UNIVERSIDAD DE GRANADA
DEPARTAMENTO DE FISIOLOGÍA VEGETAL

IDENTIFICATION, ISOLATION AND CHARACTERIZATION OF EXTRACYTOPLASMIC PROTEINