). Unkles and colleagues (Unkles et al., 2004a) using the wild type NrtA background altered R87 and R368 with seven amino acid residues, asparagines, cysteine, histidine, isoleucine, glutamate, glutamine or threonine. All these arginine substitutions at position 87 or 368 with one of a variety of amino acids having different properties and side chain volumes, resulted in no growth on nitrate. Only the positively charged lysine substitution of both conserved arginines was tolerated. Another important factor to be considered, is the presence of a part of MFS signature motif in both Tm 2 (G91 – C94) and Tm 8 (G372 – A375) (Figure 6.1). It has been reported that alteration in MFS motif residues in LacY (Jessen-Marshall et al., 1995) and NrtA (Kinghorn et al., 2005) resulted in a significant reduction in the activity of these transporters although reasons are unclear.

NrtA Tm 2 and Tm 8 are considered important in the binding and transport of nitrate due to the presence of conserved arginine residues, R87 and R368 (Unkles et al., 2004a) The prevailing model of NrtA coupled with biochemical studies also suggests that these conserved arginine residues are involved in forming a translocation pore. But the present study suggests that many residue substitutions with cysteine in both Tm 2 and Tm 8 could cause major structural disturbance probably due to misfolding of the NrtA with consequent loss-of function.

Protein expression of mutants having single cysteine alterations was consistent with those of other prokaryotic and eukaryotic single cysteine mutants (Frillingos and Kaback 1996; Frillingos and Kaback, 1997; Falke et al., 1997; Tang et al., 1999; Cao and Matherly, 2004; Karatza et al., 2006;
Kaback et al., 2007; Punginelli et al., 2007; Wang et al., 2008a) indicated that cysteine substitution did not affect the insertion of the NrtA protein in the membrane (Kinghorn et al., 2005).

6.5 Future work

As it is advantageous for topological studies of membrane proteins that such single cysteine mutants should retain their activity (Tamura et al., 2001; Wang et al., 2008a), therefore the loss-of-function single cysteine mutants (implied by the loss-of-growth) in Tm 2 or Tm 8 was not studied further. However similar studies might be successfully extrapolated to other Tms of the NrtA to map the location of individual amino acids in lipid or aqueous environment of membrane.

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Chapter Seven

Crystallography trials of bacterial nitrate transporters

7.1 Introduction

7.1.1 Background
Detailed information on three dimensional structures of membrane proteins, that play a key role in crucial cellular metabolic processes of cells, is essential for understanding their working mechanism. Membrane proteins encoding genes constitute a significant number of the sequenced genomes but due to the lack of laboratory techniques for over-expression and purification of these hydrophobic molecules that naturally occur in minute quantity, only few of them have been studied in detail by crystallography (Drew et al., 2005). The main problem in working with membrane proteins is their poor stability because once these proteins are extracted from the lipid bilayer they lose their natural folding and hence function. As extraction of the membrane protein from the lipid bilayer is essential while working with the currently available laboratory techniques therefore optimisation of the best medium that could maintain the natural integrity or stability of membrane protein is the milestone in determining the detailed structure of these proteins. Detergent containing solutions are usually employed to overcome this problem but important goal is to determine the best detergent for each protein that can be employed for its extraction in the monodispersed form and to maintain its stability (Bowie, 2001; Kawate and Gouaux, 2006).

7.1.2 Membrane protein crystallisation
X-ray diffraction studies, the most commonly used method of protein structure determination require protein crystals (Newstead et al., 2008; Leviatan et al., 2010). Conventional pre-crystallisation screening methods for membrane proteins are more time and resource consuming because such methods require microgram to milligrams of protein. However membrane proteins have low expression levels in nature (Kawate and Gouaux, 2006; Sonoda et al., 2010). Another difficulty associated with membrane proteins is usually they over-express in inclusion bodies instead of in cytoplasmic membranes. Practically it is not easy to purify proteins from inclusion bodies and also it is not possible to know beforehand whether specific protein under study will express in cytoplasmic membrane or in inclusion bodies (Drew et al., 2001). Therefore screening for membrane protein over-expression is even more laborious, unexciting and unrewarding.

To attempt to overcome the problems associated with the over-expression and purification of membrane proteins, their fusion to the green fluorescence protein (GFP) can be used as an indicator of level of protein expression (Drew et al., 2003; Kawate and Gouaux, 2006; Hammon et al., 2009; Liu and Niasmith, 2009; Hsieh et al., 2010; Chaudhary et al., 2011; Ihara et al., 2011). The 27 kDa
GFP originally identified in the jellyfish, *Aequorea victoria*, fluorescent green under UV light. GFP fused to a membrane protein allows not only the direct monitoring of the level of protein expression without purification but also differentiates whether or not the protein is expressed in the membrane or inclusion body. In this regard, if located in the inclusion body, no green fluorescence is detected (Drew *et al.*, 2001; Sonoda *et al.*, 2010). In small scale expression experimental trials, GFP fused target protein has been proved to be a very useful tool to optimise the over-expression conditions. In addition as one can easily repeat such simple experiments until reaches to desired expression level (Liu and Niasmith, 2009; Hsieh *et al.*, 2010).

7.1.3 Pre-crystallisation screening of detergent

The stability of the protein in detergent-containing solution is the key for its successful crystallisation (Kawate and Gouaux, 2006; Newstead *et al.*, 2008; Sonoda *et al.*, 2010). One crucial advantage of GFP protein fusions is the study of homogeneity or monodispersity of protein extracted in a detergent-containing solution. Fluorescence-detection size-exclusion chromatography (FSEC) is a potential approach for screening homogeneity or monodispersity as well as the stability of protein in a detergent-containing-solution (Kawate and Gouaux, 2006; Sonoda *et al.*, 2010; Ihara *et al.*, 2011). For FSEC, a fluorescence detector is attached to a size-exclusion chromatography (SEC) system that detects the GFP fluorescence signals (Figure 1.16, Chapter 1). In this way, therefore elution of GFP fusion protein under study may be monitored in cell lysate or crude membrane preparations. Because FSEC is a very sensitive technique (less than ~ 10 ng of GFP can be detected in crude membrane preparations), large scale grow ups and purification of protein is not required (Kawate and Gouaux, 2006; Liu and Niasmith, 2009; Sonoda *et al.*, 2010). As well as the fluorescence detector, FSEC set up also includes a UV detector for total proteins in cell lysates and a fraction collector to purify GFP fusion proteins on the basis of fluorescence peak profiles. Using the FSEC as pre-crystallisation tool, certain membrane proteins have been successfully crystallised (Sobolevsky *et al.*, 2009; Shaffer *et al.*, 2009; Kawate *et al.*, 2009; Gonzales *et al.*, 2009).

In this present study, covalently linked GFP to nitrate transporters from bacteria, *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa*, or *Thermus thermophilus*, were studied for over-expression and pre-crystallisation trials. Cultural conditions were optimised for their over-expression in *E. coli* competent cells in small volume cultures. Over-expressed proteins were screened for their solubility and stability in detergent containing solutions by FSEC. Based on the FSEC results, optimised conditions / detergents were selected for scale-up production and detergent extraction of proteins. As the target proteins contain histidine tags, immobilised metal ion affinity chromatography (IMAC) and gel filtration were employed for their purification.
7.2 Objectives

The objectives of this structural aspect of nitrate transport study were to attempt to optimise (i) over-expression, (ii) detergent screening for purification and (iii) crystallisation of nitrate transporter proteins.

7.3 Results

7.3.1 GFP based screening of proteins over-expression

Green fluorescence in UV light was selected as a tool to monitor the expression levels in fusion proteins. For the initial trials, GFP fused *E. coli* NarU or *T. thermophilus* NarK1 proteins were employed to optimise the over-expression of the fusion proteins in *E. coli* expression systems (host cells), BL21(DE3) and Lemo21(DE3). Host cells transformed with the plasmids encoding the transporter proteins were grown on LB medium and ZYP auto-induction medium at different incubation temperatures 20, 25, 30 and 37 ºC.

Increasing concentrations of IPTG was used to induce the protein expression in the cells grown in LB medium. It was observed that the addition of IPTG resulted in the death of BL21(DE3) and Lemo21(DE3) host cells expressing either *E. coli* NarU or *T. thermophilus* NarK1 fusion protein, as rapid decline in the cells culture OD$_{600}$ was recorded. With the 50 µM IPTG, approximately 75% cell death was recorded. It was also observed that the host cells expressing the *E. coli* NarU, grown on LB without induction showed significantly high green fluorescence in UV light as compare to those induced with 50 µM IPTG. The significantly high over-expression of *E. coli* NarU, as evaluated by visual inspection in UV light, was recorded when expressed in BL21(DE3) cells, grown on ZYP auto-induction medium. No green fluorescence was detected in the cell pellet of host cells expressing *T. thermophilus* NarK1 at least in the conditions applied.

7.3.2 SDS-PAGE of crude membranes over-expressing *E. coli* NarU fusion protein

The host cells harbouring *E. coli* NarU or *T. thermophilus* NarK1 were grown under conditions optimised for the over-expression of *E. coli* NarU fusion protein based on GFP. The crude membranes were extracted from cells and levels of expression were determined by SDS-PAGE gel. The expression of *E. coli* NarU fusion protein was very high as determined by Coomassie Blue staining (Figure 7.1). When the membrane pellets of both fusion proteins were observed in UV light, significant green fluorescence was recorded for *E. coli* NarU fusion protein but not for *T. thermophilus* NarK1 (Figure 7.2). Similar to the GFP based analyses (Section 7.3.1) the expression of *E. coli* NarU fusion protein was higher in uninduced cells as compared to the induced ones, grown in LB liquid medium. This level was approximately similar to the level of expression in the host cells grown on ZYP auto-induction medium. The *T. thermophilus* NarK1 fusion protein failed to over-
express under any of the conditions tested, as detected by SDS-PAGE gel and observing the crude membranes pellet in UV light (Figure 7.1, Figure 7.2).

Figure 7.1: SDS-PAGE of crude membranes extracted from the host cells expressing GFP fused *E. coli* NarU and *T. thermophilus* NarK1 proteins.

15 μg of total protein was run on the SDS-PAGE gel and stained with the Coomassie Blue stain. Lane 1: *T. thermophilus* NarK1, expressed in BL21(DE3) strain, grown on LB medium and induced with 50 μM IPTG; Lane 2: *T. thermophilus* NarK1, expressed in Lemo21(DE3) strain, grown on LB medium and induced with 50 μM IPTG; Lane 3: *T. thermophilus* NarK1, expressed in BL21(DE3) strain, grown on ZYP auto-induction medium; Lane 4: *E. coli* NarU, expressed in Lemo21(DE3) strain, grown on ZYP auto-induction medium; Lane 5: *E. coli* NarU, expressed in BL21(DE3) strain, grown on LB medium without induction with IPTG; Lane 6: *E. coli* NarU, expressed in BL21(DE3) strain, grown on LB medium and induced with 50 μM IPTG; Lane 7: *E. coli* NarU expressed in BL21(DE3) strain, grown on ZYP auto-induction medium; Lane 8: *E. coli* NarU expressed in Lemo21(DE3) strain and grown on ZYP auto-induction medium. Strains were grown at 23 °C and 250 rpm orbital shaking. The arrow shows the position of the *E. coli* NarU fusion protein that was confirmed by mass spectroscopy (data not shown).
Figure 7.2: GFP based analysis of *T. thermophilus* NarK1 and *E. coli* NarU fusion proteins.

The crude membranes extracted from the over-expressed BL21(DE3) host cells transformed with GFP fused *T. thermophilus* NarK1 or *E. coli* NarU. The cells were grown in LB (Uninduced or induced with 50 µM IPTG) and ZYP auto-induction medium. Green fluorescence of proteins was observed in UV light.

7.3.3 Determination of over-expression of some other prokaryotic nitrate transporter proteins

Five different prokaryotic nitrate transporter proteins from four bacterial species were studied for over-expression and detergent solubility screening. The optimised conditions under which *E. coli* NarU fusion protein over-expressed were, transformed in BL21(DE3) host cells and grown on either ZYP auto-induction medium or LB medium without IPTG induction. Therefore the similar condition applied to other nitrate transporters, *T. thermophilus* NarK2, *Pseudomonas aeruginosa* NarK1, *P. aeruginosa* NarK2 and *Bacillus cereus* Nar (all fused with GFP), their degree of expression was recorded. The expression intensity was first determined by (i) observing the cell pellet in UV light for green fluorescence, (ii) comparing the values of relative fluorometric units (RFU) determined in cell lysate (Figure 7.3) and (iii) electrophoresis of the crude membranes on SDS-PAGE gel. With the exception *T. thermophilus* NarK2, all proteins fluoresced.

From the results presented in Figure 7.3, the expression of the transporter proteins studied, except *P. aeruginosa* NarK1 where the amount of protein expressed was approximately similar in both LB and ZYP auto-induction medium, higher expression was observed in ZYP auto-induction medium grown cells. The highest RFU value and hence the level of expression was achieved for the *B. cereus* Nar fusion protein.
Figure 7.3: The over-expression screening of GFP fused nitrate transporter proteins.

Level of expression in fluorescence based screening, is presented in relative fluorometric units (RFU) determined in cell lysates (as described in Chapter 2, Section 2.7.3.3). GFP fused proteins were expressed in BL21(DE3) E. coli host cells grown in LB without induction or ZYP auto-induction broth medium under the conditions optimised for over-expression, cells were lysed and RFUs were recorded. Ec denotes E. coli; Bc, B. cereus; Pa, P. aeruginosa and Tt, T. thermophilus. Experiments were carried out in triplicate. Results of one representative experiment are presented here.

Figure 7.4: Comparison of GFP fused nitrate transporter proteins expression.

15 µg of total protein from crude membranes (containing the GFP fused over-expressed nitrate transporter proteins in BL21(DE3) expression system) were run on SDS-PAGE gel and stained with histidine tag stain. On the left hand side, the migration positions of standard protein bands are indicated in kDa. Lane 1: E. coli NarU, cells grown in LB and Lane 2: in ZYP auto-induction medium. Lane 3: B. cereus Nar, cells grown in LB and Lane 4: ZYP auto-induction medium. Lane 5: P. aeruginosa NarK1, cells grown in LB and Lane 6 in ZYP auto-induction medium. Lane 7: P. aeruginosa NarK2, cells grown in LB and Lane 8: in ZYP auto-induction medium. Lane 9: T. thermophilus NarK2, cells grown in LB and Lane 10: in ZYP auto-induction medium. All cells were grown in shake cultures at 250 rpm and 23 °C.
7.3.4 Detergent screening for protein solubilisation by FSEC

To determine which target proteins would be appropriate for crystallisation trails, fluorescence detection size-exclusion chromatography (FSEC) was employed for pre-crystallisation screening of suitable combination of protein and solubilising detergent. Test proteins that yielded high fluorescence in small scale optimisation were further screened for their stability and solubility in various micelle sized detergents. Crude membranes were extracted and solubilised in the detergent-containing solution followed by ultra centrifugation. The supernatant was loaded on to a size-exclusion chromatography column (SEC) fitted to the ÄKTA purifier. A fluorescence detector was also attached to this equipment set up to monitor GFP fused proteins visually using Unicorn software.

Figure 7.5: A representative FSEC profile for GFP fusion proteins.

A typical FSEC peak profile of GFP fusion protein, designated as Target protein-GFP-His, is presented. The small peak in the void volume represents the amount of aggregated or insoluble protein in detergent-containing solution. The peak designated as ‘GFP-His’ represents the free GFP that proteolysed from its fusion. The sharp and symmetrical Target protein-GFP-His peak is the indication of monodispersed protein in detergent solution.

7.3.4.1 Pre-crystallisation detergent screening of E. coli NarU

FSEC peak profiles of solubilised GFP fused E. coli NarU samples in various detergent solutions were different in term of their symmetry or monodispersity as shown in the Figure 7.6. It was also clear that the type of growth medium employed also affected the expression. For example, protein isolated from the cells that were grown in LB, had single and symmetrical peaks in DDM, DM and in OTG containing solutions, although the fluorescence in OTG was very low indicating the low solubility of protein in this detergent. It means the proteins were monodispersed in these detergents and no protein was found in void volume, therefore this protein did not tend to aggregate in these detergents. In other two detergents, LDAO and OG, not only the fluorescence level was very low but also the peak profiles were asymmetrical suggesting that these small micelle sized detergents were not suitable for extractions or finally crystallisation of E. coli NarU fusion protein.
The FSEC results of the *E. coli* NarU expressed in the cells grown in ZYP auto-induction medium exhibited almost the similar type of peak symmetry in DM and OTG as for the cells grown in LB medium. But for the protein, soluble in DDM, two peaks of almost equal size were found, one for GFP-fusion protein and the other one supposed to be due to free GFP (Figure 7.6) that was cleaved from its fusion.

The peaks were relatively sharper and higher for proteins extracted from the cells grown in LB medium without induction. Figure 7.7 (a&b) suggested that OTG, DM and DDM may be the promising detergents for scale-up production and extraction of protein for crystallography.
Figure 7.6: FSEC based over-expression and detergent screening of *E. coli* NarU.

The FSEC profiles on left hand panel represent the fusion protein extracted from the cells grown on LB medium and on right side from ZYP auto-induction medium. Detergent screened, is mentioned. The fluorescence indicates the elution profile of fusion protein. The UV absorbance ($A_{280}$) represents total proteins from crude cell lysate.
Figure 7.7: Comparison of FSEC profiles of *E. coli* NarU fusion protein in different detergents.

Membranes were extracted from the BL21(DE3) cells grown on (a) LB and (b) ZYP auto-induction medium. Membrane proteins were solubilised overnight in 500 µl detergent containing buffer at 4 ºC with gentle mixing. After centrifugation, 200 µl of the supernatant (soluble protein sample) was run through a Superose 6 10 / 300 column using the running buffer containing 1 CMC of detergent tested.

7.3.4.2 Pre-crystallisation screening of *B. cereus* Nar

*B. cereus* Nar fusion protein extracted from cells grown on LB medium without induction showed sharp and symmetrical FSEC peak profiles in DDM, DM and LDAO detergent although the levels of expression were very low. OG and OTG seemed to be unsuitable for this specific protein extraction as most of the protein in these detergents was eluted in the free GFP region. Results with the ZYP auto-induction medium were similar to that of LB except for the DDM, which gave a sharp and symmetrical peak with high fluorescence in ZYP auto-induction medium.
Figure 7.8: FSEC based over-expression and detergent screening of *B. cereus* Nar.

For the legends see Figure 7.6.

With regard to the *B. cereus* Nar fusion protein, it was observed that larger the detergent in the micelle size, higher was the solubility and homogeneity of the protein (Figure 7.8). Therefore by
comparing the FSEC profiles (Figure 7.9), DDM was selected for scale-up purification of *B. cereus* Nar fusion protein.

**Figure 7.9: Comparison of FSEC profiles of GFP fused *B. cereus* Nar in different detergents.**

![Fluorescence peaks](image)

Bacterial host cells expressing *B. cereus* Nar fusion protein were grown in 10 ml (a) LB and (b) ZYP auto-induction medium. The extracted crude membrane proteins were solubilised in detergent containing buffer. 200 µl of soluble protein sample (supernatant) was run through a Superose 6 10/300 column in the running buffer containing 1 CMC of detergent tested.

7.3.4.3 Pre-crystallisation screening of *P. aeruginosa* NarK1

*P. aeruginosa* NarK1 extracted from the cells grown on either LB or ZYP auto-induction medium showed significantly sharp and symmetrical fluorescence peaks in DDM, DM, LDAO and OTG. In OG, the level of expression in both grow ups was very low, also the protein extracted from the cells grown on ZYP auto-induction medium, GFP fusion from most of the protein cleaved resulted in substantial GFP peak (Figure 7.10). The fluorescence peaks associated with the *P. aeruginosa* NarK1 extracted in OTG from cells of both types of grow ups (grown in LB or ZYP auto-induction medium) were larger than the peaks of same proteins soluble in other detergent solutions (Figure 7.11).
Therefore, from the initial screening, it was concluded that *B. cereus* Nar had high solubility and monodispersity in OTG.

Figure 7.10: FSEC based over-expression and detergent screening of *P. aeruginosa* Nar K1.
Figure 7.11: Comparison of FSEC profiles of GFP fused *P. aeruginosa* Nar K1 in different detergents.

The crude membranes extracted from the cells grown on (a) LB and (b) ZYP auto-induction medium were incubated with gentle mixing overnight at 4 °C in 500 μl detergent containing solubilisation buffer. 200 μl of the soluble protein was run through a Superose 6 10 / 300 column in column running buffer containing 1 CMC of detergent tested.

7.3.4.4 Pre-crystallisation screening of *P. aeruginosa* NarK2

Like the *B. cereus*, the peak profiles from the solubilised *P. aeruginosa* NarK2 extracted from the cells grown either on LB or ZYP auto-induction medium, were asymmetrical in small sized micelle forming detergents, LDAO, OG and OTG (Figure 7.12). The FSEC peak profiles of *P. aeruginosa* NarK2 soluble in OG and OTG indicated that not only the overall protein expression level was very low in these detergents but also the substantial peak due to proteolysed GFP from the fusion was present showing that only free GFP solubilised in such detergent solutions. The *P. aeruginosa* NarK2 protein extracted from the cells grown on ZYP auto-induction medium, in DDM asymmetrical and in DM significantly sharp, symmetrical FSEC peak profiles were recorded (Figure 7.13). Also neither free GFP nor the aggregated protein in void volume of the column was observed indicating the homogeneity of the fusion protein DM.
Figure 7.12: FSEC based over-expression and detergent screening of *P. aeruginosa* NarK2.

For the legends see Figure 7.6.
Figure 7.13: Comparison of FSEC profiles of GFP fused *P. aeruginosa* NarK2 in different detergents.

Crude membranes extracted from the cells grown on 10 ml (a) LB and (b) ZYP auto-induction medium were solubilised in 500 µl of detergent containing buffer. After centrifugation, 200 µl of the supernatant (soluble protein sample) was run through Superose 6 10 / 300 column in the running buffer containing 1 CMC of detergent tested.

7.3.4.5 Pre-crystallisation screening of *T. thermophilus* NarK2

*T. thermophilus* NarK2 that did not exhibit green fluorescence under UV light was also analysed by FSEC to verify the efficiency of this technique for the target proteins that belong to the same class of MFS protein group. For the protein extracted from the cells grown on LB medium in all detergents except OTG, low expression and asymmetrical peaks were observed, also in DDM and OG considerable free GFP was eluted. For the protein, extracted from the cells grown on ZYP auto-induction medium, significantly sharp and symmetrical peaks were found in all detergents except OG solubilised protein where substantial free GFP peak profile was observed (Figure 7.14, Figure 7.15).
Figure 7.14: FSEC based over-expression and detergent screening of *T. thermophilus* NarK2.

Legends are same as for Figure 7.6.
Figure 7.15: Comparison of GFP fused *T. thermophilus* NarK2 FSEC profiles in different detergent solutions.

Membrane proteins extracted from cells grown in (a) LB and (b) ZYP auto-induction medium were solubilised in detergent-containing solution. 200 µl of the soluble protein sample was run through a Superose 6 10 / 300 column in running buffer supplemented with 1 CMC of detergent tested.

7.3.5 Large scale expression and purification of nitrate transporter fusion proteins

The FSEC results identified the detergents for nitrate transporter proteins that could be used for scale-up purification of these fusion proteins. For large scale protein production, in case of more than one detergent screened by FSEC, detergent with smaller micelle size was used. Also protein was extracted from cells grown on ZYP auto-induction to avoid any confusion with the uninduced LB grown cells.

7.3.5.1 Large scale expression and purification of *P. aeruginosa* NarK2 soluble in DM

On the basis of *P. aeruginosa* NarK2 FSEC profiles, DM was identified as promising detergents for scale-up extraction and purification of protein (Figure 7.12, Figure 7.13). Therefore DM was selected for subsequent large scale membrane protein extraction and hence for crystallisation trials. Purification of fusion protein was carried out by immobilised metal affinity chromatography (IMAC) and gel filtration (Figure 7.16) and monitored by Coomassie Blue staining of SDS gel and western
No protein band was detected in samples from the flow through and fractions of Peak A and Peak B on the western blot indicating that these peak profiles were not of the fusion protein. The peak annotated as PaNarK2-GFP-His that had highest fluorescence, contained almost all the soluble fusion protein as confirmed by Coomassie Blue staining (Figure 7.17a) and also by western blot analysis (Figure 7.17b). This fusion protein was eluted with increasing concentration of imidazole in the column running buffer.

Figure 7.16: Purification of *P. aeruginosa* NarK2 in DM.

A chromatogram showing the expression analysis and purification of *P. aeruginosa* NarK2 in DM detergent under the conditions optimised for over-expression. BL21(DE3) cells over-expressing *P. aeruginosa* NarK2 were harvested by centrifugation. Cells were resuspended in PBS buffer and lysed by the cell disrupter at a pressure 30 Kpsi. The crude membranes pellet collected by ultra centrifugation was solubilised in 1.5 % DM containing solubilisation buffer. Before running through the His-trap column using the ÄKTA purifier, the soluble protein sample was filtered through Millipore filters to remove any precipitated protein in the sample. The filtered soluble protein sample was run from one column volume of 150 ml Superloop at the flow rate of 2 ml / min. Total protein was monitored by reading the absorbance at 280 nm (UV absorbance) and GFP fused *P. aeruginosa* NarK2 by fluorescence peak profiles. The His-tagged protein was eluted from the His-trap column by imidazole in running buffer. Collected fractions were run on SDS-PAGE gel, Coomassie Blue stained and protein was also detected by western blotting. FT, denotes flow through and Pa, *P. aeruginosa*.

Western blot analysis of elution fractions of the peak, Pa NarK2-GFP-His (Figure 7.16), identified three different sized protein bands of approximately 150, 60 and 40 kDa sizes. It was hypothesised that the ~ 150 kDa protein band resulted from aggregation or oligomerisation and ~ 40 kDa band from proteolysis of fusion protein. However the fusion protein extracted (~ 60 kDa) was in low abundance and also had numerous contaminant proteins (Figure 7.17a). Therefore this protein was not
an encouraging candidate for purification and crystallisation trials at least under the conditions examined here.

Figure 7.17: SDS-PAGE and western blot analysis of elution fractions from gel filtration of

\[ P. \text{aeruginosa} \ \text{NarK2}. \]

![SDS-PAGE and western blot analysis of elution fractions from gel filtration of P. aeruginosa NarK2.](image)

Elution fractions, as discussed in the chromatogram shown in Figure 7.16, were selected from different regions of fluorescence peak profiles and (a) run on a SDS gel and protein bands stained with Coomassie Blue. (b) Position of the His-tagged fusion protein bands on a SDS gel was also verified by western blot analysis and probed with anti-His antibody.

7.3.5.2 Large scale expression and purification of \( B. \text{cereus} \) Nar soluble in DDM

Comparison of FSEC profiles of the \( B. \text{cereus} \) Nar soluble in different detergents encouraged the selection of DDM for its large scale purification (Figure 7.8, Figure 7.9). Purification of \( B. \text{cereus} \) Nar fusion protein was carried out in two steps method, IMAC and gel filtration. Each step of purification was monitored by Coomassie blue staining coupled with western blotting.

High fluorescence peak was recorded by \( B. \text{cereus} \) Nar fusion protein in flow through indicating the presence of GFP. Three small peaks (Peak A, B, C) and one high fluorescence peak was observed. When the samples from the fractions of these small peaks (Peak A, B, C) and flow through were western blotted and probed against anti-His antibody no protein band was detected. The peak named as Bc Nar-GFP-His was the actual profile of the \( B. \text{cereus} \) Nar fusion protein (Figure 7.18). The western blot analysis detected a very faint expected fusion protein sized band in only one fraction (D6). A protein band of ~ 150 kDa represented some oligomerisation of fusion protein. Also a band of ~ 40 kDa was detected that might have resulted from proteolysis of fusion protein (Figure 7.19b). The Coomassie staining of eluted protein (Figure 7.19a) also showed that the target fusion protein was extremely low in quantity and would not be a good candidate for crystallography.
Figure 7.18: Purification of *B. cereus* Nar extracted in DDM.

This chromatogram shows the DDM soluble expression analysis of *B. cereus* Nar fusion protein. The host cells, BL21(DE3) over-expressing *B. cereus* were harvested by centrifugation. The cell pellet was suspended in PBS buffer and lysed by passing through the cell disrupter at a pressure of 30 Kpsi. Crude membranes collected by ultracentrifugation were solubilised in 1 % DDM solubilisation buffer. The soluble protein sample, after filtration was run through the His-trap column fitted to ÄKTA purifier. The protein profile of the absorbance at 280 nm showed the total protein content. The His-tagged fusion protein was eluted from the His-trap column by adding imidazole in column running buffer. FT, is for flow through and Bc stands for *B. cereus*.

Figure 7.19: SDS-PAGE and western blot analysis of *B. cereus* Nar fusion protein elution fractions

(a)  
(b)  

The fusion protein was eluted from the His-trapped column by imidazole containing running buffer. The elution fractions were selected from different regions of fluorescence peaks (Figure 7.18) (a) run on SDS gel and Coomassie Blue stained (b) western blotted.
7.3.5.3 Large scale expression and purification of *E. coli* NarU in OTG

For large scale protein production bacterial cultures were grown in ZYP auto-induction medium under the conditions optimised for over-expression in small scale trials. From the FSEC profiles of *E. coli* NarU fusion protein (Figure 7.6), OTG (a detergent of smallest micelle sizes from all tested detergents) was selected for purification of fusion protein. The membrane pellet was solubilised in solubilisation buffer with 40 mM OTG, and purified by two step purification method, IMAC followed by gel filtration (Figure 7.20). When the fractions from the peak, Ec NarU-GFP-His were run on SDS gel (Figure 7.21), it was obvious that the amount of fusion protein eluted was considerably low.

Figure 7.20: Purification of *E. coli* NarU extracted in OTG.

![Graph showing purification process](image)

Over-expressed *E. coli* NarU fusion protein in host cells BL21(DE3) was extracted in OTG detergent and run through the His-trap column. Protein profile of the absorbance at 280 nm (A_{280}) showed the total protein (in crude membrane) and fluorescence indicated the GFP. His-tagged fusion protein, trapped with the His-trap column was eluted with imidazole in running buffer.

Figure 7.21: SDS-PAGE *E. coli* NarU fusion protein elution fractions extracted in OTG.

![SDS-PAGE image](image)

Fractions (numbered A_1-A_{11}) as annotated in Figure 7.20 from the high fluorescence peak as well as flow through (FT) were run on SDS gel and stained with Coomassie Blue dye.
Large scale expression and purification of *E. coli* NarU in DDM

DDM was investigated as another candidate detergent for the purification of GFP fused *E. coli* NarU. For this detergent, manual protein extraction was carried out from the crude membranes. The crude membrane pellet was dissolved in solubilisation buffer supplemented with 2.5 % DDM. The supernatant (containing the soluble protein) was incubated with the nickel affinity beads followed by protein elution by washing the nickel beads with increasing concentration of imidazole in the buffer (Figure 7.22a).

Figure 7.22: Purification of *E. coli* NarU in DDM by manual method followed by TEV cleavage.

(a) Elution of *E. coli* NarU fusion protein by imidazole. Nickel affinity beads with trapped proteins were washed and eluted with buffer containing imidazole. FT, flow through; W0, wash with 0 imidazole; W1, wash with 30 mM imidazole; W2, wash with 50 mM imidazole; E1, elution with 100 mM imidazole and E2, elution with 500 mM imidazole. (b) TEV cleavage of purified *E. coli* NarU fusion protein. ‘+’ TEV protease added, ‘—’ TEV protease not added. Arrow indicates the proteolysed GFP protein band after TEV cleavage.

The eluted protein was concentrated by spin to remove imidazole and incubated with TEV protease. After TEV proteolysis, a band of approximately 27 kDa was recovered (Figure 7.22b). The cleaved *E. coli* NarU was further purified by gel filtration (Figure 7.23). The fractions from different peaks were run on SDS gel and Coomassie Blue stained. It was observed that none of the fraction contained purified *E. coli* NarU (Figure 7.23). The cleaved protein from the GFP fusion was recovered by IMAC and purified by gel filtration (Figure 7.23). The peak fractions were run on SDS gel (Figure 7.24) and verified by mass spectroscopy. Similar to SDS gel staining (Figure 7.24), mass spectroscopy also identified contaminant proteins along in *E. coli* NarU protein.
The negative output of the purification trials with all the three fusion proteins, *P. aeruginosa* NarK2, *B. cereus* Nar and *E. coli* NarU was really surprising as the conditions for over-expression and detergent solubility were optimised beforehand. Therefore it was decided to monitor each and every step involved from protein extraction to gel filtration by western blot analysis and SDS-PAGE to find where protein was actually lost. It was clear from the western blot analysis of the samples from membrane extraction and purification steps that the protein did not solubilise in the detergent in sufficient amount and almost all protein was collected in the pellet by centrifugation after solubilisation in detergent (Figure 7.25).
Figure 7.25: SDS-PAGE and western blotting for screening the detergent solubility and purification of *E. coli* NarU in OTG.

Samples for (a) SDS and (b) western blotting were taken at every step of membrane preparation. S1, supernatant from lysed cell; P1, pelleted lysed cells; S2, supernatant after second centrifugation of S1; Solub o/n, overnight solubilisation of membrane protein pellet in detergent containing solution; PII, pellet after centrifugation of overnight soluble sample, SII, protein soluble supernatant.

7.3.6 Western blot analysis of fusion proteins

The initial over-expression trials as well as FSEC profiles identified encouraging protein expression (on the basis of GFP fluorescence) in cells grown on LB medium without inducing the protein. The results of large scale grow ups were not parallel to these initial over-expression and FSEC optimisation. Therefore, the cells were grown under the same condition as used for FSEC, lysed and western blotted to confirm the presence of His-tagged fusion proteins. As it is obvious from the Figure 7.26, that in the cells grown on LB without induction, no protein band was observed. A satisfactory level of expression of all the five nitrate transporters was recorded when the cells were grown on ZYP auto-induction medium.
Figure 7.26: Western blotting of nitrate transporter fusion proteins.

BL21(DE3) cells producing the fusion proteins were grown on LB or ZYP auto-induction medium supplemented with kanamycin, under the same condition used for FSEC. Cells were harvested by centrifugation and lysed by sonication. A portion of the lysed cells was solubilised in SDS running buffer and proteins were separated by SDS-PAGE. The His-tagged fusion proteins were detected by western blot analysis.

7.4 Discussion

Membrane proteins that are the main pharmaceutical drug targets (Zambrowicz and Sands, 2003; Overington et al., 2006) need to be characterised in detail to understand their structures and functions. Due to the hydrophobic nature of the membrane proteins they are difficult to purify from the membrane in a stable form and are mostly purified in the form of complexes with detergents. GFP fusion of membrane proteins is a potential tool to optimise detergent extraction and purification, and has been employed successfully to a number of membrane proteins (Drew et al., 2001; Drew et al., 2005; Newstead et al., 2008; Hammon et al., 2009; Leviatan et al., 2010; Chaudhary et al., 2011). Using this method protein can be over-expressed and purified using less time and resources as multiple experiments can be repeated to optimise the procedure (Liu and Niasmith, 2009).

In the present study nitrate transporter proteins from bacteria were over-produced using the nutrient rich, ZYP auto-induction medium. Hsieh et al. (2010) studied the relationship between yield and expression conditions for different membrane proteins. They suggested that the cell strain, growth medium and time of induction as being the most significant whilst concentration of inducer is the least significant variable for high protein productions. Therefore the use of the nutrient rich medium can be explained to fulfil the needs of cells as extended time is required to achieve the over-expression. Another advantage of using ZYP auto-induction medium for over-expression is the uniform induction with highly saturated cell cultures (Studier, 2005). The toxicity of over-expression
of membrane protein as observed in this present study when cells were grown on LB and induced with IPTG has also been reported previously by many workers (Studier et al., 1990; George et al., 1994; Studier, 2005). Miroux and Walker (1996) demonstrated that over-expression of membrane protein may be toxic to the host cells, BL21(DE3).

Green fluorescence in cell pellets by visual inspection under UV light was used as initial selection criteria for the over-expressed protein as GFP fluorescence in whole cell is directly proportional to the amount of protein expressed (Drew et al., 2005). Except for T. thermophilus, from which two different nitrate proteins (NarK1 and NarK2) were screened, all other four exhibited good green fluorescence. Also the SDS-PAGE gel has a very dense protein band with a considerable amount of protein. These results encouraged us to pursue for detergent screening of proteins. As larger proteins can reduce the fluorescence of GFP and as the fluorescence of GFP fusion protein is actually the amount of protein per ml, large proteins expression is usually underestimated (Liu and Niasmith, 2009) therefore one of the nitrate transporter from T. thermophilus NarK2 was also studied.

As no protein in the cells grown on LB uninduced conditions was detected (Figure 7.26), the substantial green colour observed in LB grown uninduced cells and extracted crude membranes membrane proteins may arise due to the proteolysis of the fusion protein separating the transporter protein and GFP or translation of GFP alone, as at the start of the GFP sequence methionine amino acid was present (Kawate and Gouaux, 2006). The sharp and symmetrical peaks in FSEC profiles of membrane proteins from uninduced cells were of course from the fusion protein. When the peak profiles of such proteins were compared in different detergents it was obvious that the same protein eluted in according to the micelle size of detergent, following the principle of size-exclusion chromatography. For example, the data presented in Figure 7.27, compare the elution of E. coli NarU extracted from the cells grown uninduced on LB in three different micelle sized detergents, DDM, DM and OTG. It is very clear that the DDM-NarU complex eluted first, followed by DM-NarU and finally OTG-NarU. Such results indicated that the FSEC technique is very sensitive and can detect the protein (as less) that cannot be detected with immunoblotting. As described by Kawate and Gouaux, 2006, fluorometers can detect up to ~ 10 ng of the GFP.
Figure 7.27: The elution position of *E. coli* NarU fusion protein in different detergents.

*E. coli* NarU fusion protein was extracted from the host cells grown on LB medium without induction, solubilised in detergent containing buffer and run through Superose 6 10 / 300 column. Arrows indicate the elution position of fusion protein in respective detergents.

In this present study a variety of detergents was used for solubility trials of target proteins. As recommended by Newstead *et al.* (2008), DDM, DM, OG and LDAO could be the detergents for membrane protein solubility. It is also suggested that the smaller the micelle size of a detergent more is the exposed surface area of protein and well ordered with better resolution crystals may be obtained (Warne *et al.*, 2008; Sonoda *et al.*, 2010).

Although in this present study, over-expressed proteins were screened for detergent extraction in FSEC but in scale-up experiments none of the protein was produced in sufficient amounts for crystallisation trials. Kawate and Gouaux, (2006) demonstrated that GFP tag, purity and concentration of the target protein did not affect the monodispersity of the protein as there is no difference in FSEC results and SEC results after removing the GFP tag. One of the problems associated with FSEC is that its set-up allows analysis of only one sample at a time and therefore comparative analysis of different proteins samples is not possible.
Therefore the results of this present study suggested that this specific group of proteins is difficult to purify in high concentration and there is need to improve the solubilisation conditions. By changing the constructs or detergent, the degree of solubility may be improved. Chaudhary and colleagues (2011) while working on human membrane proteins, demonstrated that different proteins have different solubility in the same detergent. In another study by Warne et al. (2008), it was reported that certain mutations maximised the stability of the protein and this increase in its stability or better solubility in short chained detergents (OTG).

Lastly, although membrane proteins do not fluoresce when in the inclusion bodies but the possibility of their fluorescence cannot be omitted.

7.5 Conclusion

This study failed to produce proteins that could realistically be taken forward to achieve the objectives set for this particular research. Structural and biochemical studies on membrane proteins is and will be a challenge and the probability of negative outcomes for crystallisation trials is high and the research even risky in terms of time and funds spend. In future work, different combinations of expression systems, vectors, target proteins and detergents should be tried to determine the best combination of these over-expression and purification variables. Mutagenesis with just one residue changes may allow better solubility and stability of membrane proteins in detergents. In the present study the applicability of FSEC for GFP fusion proteins was tested as a pre-crystallisation tool. Although none of the target protein could be purified in term of sufficient quantity and quality, notwithstanding the principle of technique is still promising. This is a simple, efficient and flexible technique for protein structural studies that can be modified by including further different vectors, tags, creation of mutant proteins, host cells, position of GFP (N- or C-terminal) and/or membrane proteins but is outside the scope of this current study.

Acknowledgment

I wish to thank Dr. Gregor Hagelueken and Ms. Haxian Huang (University of St. Andrews) for help and collaboration with this section of work.
Chapter Eight

Substrate specificity and inhibition of nitrate and nitrite permeases

8.1 Introduction

8.1.1 Nitrate and nitrite transporters
Nitrate transport is carried out solely by two transporter proteins, NrtA and NrtB, encoded by the genes nrtA and nrtB respectively in Aspergillus nidulans (Unkles et al., 2001; Kinghorn et al., 2005; Wang et al., 2008a). It was also reported that NrtA and NrtB transporters were also capable of transporting nitrite (Wang et al., 2008a). Similar nitrate and nitrite transport systems were found in other organisms, for example, Escherichia coli in which two related transporter proteins NarK and NarU, not only catalyse nitrate transport but also nitrite transport. As well as these two E. coli nitrate / nitrite transporters, a third protein NirC carries out nitrite uptake only (Clegg et al., 2002; Jia et al., 2009). A further example of such a dual transport system for nitrate and nitrite is the NarK1 and NarK2 proteins in Pseudomonas aeruginosa (Sharma et al., 2006).

8.1.2 Inhibition of nitrate transport
Several studies have indicated that chlorate (ClO\textsuperscript{3-}) is a growth inhibitor of certain bacteria and fungi when grown on nitrate as the sole source of nitrogen. The inhibition of net nitrate transport by chlorate has also been studied in plants, for instance barley (Siddiqi et al., 1992), tomato (Kosola and Bloom, 1996) and in model fungus A. nidulans (Brownlee and Arst, 1983; Unkles et al., 1991; Unkles et al., 2001; Kinghorn et al., 2005). In addition, chlorite (ClO\textsuperscript{2-}) has been recognised as a strong inhibitor of nitrate uptake in barley (Siddiqi et al., 1992) and A. nidulans (Zhou et al., 2000). Moreover nitrite was also identified as an inhibitor of nitrate transport in barley (Siddiqi et al., 1992; Aslam et al., 1992) and in A. nidulans (Wang et al., 2008a). Finally, inhibition of growth of A. nidulans nrtA mutant strains by caesium has been observed (Brownlee and Arst, 1983; Unkles et al., 1991; Unkles et al., 2001).

Glossary

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Inhibitor</td>
<td>A molecule that suppresses or prevents the other molecule from engaging in a process.</td>
</tr>
<tr>
<td>Inhibition</td>
<td>The prevention or blockage of a physiological function due to inhibitor(s).</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>The degree of susceptibility of an organism to a toxic molecule or environment.</td>
</tr>
<tr>
<td>Resistance</td>
<td>The ability of an organism to withstand the harmful physical or environmental stimulus.</td>
</tr>
</tbody>
</table>
It was established half a century ago that an analogue of a molecule may act as that molecule’s transport inhibitor, for example, in *E. coli*, the proline permease is also responsible for the influx of proline analogues such as 3,4-dehydroproline and azetidine-2-carboxylic acid, that inhibit cellular growth by integration into cellular proteins leading to their malfunction (Tristram and Neale, 1968). Similarly chlorate is an analogue of nitrate, which has been reported to be transported by a nitrate transporter (Brownlee and Arst, 1983). It was assumed that chlorite produced due to reduction of chlorate by nitrate reductase activity (Cove, 1976; LaBrie et al., 1991), was a more potent inhibitor of growth and transport activity (Siddiqi et al., 1992; Zhou et al., 2000).

Figure 8.1: Structural comparison of potential nitrate anion analogues.

Chlorate (ClO$_3^-$) has trigonal pyramid structure and is an established inhibitor of nitrate transport. Sulphite (SO$_3^{2-}$) also has the same trigonal pyramid structure as chlorate. The molecular structure of carbonate (CO$_3^{2-}$), and bicarbonate (HCO$_3^-$), common compounds in environmental water bodies, are also similar to nitrate but have carbon atom in the centre instead of nitrogen. Formate (HCOO$^-$), the trigonal planar, has carbon atom in the centre and a hydrogen atom is present instead of oxygen compared to nitrate molecule. Malonate (CH$_2$(COO)$_2$$^{2-}$) appears as two nitrate joined together and oxalate ((COO)$_2$$^{2-}$) is different from the (CH$_2$(COO)$_2$$^{2-}$) being shorter in carbon chain.

Resistance to chlorate by mutants in the nitrate assimilation pathway has been shown for a number of organisms including *A. nidulans*. Of relevance to the present study, a large number of loss-of-function
mutants in the *A. nidulans* gene *nrtA* (eg. *nrtA1, nrtA747*) were isolated on the basis of chlorate resistance with proline or various other nitrogen sources. Such chlorate resistant mutants could be distinguished from the knock-out *nrtB* mutant (*nrtB110*) on the bases of chlorate toxicity since *nrtB110* is sensitive to chlorate (Unkles *et al.*, 2001; Kinghorn *et al.*, 2005). In an electrophysiology study of the *A. nidulans* NrtA transporter, expressed in a heterologous system, *Xenopus* oocytes, Zhou and colleagues (2000) reported that actually chlorite is the true inhibitor of nitrate transport and is present in chlorate solution as a chemical contaminant. Another difference that has been reported is that loss-of-function mutant in *nrtA* could be distinguished from the *nrtB* loss-of-function mutant (*nrtB110*) on the basis of the sensitivity of *nrtA* mutants to caesium when grown on nitrate as the sole source of nitrogen (Unkles *et al.*, 2001).

In this present study, further anions with similar structures to nitrate (Figure 8.1) were examined for inhibitory effects on growth of *A. nidulans* mutant strains, on limiting level of nitrate. These molecules include chlorate, chlorite, carbonate, bicarbonate, sulphite, formate, malonate and oxalate. The cation caesium was also evaluated for its inhibition potential.

Due to the complexity of the presence of more than one transport systems for nitrate or nitrite uptake (*NrtA, NrtB* and *NitA*), the effect of an inhibitor on individual permeases was investigated using various mutants (Chapter 2, Table 2.2) in these three transporters. Such mutant strains were analysed for their resistance / sensitivity or inhibition / non-inhibition patterns to nitrate analogues (Figure 8.1) as well as caesium. This examination was carried out on (i) proline as the sole source of nitrogen and (ii) on limiting level of nitrate respectively. If a molecule was identified as a growth inhibitor of nitrate, its effect on actual transport of nitrate or nitrite was determined by net uptake assays.

### 8.2 Objective

The objective of this research was to determine the substrate specificity of individual *NrtA* and *NrtB* permeases for nitrate and nitrite transport.

### 8.3 Results

#### 8.3.1 Nitrate specificity of *NrtA* and *NrtB* proteins

Nitrate specificity of transporters, *NrtA* and *NrtB* was examined for the effects of the presence of a range of molecules (Figure 8.1) which are structurally similar to nitrate using (i) mutant strains in which only one nitrate transporter (either *NrtA* or *NrtB*) is functional and (ii) the wild type strain with the two functional nitrate transporters.
The results demonstrated that high concentrations of carbonate, bicarbonate, formate, malonate (up to 200 mM) and oxalate (up to 50 mM, due to low solubility of oxalate in water) had little or no effect on the growth of either loss-of-function \textit{nrtA1} and \textit{nrtB110} mutants or the wild type strain on 1 mM nitrate (a concentration at which growth is marginally reduced) (Figure 8.2a). Also when sensitivity of either loss-of-function \textit{nrtA1} or \textit{nrtB110} mutants and the wild type strain was evaluated for these molecules with 1 mM proline as sole source of nitrogen, more or less a similar growth response was observed for both loss-of-function mutants and the wild type strain (Figure 8.2b). The results showed unambiguously that these anions were not inhibitors of nitrate transport by either NrtA or NrtB proteins. On the other hand, chlorate, chlorite, sulphite and caesium retarded the growth of the both mutants and the wild type strain to various degrees. Therefore chlorate, chlorite, sulphite and caesium were selected for further study.

\textbf{Figure 8.2: Effect of potential structural anion analogues of nitrate on growth of mutants in \textit{nrtA} or \textit{nrtB} and the wild type strain.}

\begin{center}
\includegraphics[width=0.5\textwidth]{figure8a.png}
\end{center}

\begin{center}
\includegraphics[width=0.5\textwidth]{figure8b.png}
\end{center}

The strains were grown for 2-3 days at 37 °C on minimal medium supplemented with (a) 1 mM nitrate or (b) 1 mM proline as the sole source of nitrogen. Aqueous solutions of sodium salts of carbonate, bicarbonate and formate; oxalic acid and malonic acid (pH 6.5) was added to the minimal medium to the make the final concentration as indicated.
8.3.1.1 Growth tests of mutants in *nrtA* and *nrtB* on nitrate in the presence of chlorate

*a. Growth inhibition of the wild type and mutant strains by chlorate*

Growth of the wild type and mutant strains *nrtA* and *nrtB110* was examined on 1 mM or 10 mM nitrate in the presence of chlorate concentrations ranging from 1 to 100 mM (or until complete growth inhibition was achieved). On 1 mM nitrate, complete growth arrest of the wild type was recorded at 40 mM and that of the mutant *nrtA* at 50 mM chlorate. However, for mutant *nrtB110*, similar level of inhibition (no growth) was observed with 5 mM chlorate (and above) on 1 mM nitrate (Table 8.1).

Lack of growth inhibition up to 100 mM chlorate was recorded for the wild type and the mutant strain *nrtA* on 10 mM nitrate. However the mutant *nrtB110* failed to grow in the presence of chlorate above 50 mM, provided with 10 mM nitrate as the sole source of nitrogen (Figure 8.3).

**Table 8.1: Growth responses of strains on 1 mM nitrate to the presence of chlorate.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>0</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>nrtA</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>nrtB110</em></td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Strains were grown on minimal medium containing 1 mM nitrate as sole source of nitrogen at 37 ºC. Aqueous solution of KClO₃ (pH 6.5) was added to the medium to the final concentration as indicated. Growth responses are scored as; +, cells growing normal similar to the non-treated strain, +/- minimal (intermediate or poor) growth and - no growth. Each treatment was carried out in replicates of five.

**Figure 8.3: Growth inhibition of the wild type and mutant strains by chlorate.**

![Growth inhibition of wild type and mutant strains by chlorate](image)

The strains were grown on minimal medium containing 10 mM nitrate as the sole source of nitrogen. Chlorate in the form of aqueous solution of KClO₃ (pH 6.5) was added to the medium to make the final concentration as indicated. Each treatment was carried out in replicates of five.

*b. Resistance or sensitivity of the wild type and mutants to chlorate*

To assess resistance or sensitivity to chlorate, the wild type and mutant strains were grown on 1 mM proline as the sole nitrogen source with chlorate. Growth test results demonstrated that the *nrtA*
mutant was resistant up to 100 mM chlorate. Whereas 5 mM chlorate reduced the growth of the wild type strain and mutant *nrtB110* and no growth was observed at 10 mM chlorate or above (Table 8.2).

Table 8.2: Growth responses on 1 mM proline to the presence of chlorate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>KClO₃ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0  2.5  5  10  20  30  40  50  100  200</td>
</tr>
<tr>
<td>Wild type</td>
<td>+ + +/− − − − − − − −</td>
</tr>
<tr>
<td><em>nrtA1</em></td>
<td>+ + + + + + + +/− − − − − − −</td>
</tr>
<tr>
<td><em>nrtB110</em></td>
<td>+ + +/− − − − − − − −</td>
</tr>
</tbody>
</table>

Legends same as for Table 8.1, except that the strains were grown on 1 mM proline instead of 1 mM nitrate as the sole source of nitrogen. Growth is scored as described in Table 8.1.

Similar to 1 mM proline growth test results, the *nrtA1* mutant grown on 10 mM proline was resistant up to 100 mM chlorate. In contrast to the *nrtA1* mutant, growth of the wild type strain and mutant *nrtB110* on 10 mM proline was sensitive to 2.5 mM chlorate (Figure 8.4).

Figure 8.4: Resistance or sensitivity of the wild type and mutant strains to chlorate.

Transport assays - Inhibition of net nitrate transport by chlorate

In contrast to the 10 mM nitrate growth test results, where chlorate did not inhibit the growth of the wild type or mutant *nrtA1*, the effect of chlorate on the rate of net nitrate transport by the wild type strain and mutant *nrtB110* was similar (Figure 8.5). The Kᵢ values determined for wild type and mutant *nrtB110* were found to be 27.03 ± 3.90 and 24.90 ± 0.20 mM respectively. Lower concentrations of chlorate did not reduce the net nitrate transport by the mutant *nrtA1* which retained 82.29 ± 3.4 % transport activity in the presence of 30 mM chlorate. However including 50 mM chlorate in the assay medium reduced net nitrate transport by the mutant *nrtA1* to 28.79 ± 6.3 %. This value is somewhat similar to the wild type (25.90 ± 0.69 %) and mutant *nrtB110* (26.85 ± 3.3 %) at the same concentration of chlorate.
concentration of chlorate. The nrtA1 mutant (possessing a NrtB functional protein) gave a 41.68 ± 3.76 mM \( K_i \) value for chlorate (Table 8.3).

Figure 8.5: Effect of chlorate on net nitrate transport by the wild type and mutant strains.

![Graph showing effect of chlorate on net nitrate transport](image)

The wild type (——), mutant nrtA1 (——) and mutant nrtB110 (——) were grown on minimal medium supplemented with 5 mM urea for 6.5 h and induced by 10 mM nitrate for 100 min prior to harvesting the cells. Net nitrate transport was determined by the depletion of nanomoles of nitrate (from the 500 µM NaNO\(_3\) in the assay medium) per mg mycelium dry weight per hour (nmol/mg DW/h) and presented as the mean ± SE (standard error) of at least three independent experiments. All strains exhibited binomial kinetics. \( R^2 \) for regression was 0.978 for the wild type strain, 0.963 for the mutant nrtA1 and 0.908 for mutant nrtB110.

Table 8.3: Kinetic constants for nitrate transport and inhibition by the A. nidulans wild type strain, mutant nrtA1 (possessing NrtB functional) and nrtB110 (possessing NrtA functional) transport systems by chlorate, chloride, sulphite and caesium.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Functional nitrate transporter(s)</th>
<th>( K_m ) (µM)</th>
<th>( K_i ) (mM)</th>
<th>Chlorate</th>
<th>Chlorite</th>
<th>Sulphite</th>
<th>Caesium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>NrtA, NrtB</td>
<td>~110</td>
<td>27.03 ± 3.90</td>
<td>0.32 ± 0.03</td>
<td>9.2 ± 0.72</td>
<td>45.59 ± 3.32</td>
<td></td>
</tr>
<tr>
<td>nrtA1</td>
<td>NrtB</td>
<td>~10</td>
<td>41.68 ± 3.76</td>
<td>0.29 ± 0.03</td>
<td>7.49 ± 0.13</td>
<td>1.95 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>nrtB110</td>
<td>NrtA</td>
<td>~100</td>
<td>24.90 ± 0.20</td>
<td>0.28 ± 0.03</td>
<td>8.48 ± 0.11</td>
<td>56.97 ± 1.3</td>
<td></td>
</tr>
</tbody>
</table>

Strains were grown and induced as described in Figure 8.5. For each experiment, aliquots of young mycelial cells from the same grow up were taken for inhibition analyses by different concentrations of an inhibitor to minimise the differences in growth conditions or stage. The \( K_i \) (inhibition constant) value was determined by plotting nitrate flux values against the log of the inhibitor concentrations and then determining the concentration that reduced net nitrate transport rate to 50 % (Appendices I, II, III). The values of \( K_i \) given are the mean ± SE of at least three independent experiments. The \( K_m \) values (affinity constant for nitrate) of the wild type and the mutant strains were obtained from Unkles et al. (2001) and compared with their respective \( K_i \) for each inhibitor to identify affinity of an inhibitor for individual transporters. Please note that the nrtA mutant strain that Unkles and colleagues studied was nrtA747 but both mutants are deletions and therefore equivalent.
8.3.1.2 Growth tests of mutants in *nrtA* or *nrtB* on nitrate in the presence of chlorite

*a. Growth inhibition of the wild type and mutant strains by chlorite*

Similar growth inhibition response was observed for the wild type strain and mutants *nrtA1* and *nrtB110* to chlorite on 1 mM nitrate. Both mutants as well as the wild type strain did not grow in the presence of 0.1 mM chlorite (Table 8.4). At 10 mM concentration, nitrate slightly overcome the growth inhibitory effect by chlorite, however all strains (wild type and both mutants) failed to grow on 0.5 mM chlorite even after providing prolonged incubation period (Figure 8.6).

<table>
<thead>
<tr>
<th>Strain</th>
<th>NaClO2 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Wild type</td>
<td>+</td>
</tr>
<tr>
<td><em>nrtA1</em></td>
<td>+</td>
</tr>
<tr>
<td><em>nrtB110</em></td>
<td>+</td>
</tr>
</tbody>
</table>

The strains were grown on 1 mM nitrate as the sole source of nitrogen. Aqueous solution of NaClO2 (pH 6.5) was added to the medium to the final concentration of chlorite as indicated. The growth responses are scored as described in Table 8.1. Each experiment was carried out in replicates of five.

*b. Resistance or sensitivity of the wild type and mutants to chlorite*

Growth tests on 1 mM proline as the sole source of nitrogen revealed that the wild type as well as both mutant strains *nrtA1* or *nrtB110* were sensitive to 0.3 mM chlorite and above (Table 8.5). When these strains (wild type and both mutants) were grown on 10 mM proline as the sole source of nitrogen, increase in resistance to chlorite was observed and complete cessation of growth for the mutants and the wild type strain was achieved at 5 mM chlorite concentration (Figure 8.7).
Table 8.5: Growth responses on 1 mM proline to the presence of chlorite.

<table>
<thead>
<tr>
<th>Strain</th>
<th>NaClO₂ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Wild type</td>
<td>+</td>
</tr>
<tr>
<td>nrtA1</td>
<td>+</td>
</tr>
<tr>
<td>nrtB110</td>
<td>+</td>
</tr>
</tbody>
</table>

1 mM proline was used as the sole source of nitrogen to study the resistance or sensitivity of strains to chlorite. Growth responses are scored as described in Table 8.1. For each treatment, strains were grown in replicate of five.

Figure 8.7: Resistance or sensitivity of the wild type and mutant strains to chlorite.

Strains were grown on minimal medium supplemented with 10 mM proline as the sole source of nitrogen in the presence of 0 to 5 mM NaClO₂. Aqueous solutions of NaClO₂ (pH 6.5) was prepared fresh before use. The phenotype of the strains was recorded after 2-3 days growth at 37 °C.

c. Transport assays - Inhibition of net nitrate transport by chlorite

Net nitrate transport by the mutants nrtA1 and nrtB110 as well as the wild type strain was determined in the presence of increasing concentrations of chlorite. The inhibition pattern for both of the mutants was similar to the wild type strain and appeared concentration dependent (Figure 8.8). In contrast to chlorate inhibition profiles, extremely low concentrations of chlorite inhibited nitrate transport to a substantial degree and 1 mM chlorite was sufficient to prevent transport of nitrate completely. Kinetic analysis of inhibition by chlorite (Table 8.3) provided almost similar Kᵢ values (~ 300 µM) for the wild type and both mutant strains.
Figure 8.8: Effect of chlorite on net nitrate transport by the wild type and mutant strains.

A comparison of % net nitrate transport by the wild type strain (---), mutant nrtA1 (---) and mutant nrtB110 (---) in the presence of chlorite. Strains were grown on minimal medium supplemented with 5 mM urea and induced with 10 mM nitrate. Net nitrate transport was determined in young mycelium by the rate of depletion of nitrate (nmol/mg DW/h) providing 500 µM NaNO₃ in the assay medium. Error bars represent the standard error of net nitrate transport of at least three independent experiments. Binomial kinetics was applied to the results. Regression R² for the wild type strain = 0.955, mutant nrtB110 R² = 0.981 and for the mutant nrtA1 R² = 0.995.

8.3.1.3 Growth tests of mutants in nrtA and nrtB in the presence of sulphite

a. Growth inhibition of the wild type and mutant strains by sulphite

Equivalent levels of growth inhibition of the wild type and mutant nrtA1 by sulphite was observed on 1 mM nitrate however mutant nrtB110 exhibited reduced growth. The concentration of sulphite, as high as 35 mM was required to inhibit the growth on 1 mM nitrate for all the three strains (Table 8.6). At 10 mM nitrate, 20 mM sulphite inhibited the growth of the wild type as well as both mutant strains (Figure 8.9).

Table 8.6: Growth responses on 1 mM nitrate to the presence of sulphite.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Na₂SO₃ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Wild type</td>
<td>+</td>
</tr>
<tr>
<td>nrtA1</td>
<td>+</td>
</tr>
<tr>
<td>nrtB110</td>
<td>+</td>
</tr>
</tbody>
</table>

The strains were grown on 1 mM nitrate as the sole source of nitrogen in the presence of Na₂SO₃. The growth responses to sulphite were scored as described in Table 8.1. For each treatment, five replicates of each strain were grown.
Figure 8.9: Growth inhibition of the wild type and mutant strains by sulphite.

The strains were grown for 2-3 days at 37 °C on different concentrations of sulphite (aqueous solution of Na₂SO₃, pH 6.5) providing 10 mM nitrate as the sole source of nitrogen.

b. Resistance or sensitivity of the wild type and mutants to sulphite

The wild type strain and mutant \( nrtA1 \) were found to be equally resistant to sulphite up to 25 mM concentration however further increase in sulphite concentration to 35 mM ceased the growth of the wild type and \( nrtA1 \) mutant on 1 mM proline. To some extent, the mutant strain \( nrtB110 \), was sensitive to 15 mM sulphite on 1 mM proline however 35 mM sulphite or above was required to stop growing the strains (Table 8.7). At 10 mM proline, the concentration of sulphite required to completely prevent the growth of the wild type and mutant \( nrtA1 \) was 20 mM while the mutant \( nrtB110 \) was sensitive to 10 mM sulphite (Figure 8.10).

Table 8.7: Growth responses on 1 mM proline to the presence of sulphite.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Na₂SO₃ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Wild type</td>
<td>+</td>
</tr>
<tr>
<td>( nrtA1 )</td>
<td>+</td>
</tr>
<tr>
<td>( nrtB110 )</td>
<td>+</td>
</tr>
</tbody>
</table>

Legends same as for Table 8.6 except that 1 mM proline was provided as the sole source of nitrogen.

Figure 8.10: Resistance or sensitivity of the wild type strain and the mutants to sulphite.

The strains were grown at 37 °C for 2-3 days on 10 mM proline in the presence of sulphite as indicated. The treatments were carried out on five replicates of each strain.
c. Transport assays - Inhibition of net nitrate transport by sulphite

The results of net nitrate transport indicated that sulphite inhibited the net nitrate transport by NrtB protein (expressed in mutant nrtA1) more adversely than the wild type and mutant strain nrtB110. 100% inhibition of net nitrate transport by the mutant nrtA1 was observed at 10 mM sulphite (Figure 8.11b). Sulphite inhibition of net nitrate transport by the wild type strain and mutant nrtB110 was similar and appeared to be concentration dependent (Figure 8.11a) obviously due to a functional NrtA transporter. Kinetic analysis of net nitrate transport inhibition by sulphite yielded $K_i$ values of $7.49 \pm 0.13$ and $8.48 \pm 0.11$ mM for the mutants nrtA1 and nrtB110 respectively. These were very close to the wild type $K_i$ value, $9.2 \pm 0.72$ mM (Table 8.3).

Figure 8.11: Effect of sulphite on net nitrate transport by the wild type and mutant strains.

Effect of different concentrations of sulphite was determined on the net nitrate transport by (a) the wild type (—) and mutant nrtB110 (—), (b) mutant nrtA1. Net nitrate transport was measured on the basis of the exhaustion of nitrate (nmol/mg DW/h) providing 500 µM NaNO$_3$ in the assay medium. All strains exhibited binomial regression with $R^2$ for the wild type strain = 0.981, mutant nrtB110 = 0.922 and mutant nrtA1 = 0.954.
8.3.1.4 Growth tests of \textit{nrtA} and \textit{nrtB} mutants on nitrate in the presence of caesium

\textit{a. Growth inhibition of the wild type and mutant strains by caesium}

The growth of the mutant \textit{nrtA1} was completely inhibited by 15 mM caesium on 1 mM nitrate. In contrast, the wild type and mutant strain \textit{nrtB110} grew in the presence of up to 40 mM caesium on 1 mM nitrate.

\begin{table}[h]
\centering
\begin{tabular}{lcccccccc}
\hline
Strain & CsCl (mM) & 0 & 5 & 10 & 15 & 20 & 30 & 40 & 50 \\
\hline
Wild type & + & + & + & + & + & + & + & + & - \\
\textit{nrtB110} & + & + & + & + & + & + & + & - & - \\
\hline
\end{tabular}
\caption{Growth responses on 1 mM nitrate to the presence of caesium.}
\end{table}

Aqueous solution of CsCl (pH 6.5) was added to 1 mM nitrate containing medium to the final concentration of caesium as indicated. Growth responses were scored as described in Table 8.1. Each treatment was carried out in replicates of five.

On 10 mM nitrate, 10 mM caesium was sufficient to reduce the growth and 20 mM caesium completely inhibited the growth of the mutant \textit{nrtA1}. In contrast to the mutant \textit{nrtA1}, the wild type and mutant \textit{nrtB110} grew up to 50 mM caesium (the highest concentration tested) with 10 mM nitrate as the sole source of nitrogen (Figure 8.12).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure8_12.png}
\caption{Growth inhibition of the wild type and the mutant strains by caesium.}
\end{figure}

The strains were grown on minimal medium with 10 mM nitrate and CsCl as indicated. All treatments were assessed in five replicates.

\textit{b. Resistance or sensitivity of the wild type and mutants to caesium}

Almost similar growth responses of the wild type as well as mutants \textit{nrtA1} and \textit{nrtB110} were observed on 1 mM proline with caesium. A concentration of caesium, as high as 50 mM was required to inhibit the growth of all the three strains grown on 1 mM proline as the sole source of nitrogen.
Table 8.9: Growth responses on 1 mM proline to the presence of caesium.

<table>
<thead>
<tr>
<th>Strain</th>
<th>CsCl (mM)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>nrtA1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>nrtB110</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Legends same as for Table 8.9 except that 1 mM proline was provided as the sole source of nitrogen.

The wild type strain, mutant nrtA1 and mutant nrtB110 were considerably resistant to caesium provided with 10 mM proline as the sole nitrogen source (Figure 8.13) and no growth inhibition of the wild type and both mutant strains was observed up to 50 mM of caesium (Figure 8.13).

Figure 8.13: Resistance or sensitivity of the wild type and mutants to caesium.

The strains were grown at 37 °C for 2-3 days on 10 mM proline, in the presence of different concentrations of CsCl as indicated. Experiments were carried out with five replicates of each strain.

c. **Transport assays - Inhibition of net nitrate transport by caesium**

The effect of caesium on net nitrate transport by the mutant nrtB110 and the wild type strain is presented in Figure 8.14a. The mutant strain nrtB110 retained 98.46 ± 0.8 % transport activity at 20 mM and 59.73 ± 0.6 % at 50 mM caesium, comparing the nitrate transport rate by the wild type strain, 80.21 ± 4.2 % at 20 mM and 45.65 ± 1.8 % at 50 mM caesium. The Kᵢ values for mutant nrtB110 and the wild type strains were 56.97 ± 1.3 and 45.59 ± 3.32 mM respectively (Table 8.3). In contrast to the wild type and mutant nrtB110, net nitrate transport by the mutant nrtA1, reduced to 54.29 ± 5.4 % with just 2 mM and 6.88 ± 2.1 % by 5 mM caesium (Figure 8.14b). Kᵢ of the mutant nrtA1 for caesium was 1.95 ± 0.07 mM.
Figure 8.14: Effect of caesium on net nitrate transport by the wild type and mutant strains.

Net nitrate transport was determined by depletion of nitrate (500 µM) from the assay medium in the presence of CsCl. All strains displayed binomial regression (a) the wild type (—) $R^2 = 0.985$, mutant nrtB110 (—) $R^2 = 0.809$ and (b) mutant nrtA1 $R^2 = 0.987$.

8.3.2 Nitrite transport by NrtA and NrtB proteins
Glass and colleagues (Wang et al., 2008a) described nitrite transport in single mutants, nrtA747 or nrtB110 retaining functional NrtB or NrtA transporters respectively. As both of these mutants (nrtA747 and nrtB110) possessed a functional NitA (exclusively nitrite transporter), the role of individual NrtA and NrtB proteins in nitrite transport could not be rigorously assessed. Therefore, nrtA mutant strain, T12048 (possessing a functional NrtB protein) and nrtB mutant strain, T12200 (possessing a functional NrtA protein), were generated in a NitA mutant deleted genetic background to circumvent NitA transport activity and interference. Such mutants allowed the study of the role and relative contribution to nitrite transport by NrtA or NrtB proteins individually. Furthermore, to compare the effect of inhibitors on individual proteins, growth of mutant strains T12048 and T12200 harbouring a nitA mutant deletion was compared with the wild type strain (containing NrtA, NrtB and
NitA functional proteins), mutant \textit{nrtA1} (possessing NrtB and NitA functional proteins) and mutant T110 (possessing only functional NitA).

8.3.2.1 Substrate affinity

Net nitrite transport by the strains T12048 (studying NrtB) and T12200 (following NrtA) was determined by initially providing the 100 µM nitrite in the influx medium as previously described for the wild type strain and \textit{nrtA}, \textit{nrtB} mutants (Unkles \textit{et al.}, 2001), and double mutant T110 (\textit{nrtA747} and \textit{nrtB110}) (Wang \textit{et al.}, 2008a). Rate of net nitrite transport by the strain T12200 (possessing functional NrtA) was 40.71 ± 17.39 nmol/mg DW/h. Due to very low nitrite transport rate combined with the sensitivity of the assay method, it was practically difficult to find out the substrate affinity (K\textsubscript{m}) using a range of nitrite concentrations and any reduction in transport by inhibitors for the strain T12200. Preliminary experiments were carried out to determine the rate of nitrite transport by the strain T12200 at different concentrations of nitrite as well as the effect of inhibitors (e.g. chlorate) but no conclusive results were obtained.

Net nitrite transport by NrtB (expressed in strain T12048) is presented in Figure 8.15. Measured rate of nitrite transport by this strain was found to be 192.77 ± 9.83 nmol/mg DW/h. Maximum transport rate, V\textsubscript{max} and affinity constant, K\textsubscript{m} (substrate concentration that gives half of the V\textsubscript{max}) was determined by extrapolating the rate of nitrite transport (v) to infinite nitrite concentration using Eadie-Hoffstee analysis (v plotted against v/s) and Lineweaver-Burk double reciprocal analysis (1/v plotted against 1/s). The values obtained by both analyses are given in Table 8.10.
Figure 8.15: Nitrite affinity by the NrtB protein.

(a) Concentration dependence of strain T12048 (NrtB) for net nitrite transport per mg dry weight of mycelium per hour. Shown within the graph, are the mean values of net nitrite transport ± SE of three independent experiments. Data was analysed by (b) Eadie-Hoffstee analysis ($R^2 = 0.962$) and (c) Lineweaver-Burk double reciprocal analysis ($R^2 = 0.997$). The strain was grown on minimal medium supplemented with 5 mM urea for 5 h followed by 100 min induction with 10 mM NaNO$_3$. Nitrite transport assays were carried out for 20 min in the presence of 10 to 250 µM NaNO$_2$. A summary of the $K_m$ and $V_{max}$ values is given in Table 8.10.

Table 8.10: Kinetic constants for the net nitrite transport by the NrtB protein.

<table>
<thead>
<tr>
<th>Functional nitrite transporter</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/mg DW/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eadie-Hoffstee analysis</td>
<td>Lineweaver-Burk analysis</td>
</tr>
<tr>
<td>NrtB</td>
<td>44.43 ± 0.29</td>
<td>46.67 ± 3.91</td>
</tr>
</tbody>
</table>

The above kinetic constants for the strain T12048 were computed as described for Figure 8.14 from three independent experiments.
8.3.2.2 Effect of chlorate on nitrite uptake by NrtA and NrtB proteins

   a. Growth responses of the wild type and mutant strains to chlorate on nitrite

   The effect of chlorate on the mutant strains T12200 (NrtA) and T12048 (NrtB) was investigated by evaluating and comparing their phenotypic response to chlorate with 2 mM nitrite as the sole nitrogen source, a concentration that begins to limit wild type growth (Figure 8.16). The wild type, mutant nrtA1 and the double mutant strain T110 (nrtA747 and nrtB110) were included as controls. Chlorate strongly inhibited the growth of mutants having either NrtA or NrtB as the only functional nitrite transporter. It was also obvious from the results that NitA (exclusively a nitrite transporter) was not involved in the uptake of chlorate. Interestingly mutant strain nrtA1 showed resistance up to 30 mM chlorate but the wild type strain, mutants T12048 and T12200 were sensitive to less than 10 mM chlorate.

   Figure 8.16: Effect of chlorate on the growth of the wild type and mutant strains on nitrite.

   ![Figure 8.16: Effect of chlorate on the growth of the wild type and mutant strains on nitrite.](image)

   The strains were grown on minimal medium supplemented with 2 mM NaNO₂ as the sole nitrogen source (2 mM nitrite is just sufficient to sustain reasonable growth). Freshly prepared aqueous solution of KClO₃ (pH 6.5) was added to make the final concentration as indicated. Experiments included five replicates for each treatment.

   b. Transport assays – Inhibition of NrtB net nitrite transport by chlorate

   The results showed that net nitrite transport by the strain T12048 (NrtB protein) was reduced to 47.61 ± 3.56 % by 1 mM chlorate and 25.15 ± 5.49 % by 2 mM chlorate (Figure 8.17). The Kᵢ value, 0.83 ± 0.06 mM, for NrtB transport inhibition of nitrite by chlorate was very close to the Kᵣ (~ 0.45 mM) (Table 8.10) of this transporter for nitrite.
Figure 8.17: Effect of chlorate on net nitrite transport by the NrtB transporter.

The strain were grown on minimal medium (as for nitrate transport assays), induced with 10 mM NaNO₃ for 100 min. Net nitrite transport was determined measuring the depletion of nitrite from the assay medium. The values presented by this graph are mean of three independent experiments, (R² = 0.97).

8.3.2.3 Interaction of chlorite or sulphite with nitrite

Perhaps surprisingly, chlorite that was reported as a potent growth and transport inhibitor of nitrate (section 8.3.1.2) by the wild type and mutant strains (nrtA1 and nrtB110), did not affect the growth of mutants as well as the wild type strain on 2 mM nitrite, up to 0.5 mM chlorite (Figure 8.18a). Similar results were obtained when the strains were grown on sulphite containing minimal medium with 2 mM nitrite and no toxic effect of sulphite was detected up to 30 mM concentration levels (Figure 8.18b).

Figure 8.18: Effect of chlorite and sulphite on the growth of the wild type and mutant strains on nitrite.
Strains were grown on minimal medium with 2 mM nitrite as the sole source of nitrogen and (a) 0 to 5 mM NaClO₂, (b) 0 to 30 mM Na₂SO₃.

Unfortunately net nitrite transport by the strain T12048 could not be determined in the presence of chlorite and sulphite due to chemical interaction between highly reactive nitrite and chlorite / sulphite. When the indicator solution was added to the nitrite assay filtrate (Chapter 2, Section 2.5.3), a pink color did not develop, indicating the absence of nitrite in the solution. Instead the presence of considerable amount of nitrate was confirmed in the same assay filtrate. These results suggested the oxidation of nitrite to nitrate by chlorite or sulphite.

8.3.2.4 Effect of caesium on nitrite uptake by NrtA and NrtB proteins

a. Growth responses of the wild type and mutant strains to caesium on nitrite

Growth test results on 2 mM nitrite showed that the wild type and mutant strains T110, nrtA1, T12048 and T12200 were resistant to caesium and approximately 50 mM concentration of caesium was required to stunt the growth completely (Figure 8.19).

Figure 8.19: Growth response of the wild type and mutants to caesium.
b. Transport assays - Inhibition of NrtB net nitrite transport by caesium

Presence of caesium in the transport assay medium could not interfere much with the net nitrite transport by mutant T12048 (with the functional NrtB protein). The concentration of caesium, as high as 50 mM could only reduce the transport rate by 57.57 ± 3.3 % (Figure 8.20). The $K_i$ value calculated for caesium inhibition of nitrite transport by mutant T12048 was 61.49 ± 6.95 mM indicating very low affinity of caesium to NrtB that has $K_m$ (affinity for nitrite) ~ 45 µM.

Figure 8.20: Effect of caesium on net nitrite transport by the strain T12048.

Net nitrite transport was determined in the presence of 0 - 50 mM CsCl by the depletion of nitrite with 100 µM NaNO₂ in the assay medium. Data represents the mean value of three independent experiments ($R^2=0.97$).

8.3.2.5 Competition between nitrate and nitrite for NrtA and NrtB transporters

a. Growth tests

The wild type and mutant strains, T110, nrtA1, T12048 and T12200 were grown on 1 mM nitrate in the presence of 0 to 100 mM nitrite to study the interaction of both of these anions. Growth of the wild type, mutant T110 and mutant nrtA1 was not inhibited by nitrite up to 50 mM concentration however the growth of the strains T12048 and T12200 was sensitive to 10 mM nitrite.

At 10 mM nitrate (Figure 8.21) the growth responses of all strains were similar as recorded for 1 mM nitrate. Nitrite growth inhibition of the strains T12048 and T12200 was of course unexplainable except if they were different from the wild type and two other mutants, T110 and nrtA1, in their auxotrophic marker and had p-aminobenzoic acid (paba) vitamin requirement for their growth. Auxotrophic markers present in these strains were determined and results are presented in Appendix V. The results showed that the wild type and mutant strains, T110, nrtA1 required vitamin biotin whereas the strains T12048 and T12200 required vitamin paba.
Figure 8.21: Effect of nitrate / nitrite interaction on the growth of wild type and mutant strains.

![Image of wild type and mutant strains growth](image)

Strains were grown on minimal medium containing 10 mM nitrate and 0 to 100 mM NaNO₂.

c. *Inhibition of NrtB nitrite transport by nitrate*

Nitrate transport by the NrtB (Figure 8.22) was reduced to 25.9 ± 0.08 % in the presence of just 1 mM nitrate. Kᵢ of NrtB for nitrite transport inhibition by nitrate was 0.51 mM compared to the Kᵢ ~ 0.45 mM (Table 8.10).

Figure 8.22: Effect of nitrate on net nitrite transport by the strain T12048.

![Image of nitrite transport by strain T12048](image)

Young mycelium of strains grown on minimal medium (as for nitrate transport assay) and induced with 10 mM nitrate were used to determine the effect of nitrate on nitrite transport by the depletion of nitrite in transport assay medium.

### 8.4 Discussion

The generation of mutants in nitrate / nitrite transporter NrtA, NrtB and NitA encoding genes has made it possible to study in finer detail, nitrate / nitrite transport and inhibition. First regarding the important question of methodology, nitrate (and nitrite) transport can either be determined indirectly
by its depletion from the assay medium (Brownlee and Arst, 1983, Unkles et al., 2001) or directly by using tracer $^{13}$NO$_3^-$ (Wang et al., 2007; Wang et al., 2008a). The latter experimentation is somewhat limited since it can only be carried out at UBC, Vancouver, Canada, is very expensive and only a few assays can be performed per day. Therefore the net nitrate (and nitrite) transport assay method was used in this present study. The results from this present study demonstrated that NrtA is mainly a nitrate transporter because the rate of net nitrate transport by the NrtA transporter protein (expressed in the nrtB110 mutant) was more than a two fold higher than the rate of nitrate transport by the NrtB transporter (expressed in the mutant nrtA1). It was also observed that the rate of nitrite transport by the NrtA protein (expressed in the strain T12200) was significantly lower than the rate of nitrate transport by the NrtB protein (expressed in the strain T12048). Interestingly the NrtB protein (expressed in strain T12048) transports nitrite at a higher rate than the NitA protein, an exclusively nitrite transporter. In contrast, the affinity of NrtB for nitrite transport is 10 times less than that of the NitA protein affinity for nitrite transport. Therefore it is suggested that NrtB is mainly a nitrite transporter although its affinity for nitrate is more than that for nitrite transport.

This present study revealed that the structurally similar anions carbonate, bicarbonate, formate, malonate and oxalate failed to inhibit the growth of wild type as well as the mutant strains nrtA1 and nrtB110 with nitrate as the sole source of nitrogen. The possible reasons for this lack of growth inhibition and by extension lack of inhibition of nitrate transport of the wild type and mutant strains (hence both NrtA and NrtB transporters) by malonate or oxalate might be that these are larger sized molecules than nitrate. In Chapter Five of this thesis, the results of NrtA thiol cross-linking at the positions R87 (Tm 2) and R368 (Tm 8) revealed that these two essential arginine residues that are involved in nitrate binding / translocation, are very close (~ 0.4 Å) with each other. In the same study, it was also observed that these arginine residues moved approximately 2 Å apart in the presence of nitrate hence providing the space for the nitrate molecule which is approximately 2.6 Å in size. The molecule structures of both oxalate and malonate (Figure 8.1) are similar to two nitrate molecules joined together to make a single molecule with more or less double in size than the nitrate molecule alone. Therefore both oxalate and malonate could not permeate the cells via NrtA and / or NrtB proteins that are specialised for nitrate (and nitrite) transport due to their larger sizes. A similar explanation was suggested by Tristram and Neale (1968) for the non-inhibitory effect of the structural analogue of proline, pipecolic acid on the proline uptake by E. coli. They suggested that due to larger ring structure than proline, pipecolic acid failed to enter the cell and hence no inhibitory effect on proline transport was observed.
Formate, a highly toxic by-product of certain cellular metabolic processes needs to be excreted from most cells. It is also well established that nitrite and formate are transported by different transporters of same transporter family (the FNT protein family) (Suppmann and Sawers, 1994; Waight et al., 2010; Unkles et al., 2011). Considering the toxic nature of formate and resistance of the wild type and both nrtA1 and nrtB110 mutant strains towards formate, it is obvious that formate would not be transported by membrane proteins. Structural crystallographic analyses of the formate transporter, FocA, from E. coli (Wang et al., 2009; Falke et al., 2010) and Vibrio cholerae (Waight et al., 2010) revealed that FocA proteins consists of pentameric formate transport channel and highly selective for the substrate, formate and no evidence of nitrite transport by the FocA protein. Electrophysiological studies on heterologously expressed A. nidulans NrtA in Xenopus oocytes revealed that bicarbonate was not transported by NrtA protein (Zhou et al., 2000) (transport of bicarbonate via NrtB was not studied). Nar1.2, a nitrite transporter of Chlamydomonas reinhardtii, was reported to be capable of bicarbonate ion transport as well (Mariscal et al., 2006). In this regard, the wild type and mutant strains nrtA1 and nrtB110 used for this present study have functional NitA protein. Therefore it is possible that NitA might be capable of bicarbonate uptake. Therefore it is unclear if carbonate or bicarbonate was transported by NrtA, NrtB or NitA protein or not. However if carbonate or bicarbonate entered into the cells by another route these molecules are non-inhibitory to nitrate or nitrite uptake.

In a number of studies, carried out on bacteria, fungi and plants (Cove, 1976; Brownlee and Arst, 1983; Unkles et al., 1991; Siddiqi et al., 1992; Kosola and Bloom, 1996; Unkles et al., 2001; Clegg et al., 2002; Kinghorn et al., 2005; Kucera, 2006), chlorate was suggested as an inhibitor of growth and / or nitrate transport. In this present study sensitivity responses of the wild type and mutant nrtB110 (both possessing the functional NrtA protein) as well as the resistance of mutant nrtA1 (with functional NrtB protein) to chlorate toxicity, suggested that chlorate and nitrate share NrtA for uptake activity. The high resistance of nrtA1 mutant (with a functional NrtB protein) to chlorate can be explained by loss-of-function of nitrate reductase to reduce chlorate to chlorite which is more toxic (Hoff et al., 1994). Comparative kinetics of net nitrate transport inhibition by chlorate indicated that although the affinity of chlorate was higher for NrtA than the NrtB protein, the K_i values for chlorate for both transporters were much higher than the apparent K_m for nitrate transport (Table 8.3). Therefore it is concluded that chlorate is a weak analogue of nitrate transport. In a previous study on barley, Siddiqi et al., (1992) demonstrated chlorate to be a weak or non-competitive inhibitor of nitrate with a K_i of 16 mM. In another study of the tomato plants (Kosola and Bloom, 1996), it was observed that nitrate could inhibit the uptake of chlorate by plant roots although the presence of
chlorate did not affect nitrate transport. In conclusion, although chlorate has a very low affinity for \textit{A. nidulans} NrtA protein, it is transported by NrtA protein. Kinetic analysis of nitrite uptake by NrtB suggested significantly higher affinity of NrtB for chlorate.

The data obtained in this present study of nitrate transport inhibition by chlorate contradict the results of Brownlee and Arst, (1983). They reported 80 \% reduction of nitrate transport in the wild type strain and \textit{crn}A1 (\textit{nrt}A1) mutant by 1 mM chlorate in 6 or 17 h grown cells. In the current study, very little reduction in net nitrate transport was observed even with certainly higher concentrations of chlorate. A potential reason for such experimental variation could be differences in the purity of the commercially available KClO$_3$, used by the two research groups or the use of old chemicals or old stock solution of chlorate. pH of the chlorate solution is another important factor that greatly influences the level of inhibition by chlorate (Brownlee and Arst, 1983). For this present study, ultra pure KClO$_3$ was used and always prepared fresh prior to each experiment to avoid reduction to chlorite or contamination or any change in pH which was adjusted if required before transport assays were performed.

\textit{A. nidulans} NrtA defective mutants were originally isolated on the basis of chlorate resistance (Tomsett and Cove, 1979) and later characterised by Brownlee and Arst (1983). Electrophysiological studies carried out on \textit{A. nidulans} NrtA transporter expressed heterologously in \textit{Xenopus} oocytes, revealed that if chlorate solutions prepared freshly, no transport of chlorate occurred via the NrtA nitrate transporters as judged by differences in electrophysiological currents. In contrast when the \textit{Xenopus} oocytes were treated with a freshly prepared chlorite solution, a current was detected that was similar in strength to nitrate induced currents suggesting the transport of chlorite (but not chlorate) by the NrtA protein (Zhou \textit{et al.}, 2000). There are still questions arising regarding their finding of chlorite transport by NrtA because Zhou and colleagues did not provide details of the purity of chlorate and chlorite salts that they used for their study. Unkles \textit{et al.} (2001) while discussing their results of chlorate resistance or sensitivity of the NrtA protein to chlorate, suggested that the findings of Zhou and colleagues (2000) of chlorite transport (not chlorate) by NrtA are enigmatic due to the presence of 20 \% contaminants in commercially available NaClO$_2$.

In this study, Alfa Aesar 80 \% pure NaClO$_2$ was used, with 10 \% NaCl, 3\% NaClO$_3$, 3 \% NaOH and 2 \% Na$_2$CO$_3$ chemical contamination. Initially, to rule out the possibility that the observed “chlorite inhibition” was due to a chemical contaminant(s) present in the chlorite 80 \% pure product (particularly chlorate), a ‘contaminant cocktail’ solution in the ratio of 10:3:3:2 (NaCl, NaClO$_3$, NaOH and Na$_2$CO$_3$) was prepared, from individual 99 \% pure grade chemicals. The pH of this solution was
adjusted to 6.5 and aliquots incorporated into minimal medium (with 10 mM nitrate or proline as the sole nitrogen source) at equivalent final concentrations to those present in a 10 mM chlorite agar medium (i.e. 1.56 mM NaCl, 0.28 mM NaClO₃, 0.68 mM NaOH and 0.17 mM Na₂CO₃). The chlorite concentration chosen (10 mM) is vastly in excess of minimal chlorite inhibitory concentrations, 0.5 mM on 10 mM nitrate (Section 8.3.1.2). The results (data not shown) showed that there was no evidence of growth inhibition on plates by the ‘contamination cocktail’ and thereby demonstrating unequivocally that it was indeed chlorite in the 80 % chlorite product that was the sole inhibitory factor. Comparison of Kₘ values of net nitrate transport by the NrtA and NrtB proteins with their respective Kᵢ suggested that chlorite has high affinity for both NrtA and NrtB transporters.

From the growth inhibition test results it was concluded that sulphite is a poor analogue of nitrate transport by NrtA (expressed in nrtB110). The Kᵢ for sulphite by all three strains was higher than their Kₘ that suggested the low affinity of NrtA and NrtB transporters for sulphite.

Inhibition of A. nidulans nrtA mutant growth by caesium had been established during previous studies (Brownlee and Arst, 1983; Unkles et al., 2001). In this present study, growth of the mutant nrtA1 (possessing the functional NrtB) was inhibited significantly on nitrate and the wild type as well as nrtB110 (both possessed functional NrtA) resumed their normal growth. It was observed that caesium did not inhibit the growth of the nrtA1 mutant provided with proline as the sole source of nitrogen (Brownlee and Arst, 1983; Unkles et al., 1991).

8.5 Conclusion

The main findings of this present study demonstrated that

i. NrtA is mainly a nitrate transporter.

ii. NrtB transports nitrite at a higher rate than nitrate but has a higher affinity for nitrate than nitrite.

iii. Both NrtA and NrtB have low affinity for chlorate and NrtA takes up chlorate and NrtB does not.

iv. Caesium is an inhibitor of nitrate transport by NrtB but not for nitrite transport.

v. Sulphite is an inhibitor of nitrate transport by both transporters, NrtA and NrtB.

Acknowledgment

I wish to thank Professor Tony Glass (University of British Columbia, Canada) for help and collaboration with this section of work.
Concluding remarks and future work

The overall objective of the work carried out during the course of this thesis was to delve deeper into the crucial environmental and agriculturally important process of nitrate conversion to ammonium. A modest level of productivity and exploration has been achieved on several aspects of nitrate metabolism and which may be pursued to greater depth by future studies.

Novel genes \(cnxL, cnxK, niaB\) and \(niaC\) were discovered that may be involved in nitrate metabolism in some way, inferred from their high protein sequence homology with functional counterparts and conservation of known essential residues. In addition, the clustering of \(cnxL, cnxK\) and \(niaB\) genes in \(Aspergillus\) \textit{nidulans} and conservation of such a homologous gene cluster in other \(Aspergillus\) species (and also in other groups of fungi), suggests a functional role and this is likely to be in nitrate metabolism. However, from the evidence presented in this thesis, it is not possible to come to this conclusion with a degree of certainty. Either the lack of transcript detection was due to the failure in identifying the cultural conditions required for their expression or they are the pseudogenes which have lost their function during the course of evolution. Clearly there is a need to study these genes further to determine their possible metabolic role (if any). Further research work might include growing knock-out mutants (and more regulatory mutants) under a wider range of growth conditions. For instance, growth and treatments of mutants under further aerobic, more exacting anaerobic conditions and perhaps a concentration range of different nitrogen sources might shed light on this research topic. Another line of research would be to make knock-out mutants in other species and search for a growth phenotype which might be more obvious in another fungus, for example in \textit{A. fumigatus} rather than \textit{A. nidulans}. Finally and with regard to the absence of mRNA evidence, it is possible that transcript levels during the present investigation were too low to be detected by northern blotting. Therefore real time PCR should be used as an alternative method for the detection of low expression levels.

A NrtA structure model was developed based on low residue homology with the \textit{E. coli} GlpT protein, another anion transporter, since no NrtA crystal structure was available. Thiol cross-linking of three double cysteine mutants, \(R87C/R368C, N168C/R368C\) and \(R87C/N459C\), was carried out as an indirect method to study the proximity of four NrtA essential residues, \(R87\) (Tm 2), \(R368\) (Tm 8), \(N168\) (Tm 5) and \(N459\) (Tm 11). The results indicated, if the paired residues are facing each other, that the distance between \(R87\) and \(R368\) is \(\sim 0.4\) Å, \(R368\) and \(N168\) \(\sim 6.2\) Å, \(R87\) and \(N459\) \(\sim 2.2\) Å. However these molecular distances do not agree with the tentative positions of residues predicted by our current NrtA model. According to this NrtA model, \(N168\) and \(R368\) are very close (or tightly packed) while the conserved arginine residues at position 87 and 368 (i.e. the nitrate binding residues)
are 26 Å apart. Another important observation is the change in the confirmation of Tm 2 and Tm 8 that takes place in the presence of substrate, nitrate. This switch resulted in an increase of ~ 2 Å gap between R87 and R368 (to give a total distance of ~ 2.4 Å), presumably to accommodate the nitrate molecule which is approximately 2.6 Å in size. Therefore the existing model is currently being modified to take into account these physical distances using such molecular rulers. The present investigation of residue proximity is just the beginning of obtaining information on residue positions and helix packing in the NrtA protein. Residues of Tm 2 and Tm 8 as well as all Tms may be studied to build up a comprehensive and meaningful molecular picture regarding the static or dynamic state of this protein. It must be noted however that the mutant residues to cysteine that were chosen to undergo thiol cross-linking procedures in this study, being essential, resulted in non-functional proteins being produced. Therefore we cannot be sure about the inherent stability and correct folding of such mutant proteins. Future studies should include functional mutants (i.e. in non-essential residues) to confirm the applicability of this linker technique in terms of providing meaningful results for NrtA.

A crop of single cysteine replacement mutants of natural residues belonging to Tm 2 and Tm 8 of the NrtA protein was generated to study residue positions and the surrounding environment (whether aqueous or hydrophobic) within these Tms, by thiol chemistry technology. It was a considerable effort to generate 42 single copy transformants harbouring cysteine mutations in both Tms. The expression of NrtA protein was observed for these mutants but growth tests results indicated that 31 mutants out of 42 had lost their ability to use nitrate as the nitrogen source. The high level of loss-of-function mutants within these two Tms was a concern. The reasons for caution are discussed above. Consequently and reluctantly, Tm 2 and Tm 8 mutants were not studied further. Clearly, more studies need to be carried out using mutants in other Tm residues to be confident of the applicability of this general approach to NrtA.

GFP fusion to membrane proteins has been used as a potential tool to optimise detergent extraction and purification, resulting in crystal structures for a number of membrane proteins such as zebrafish ATP-gated P2X(4) receptor and chicken acid-sensing ion-channel (ASIC). In this present study bacterial nitrate transporter proteins were studied and over-expressed in auto-induction nutrient rich medium. Pre-crystallisation screening of detergent solubility and stability of over-expressed proteins was carried out by fluorescence detection size-exclusion chromatography (FSEC). It was observed that whereas the detergent soluble proteins showed sharp and symmetrical FSEC profiles, they could not be detected by western blotting. Sufficient amounts of proteins are required for crystallisation trails and we could not extract the necessary protein levels probably due to low solubility of the fusion proteins in detergent solutions. Such results were surprising and disappointing. Consequently, this particular purification approach was abandoned. As FSEC is a relatively simple, efficient and successful technique for pre-crystallisation screening and determination of detergent solubility and homogeneity of membrane proteins, modifications to our approach could be made. These could
include, using different vector constructs, expression systems, other detergents, wild type or mutant nitrate transporters from further bacteria fused with GFP in various combinations to increase protein levels for further crystallisation trials, and hopefully achieve success.

More than one transport system (NrtA and NrtB) for nitrate is present in *A. nidulans*. The substrate specificity and inhibition of NrtA and NrtB was explored in mutants in these transporter proteins. The results indicated that NrtA and NrtB transporter proteins have high affinity for nitrate. NrtA and NrtB net nitrate transport inhibition was observed, for example net nitrate NrtA and NrtB transport inhibition by chlorite and NrtB nitrate transport inhibition by caesium. To determine the kinetic relationships of such inhibitions (i.e. either competitive or non-competitive) there is a need to assess the affinity constant (*K*<sub>m</sub>) for nitrate in the presence of various concentrations of inhibitor that reduce the net nitrate transport by NrtA or NrtB.

Finally and although not directly connected with nitrate assimilation research, results obtained during this work supported the notion that wild type *A. nidulans* is a safe laboratory organism. However, and somewhat surprisingly, *Aspergillus oryzae* and *Aspergillus sojae*, fungi commonly used in the Japanese fermented food and alcohol industries were identified as highly pathogenic, at least for insects. Further research work will be carried out to confirm the true identity of these organisms. Corroboration will be attempted by DNA sequencing the ITS gene, an internal transcribed sequence of ribosomal RNA as well as evaluating more *A. oryzae* and *A. sojae* strains of these species for insect pathogenicity.
Appendix I (A): $K_i$ of net nitrate transport by the wild type strain for chlorate.

### Experiment 1

<table>
<thead>
<tr>
<th>KClO₃ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
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<tr>
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<td>11.14</td>
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<td>1.3010</td>
<td>6.65</td>
<td>5.57</td>
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<td>50</td>
<td>1.6990</td>
<td>2.84</td>
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</tr>
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\[
y = -2.255x^2 - 1.104x + 11.12
\]

\[y = 5.57\]

\[x = 1.34\]

$K_i = \text{antilog} 1.34$

\[K_i = 21.87 \text{ mM}\]

### Experiment 2

<table>
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<tr>
<th>KClO₃ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
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<tr>
<td>50</td>
<td>1.6989</td>
<td>2.82</td>
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\[
y = -2.231x^2 - 0.5607x + 10.347
\]

\[y = 5.19\]

\[x = 1.39\]

$K_i = \text{antilog} 1.39$

\[K_i = 24.54 \text{ mM}\]

### Experiment 3

<table>
<thead>
<tr>
<th>KClO₃ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
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<td>1.3010</td>
<td>8.1</td>
<td>5.905</td>
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<td>1.4771</td>
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<tr>
<td>50</td>
<td>1.6989</td>
<td>4.65</td>
<td></td>
</tr>
</tbody>
</table>

\[
y = -2.8137x^2 + 0.5497x + 11.801
\]

\[y = 5.69\]

\[x = 1.54\]

$K_i = \text{antilog} 1.54$

\[K_i = 34.67 \text{ mM}\]
Appendix I (B): \( K_i \) of net nitrate transport by the wild type strain for chlorite.

### Experiment 1

<table>
<thead>
<tr>
<th>( \text{NaClO}_2 ) Conc. (( \mu )M)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>11.19</td>
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</tr>
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<td>1.6989</td>
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\[
y = -1.965x^2 + 2.494x + 11.26 \\
y = 5.595 \\
x = 2.447 \\
K_i = \text{antilog}2.447 \\
K_i = 279.9 \mu M
\]

### Experiment 2

<table>
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<tr>
<th>( \text{NaClO}_2 ) Conc. (( \mu )M)</th>
<th>Log of Conc.</th>
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</tr>
<tr>
<td>1000</td>
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<td>1.27</td>
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</table>

\[
y = -1.627x^2 + 1.548x + 12.51 \\
y = 6.295 \\
x = 2.4877 \\
K_i = \text{antilog}2.4877 \\
K_i = 307.4 \mu M
\]

### Experiment 3

<table>
<thead>
<tr>
<th>( \text{NaClO}_2 ) Conc. (( \mu )M)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
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\[
y = -1.9526x^2 + 2.8631x + 11.017 \\
y = 5.505 \\
x = 2.5646 \\
K_i = \text{antilog}2.5646 \\
K_i = 367.07 \mu M
\]
Appendix I (C): $K_i$ of net nitrate transport by the wild type strain for sulphite.

### Experiment 1

<table>
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<th>$\text{Na}_2\text{SO}_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
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\[
y = -10.83x^2 + 4.730x + 12.04
\]

\[
y = 6.02 \\
x = 0.995 \\
K_i = \text{antilog} 0.995 \\
K_i = 9.89 \text{ mM}
\]

### Experiment 2

<table>
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<tr>
<th>$\text{Na}_2\text{SO}_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
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</tr>
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<td>20</td>
<td>1.3010</td>
<td>0.89</td>
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</tbody>
</table>

\[
y = -9.727x^2 + 3.829x + 11.71
\]

\[
y = 5.855 \\
x = 0.9975 \\
K_i = \text{antilog} 0.9975 \\
K_i = 9.94 \text{ mM}
\]

### Experiment 3

<table>
<thead>
<tr>
<th>$\text{Na}_2\text{SO}_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>0</td>
<td>11.75</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.6989</td>
<td>7.33</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>5.42</td>
<td>5.875</td>
</tr>
<tr>
<td>15</td>
<td>1.1760</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.3010</td>
<td>0.9</td>
<td></td>
</tr>
</tbody>
</table>

\[
y = -4.374x^2 - 2.654x + 11.70
\]

\[
y = 5.875 \\
x = 0.89 \\
K_i = \text{antilog} 0.89 \\
K_i = 7.76 \text{ mM}
\]
Appendix I (D): $K_i$ of net nitrate transport by the wild type strain for caesium.

### Experiment 1

<table>
<thead>
<tr>
<th>CsCl Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>11.07</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>11.90</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.1760</td>
<td>10.56</td>
<td>5.535</td>
</tr>
<tr>
<td>20</td>
<td>1.3010</td>
<td>9.22</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1.4771</td>
<td>8.46</td>
<td></td>
</tr>
</tbody>
</table>

$y = -5.251x^2 + 5.777x + 11.09$

$y = 5.535$

$x = 1.71$

$K_i = \text{antilog} 1.71$

$K_i = 51.29$ mM

### Experiment 2

<table>
<thead>
<tr>
<th>CsCl Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>11.43</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>10.98</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.1760</td>
<td>9.75</td>
<td>5.715</td>
</tr>
<tr>
<td>20</td>
<td>1.3010</td>
<td>9.76</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1.4771</td>
<td>7.30</td>
<td></td>
</tr>
</tbody>
</table>

$y = -4.741x^2 + 4.400x + 11.41$

$y = 5.715$

$x = 1.654$

$K_i = \text{antilog} 1.654$

$K_i = 45.08$ mM

### Experiment 3

<table>
<thead>
<tr>
<th>CsCl Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>12.52</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>10.21</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.1760</td>
<td>9.89</td>
<td>6.26</td>
</tr>
<tr>
<td>20</td>
<td>1.3010</td>
<td>9.01</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1.4771</td>
<td>7.10</td>
<td></td>
</tr>
</tbody>
</table>

$y = -3.292x^2 + 1.381x + 12.49$

$y = 6.26$

$x = 1.6$

$K_i = \text{antilog} 1.6$

$K_i = 39.81$ mM
Appendix II (A): $K_i$ of net nitrate transport by the mutant *nrtA1* for chlorate.

### Experiment 1

<table>
<thead>
<tr>
<th>KClO$_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.72</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>3.42</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.3010</td>
<td>3.12</td>
<td>1.86</td>
</tr>
<tr>
<td>30</td>
<td>1.4771</td>
<td>2.88</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.6989</td>
<td>1.14</td>
<td></td>
</tr>
</tbody>
</table>

$y = -1.947x^2 + 2.005x + 3.681$

$y = 1.86$

$x = 1.6$

$K_i = \text{antilog} 1.6$

$K_i = 39.8$ mM

### Experiment 2

<table>
<thead>
<tr>
<th>KClO$_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>2.82</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>2.75</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.3010</td>
<td>2.41</td>
<td>1.41</td>
</tr>
<tr>
<td>30</td>
<td>1.4771</td>
<td>2.27</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.6987</td>
<td>0.48</td>
<td></td>
</tr>
</tbody>
</table>

$y = -2.0418x^2 + 2.3127x + 2.7819$

$y = 1.41$

$x = 1.56$

$K_i = \text{antilog} 1.56$

$K_i = 36.3$ mM

### Experiment 3

<table>
<thead>
<tr>
<th>KClO$_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>2.17</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>1.99</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.3010</td>
<td>1.62</td>
<td>1.085</td>
</tr>
<tr>
<td>30</td>
<td>1.4771</td>
<td>1.93</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.6989</td>
<td>0.84</td>
<td></td>
</tr>
</tbody>
</table>

$y = -0.844x^2 + 0.794x + 2.151$

$y = 1.085$

$x = 1.69$

$K_i = \text{antilog} 1.69$

$K_i = 48.93$ mM
Appendix II (B): $K_i$ of net nitrate transport by the mutant *nrtAI* for chlorite.

### Experiment 1

<table>
<thead>
<tr>
<th>NaClO$_2$ Conc. (µM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.62</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.6989</td>
<td>3.74</td>
<td>1.81</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>3.02</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>2.3979</td>
<td>2.19</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.6989</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

$y = -0.886x^2 + 1.524x + 3.624$

$y = 1.81$

$x = 2.529$

$K_i = \text{antilog}2.529$

$K_i = 337.9$ µM

### Experiment 2

<table>
<thead>
<tr>
<th>NaClO$_2$ Conc. (µM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.58</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.6989</td>
<td>3.23</td>
<td>1.79</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>2.75</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>2.3979</td>
<td>2.45</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.6989</td>
<td>0.96</td>
<td></td>
</tr>
</tbody>
</table>

$y = -0.736x^2 + 1.116x + 3.564$

$y = 1.79$

$x = 2.485$

$K_i = \text{antilog}2.485$

$K_i = 305.63$ µM

### Experiment 3

<table>
<thead>
<tr>
<th>NaClO$_2$ Conc. (µM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.66</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.6989</td>
<td>3.13</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>2.65</td>
<td>1.83</td>
</tr>
<tr>
<td>250</td>
<td>2.3979</td>
<td>2.02</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.6989</td>
<td>0.73</td>
<td></td>
</tr>
</tbody>
</table>

$y = -0.769x^2 + 1.054x + 3.649$

$y = 1.83$

$x = 2.36$

$K_i = \text{antilog}2.36$

$K_i = 233.56$ µM
Appendix II (C): $K_i$ of net nitrate transport by the mutant *nrtAI* for sulphite.

### Experiment 1

<table>
<thead>
<tr>
<th>$\text{Na}_2\text{SO}_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.11</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>2.43</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.6989</td>
<td>2.71</td>
<td>1.555</td>
</tr>
<tr>
<td>7.5</td>
<td>0.8750</td>
<td>1.64</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

$y = -9.081x^2 + 6.421x + 2.766$

$y = 1.555$

$x = 0.86$

$K_i = \text{antilog} 0.86$

$K_i = 7.28$ mM

### Experiment 2

<table>
<thead>
<tr>
<th>$\text{Na}_2\text{SO}_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>2.11</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>1.77</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.6989</td>
<td>2.23</td>
<td>1.055</td>
</tr>
<tr>
<td>7.5</td>
<td>0.8750</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

$y = -7.906x^2 + 6.022x + 1.938$

$y = 1.055$

$x = 0.8875$

$K_i = \text{antilog} 0.8875$

$K_i = 7.72$ mM

### Experiment 3

<table>
<thead>
<tr>
<th>$\text{Na}_2\text{SO}_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>2.62</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>2.32</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.6989</td>
<td>2.32</td>
<td>1.31</td>
</tr>
<tr>
<td>7.5</td>
<td>0.8750</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

$y = -7.741x^2 + 5.437x + 2.465$

$y = 1.31$

$x = 0.874$

$K_i = \text{antilog} 0.874$

$K_i = 7.47$ mM
Appendix II (D): $K_i$ of net nitrate transport by the mutant nrtA1 for caesium.

**Experiment 1**

<table>
<thead>
<tr>
<th>CsCl Conc. (µM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.46</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.6989</td>
<td>3.22</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>2.33</td>
<td>1.73</td>
</tr>
<tr>
<td>2000</td>
<td>3.3010</td>
<td>2.25</td>
<td></td>
</tr>
<tr>
<td>5000</td>
<td>3.6989</td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

$y = -0.768x^2 + 2.012x + 3.452$

$y = 1.73$

$x = 3.3$

$K_i = \text{antilog}3.3$

$K_i = 1.99$ mM

**Experiment 2**

<table>
<thead>
<tr>
<th>CsCl Conc. (µM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.59</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.6989</td>
<td>3.59</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>2.69</td>
<td>1.795</td>
</tr>
<tr>
<td>2000</td>
<td>3.3010</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>5000</td>
<td>3.6989</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

$y = -0.847x^2 + 2.261x + 3.59$

$y = 1.795$

$x = 3.31$

$K_i = \text{antilog}3.31$

$K_i = 2.04$ mM

**Experiment 3**

<table>
<thead>
<tr>
<th>CsCl Conc. (µM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.27</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.6989</td>
<td>3.04</td>
<td></td>
</tr>
<tr>
<td>1000</td>
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<td></td>
</tr>
<tr>
<td>5000</td>
<td>3.6989</td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

$y = -0.755x^2 + 1.959x + 3.269$

$y = 1.635$

$x = 3.26$

$K_i = \text{antilog}3.26$

$K_i = 1.81$ mM
Appendix III (A): $K_i$ of net nitrate transport by the mutant *nrtB110* for chlorate.

### Experiment 1

<table>
<thead>
<tr>
<th>KClO$_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>8.79</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>5.38</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.301</td>
<td>5.00</td>
<td>4.395</td>
</tr>
<tr>
<td>30</td>
<td>1.4771</td>
<td>4.42</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.6989</td>
<td>2.93</td>
<td></td>
</tr>
</tbody>
</table>

$y = -0.365x^2 - 2.6269x + 8.748$

$y = 4.395$

$x = 1.39$

$K_i = \text{antilog} 1.39$

$K_i = 24.5$ mM

### Experiment 2

<table>
<thead>
<tr>
<th>KClO$_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>9.09</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>6.34</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.301</td>
<td>5.68</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1.4771</td>
<td>5.07</td>
<td>4.545</td>
</tr>
<tr>
<td>50</td>
<td>1.6989</td>
<td>2.27</td>
<td></td>
</tr>
</tbody>
</table>

$y = -2.1958x^2 + 0.0679x + 9.024$

$y = 4.545$

$x = 1.4$

$K_i = \text{antilog} 1.4$

$K_i = 25.1$ mM

### Experiment 3

<table>
<thead>
<tr>
<th>KClO$_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>9.12</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>5.89</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.301</td>
<td>4.97</td>
<td>4.56</td>
</tr>
<tr>
<td>30</td>
<td>1.4771</td>
<td>5.85</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.6989</td>
<td>2.03</td>
<td></td>
</tr>
</tbody>
</table>

$y = -1.534x^2 - 0.9849x + 9.032$

$y = 4.56$

$x = 1.4$

$K_i = \text{antilog} 1.4$

$K_i = 25.11$ mM
Appendix III (B): $K_i$ of net nitrate transport by the mutant \textit{nrtB110} for chlorite.

### Experiment 1

<table>
<thead>
<tr>
<th>NaClO$_2$ Conc. (µM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
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<td>9.11</td>
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<td>7.5</td>
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<tr>
<td>250</td>
<td>2.3979</td>
<td>4.35</td>
<td>4.555</td>
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<td>500</td>
<td>2.6989</td>
<td>4.12</td>
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</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

$$y = -2.459x^2 + 4.776x + 9.172$$

$$y = 4.59$$

$$x = 2.4$$

$K_i = \text{antilog}2.4$

$K_i = 251$ µM

### Experiment 2

<table>
<thead>
<tr>
<th>NaClO$_2$ Conc. (µM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>9.39</td>
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</tr>
<tr>
<td>100</td>
<td>2</td>
<td>6.78</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>2.3979</td>
<td>4.42</td>
<td>4.695</td>
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<td>2.6989</td>
<td>4.07</td>
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<tr>
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<td></td>
</tr>
</tbody>
</table>

$$y = -1.713x^2 + 2.223x + 9.365$$

$$y = 4.695$$

$$x = 2.4$$

$K_i = \text{antilog}2.4$

$K_i = 251.1$ µM

### Experiment 3

<table>
<thead>
<tr>
<th>NaClO$_2$ Conc. (µM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>6.46</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>2.3979</td>
<td>5.59</td>
<td>4.15</td>
</tr>
<tr>
<td>500</td>
<td>2.6989</td>
<td>4.04</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

$$y = -1.974x^2 + 3.421x + 8.247$$

$$y = 4.15$$

$$x = 2.54$$

$K_i = \text{antilog}2.54$

$K_i = 346.7$ µM
Appendix III (C): $K_i$ of net nitrate transport by the mutant *nrtB110* for sulphite.

### Experiment 1

<table>
<thead>
<tr>
<th>Na$_2$SO$_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>8.37</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.6989</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>4.88</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.1760</td>
<td>2.68</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.3010</td>
<td>0.65</td>
<td></td>
</tr>
</tbody>
</table>

\[ y = -2.847x^2 - 1.632x + 8.238 \]
\[ y = 4.185 \]
\[ x = 0.94 \]
\[ K_i = \text{antilog } 0.94 \]
\[ K_i = 8.7 \text{ mM} \]

### Experiment 2

<table>
<thead>
<tr>
<th>Na$_2$SO$_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>8.63</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.6989</td>
<td>4.79</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>4.61</td>
<td>4.315</td>
</tr>
<tr>
<td>15</td>
<td>1.1760</td>
<td>3.31</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.3010</td>
<td>0.12</td>
<td></td>
</tr>
</tbody>
</table>

\[ y = -2.881x^2 - 1.861x + 8.476 \]
\[ y = 4.315 \]
\[ x = 0.922 \]
\[ K_i = \text{antilog } 0.922 \]
\[ K_i = 8.35 \text{ mM} \]

### Experiment 3

<table>
<thead>
<tr>
<th>Na$_2$SO$_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>7.92</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.6989</td>
<td>4.29</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.1760</td>
<td>2.48</td>
<td>3.96</td>
</tr>
<tr>
<td>20</td>
<td>1.3010</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

\[ y = -3.182x^2 - 1.168x + 7.758 \]
\[ y = 3.96 \]
\[ x = 0.924 \]
\[ K_i = \text{antilog } 0.924 \]
\[ K_i = 8.39 \text{ mM} \]
Appendix III (D): \( K_i \) of net nitrate transport by the mutant \( nrtB110 \) for caesium.

### Experiment 1

<table>
<thead>
<tr>
<th>CsCl Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>7.74</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>7.74</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.301</td>
<td>7.74</td>
<td>3.87</td>
</tr>
<tr>
<td>30</td>
<td>1.4771</td>
<td>4.88</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.6989</td>
<td>4.71</td>
<td></td>
</tr>
</tbody>
</table>

\[
y = -3.109x^2 + 3.334x + 7.736
\]
\[
y = 3.87
\]
\[
x = 1.77
\]
\[
K_i = \text{antilog} 1.77
\]
\[
K_i = 58.88 \text{ mM}
\]

### Experiment 2

<table>
<thead>
<tr>
<th>CsCl Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>8.50</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>8.56</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.301</td>
<td>8.33</td>
<td>4.25</td>
</tr>
<tr>
<td>30</td>
<td>1.4771</td>
<td>5.54</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.6989</td>
<td>5.02</td>
<td></td>
</tr>
</tbody>
</table>

\[
y = -3.534x^2 + 3.817x + 8.494
\]
\[
y = 4.25
\]
\[
x = 1.76
\]
\[
K_i = \text{antilog} 1.76
\]
\[
K_i = 57.5 \text{ mM}
\]

### Experiment 3

<table>
<thead>
<tr>
<th>CsCl Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrate transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>8.77</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>9.71</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.301</td>
<td>8.54</td>
<td>4.385</td>
</tr>
<tr>
<td>30</td>
<td>1.4771</td>
<td>6.02</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.6989</td>
<td>5.2</td>
<td></td>
</tr>
</tbody>
</table>

\[
y = -4.5279x^2 + 5.372x + 8.7917
\]
\[
y = 4.385
\]
\[
x = 1.74
\]
\[
K_i = \text{antilog} 1.74
\]
\[
K_i = 54.95 \text{ mM}
\]
Appendix IV (A): $K_i$ of net nitrite transport by the strain T12048 for chlorate.

**Experiment 1**

<table>
<thead>
<tr>
<th>KClO$_3$ Conc. (µM)</th>
<th>Log of Conc.</th>
<th>Net nitrite transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.32</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.6989</td>
<td>1.97</td>
<td>1.66</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>1.44</td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>3.3010</td>
<td>0.57</td>
<td></td>
</tr>
</tbody>
</table>

\[ y = -0.482x^2 + 0.790x + 3.312 \]
\[ y = 1.66 \]
\[ x = 2.84 \]
\[ K_i = \text{antilog } 2.84 \]
\[ K_i = 691.83 \text{ µM} \]

**Experiment 2**

<table>
<thead>
<tr>
<th>KClO$_3$ Conc. (µM)</th>
<th>Log of Conc.</th>
<th>Net nitrite transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>2.78</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.6989</td>
<td>1.56</td>
<td>1.39</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>1.52</td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>3.3010</td>
<td>0.63</td>
<td></td>
</tr>
</tbody>
</table>

\[ y = -0.465x^2 + 0.896x + 2.785 \]
\[ y = 1.39 \]
\[ x = 2.94 \]
\[ K_i = \text{antilog } 2.94 \]
\[ K_i = 870.96 \text{ µM} \]

**Experiment 3**

<table>
<thead>
<tr>
<th>KClO$_3$ Conc. (µM)</th>
<th>Log of Conc.</th>
<th>Net nitrite transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>2.97</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.6989</td>
<td>1.66</td>
<td>1.485</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>1.33</td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>3.3010</td>
<td>1.06</td>
<td></td>
</tr>
</tbody>
</table>

\[ y = -0.385x^2 + 0.634x + 2.989 \]
\[ y = 1.485 \]
\[ x = 2.96 \]
\[ K_i = \text{antilog } 2.96 \]
\[ K_i = 912.01 \text{ µM} \]
Appendix IV (B): $K_i$ of net nitrite transport by the strain T12048 for caesium.

### Experiment 1

<table>
<thead>
<tr>
<th>CsCl Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrite transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>2.73</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.3010</td>
<td>1.95</td>
<td>1.365</td>
</tr>
<tr>
<td>30</td>
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<td></td>
</tr>
<tr>
<td>50</td>
<td>1.6989</td>
<td>1.41</td>
<td></td>
</tr>
</tbody>
</table>

\[ y = -0.904x^2 + 0.736x + 2.740 \]
\[ y = 1.365 \]
\[ x = 1.7 \]
\[ K_i = \text{antilog}1.7 \]
\[ K_i = 50.12 \text{ mM} \]

### Experiment 2

<table>
<thead>
<tr>
<th>CsCl Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrite transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.68</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>3.37</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.3010</td>
<td>3.23</td>
<td>1.84</td>
</tr>
<tr>
<td>30</td>
<td>1.4771</td>
<td>2.43</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.6989</td>
<td>2.13</td>
<td></td>
</tr>
</tbody>
</table>

\[ y = -1.018x^2 + 0.797x + 3.675 \]
\[ y = 1.84 \]
\[ x = 1.78 \]
\[ K_i = \text{antilog}1.78 \]
\[ K_i = 60.25 \text{ mM} \]

### Experiment 3

<table>
<thead>
<tr>
<th>CsCl Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrite transport (nmol/min/mg DW)</th>
<th>50 % flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.15</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>2.99</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.30103</td>
<td>2.84</td>
<td>1.575</td>
</tr>
<tr>
<td>30</td>
<td>1.4771213</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.69897</td>
<td>1.99</td>
<td></td>
</tr>
</tbody>
</table>

\[ y = -0.849x^2 + 0.754x + 3.146 \]
\[ y = 1.575 \]
\[ x = 1.87 \]
\[ K_i = \text{antilog}1.87 \]
\[ K_i = 74.1 \text{ mM} \]
Appendix IV (C): $K_i$ of net nitrite transport by the strain T12048 for nitrate.

### Experiment 1

<table>
<thead>
<tr>
<th>NaNO$_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrite transport (nmol/min/mg DW)</th>
<th>$50%$ flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.49</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.6989</td>
<td>0.56</td>
<td>1.745</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>0.26</td>
<td></td>
</tr>
</tbody>
</table>

$y = 0.578x^2 - 2.681x + 3.362$

$x = 0.712$

$K_i = \text{antilog}0.712$

$K_i = 5.16/10$

$K_i = 0.516$ mM

### Experiment 2

<table>
<thead>
<tr>
<th>NaNO$_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrite transport (nmol/min/mg DW)</th>
<th>$50%$ flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.34</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.69897</td>
<td>0.56</td>
<td>1.67</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>0.28</td>
<td></td>
</tr>
</tbody>
</table>

$y = 0.565x^2 - 2.595x + 3.224$

$x = 0.708$

$K_i = \text{antilog}0.708$

$K_i = 5.077/10$

$K_i = 0.51$ mM

### Experiment 3

<table>
<thead>
<tr>
<th>NaNO$_3$ Conc. (mM)</th>
<th>Log of Conc.</th>
<th>Net nitrite transport (nmol/min/mg DW)</th>
<th>$50%$ flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3.44</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.69897</td>
<td>0.44</td>
<td>1.72</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>0.29</td>
<td></td>
</tr>
</tbody>
</table>

$y = 0.579x^2 - 2.682x + 3.324$

$x = 0.705$

$K_i = \text{antilog}0.705$

$K_i = 5.055/10$

$K_i = 0.51$ mM

$R^2 = 0.9521$

$R^2 = 0.9554$

$R^2 = 0.9629$
Appendix V: Determination of auxotrophic marker of mutant strains.

Due to the growth inhibition of mutant strains T12048 and T12200 on nitrite, a question arose if these strains were different from other mutants strains and the wild type in their auxotrophic marker. So this additional gene marker was determined by growing the strains on minimal medium with one or more vitamins as described in the Table below that could be their possible selection marker(s). These vitamins include biotin (bio), p-aminobenzoic acid (paba) and pyridoxine (pyro). Phenotype analysis suggested that the wild type strain, mutants T110 and nrtA1 were biotin requiring strains and the strains T12048 and T12200 have paba auxotrophic marker.

Table: Vitamin requirement of the wild type and mutant strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>no vitamin</th>
<th>bio + paba</th>
<th>bio + paba</th>
<th>paba + pyro</th>
<th>bio + pyro</th>
<th>bio</th>
<th>paba</th>
<th>pyro</th>
<th>Auxotrophic marker</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>biotin</td>
<td>biA1</td>
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Strains were grown at 37 ºC for 2-3 days on minimal medium supplemented with 5 mM ammonium and vitamin(s) as described.
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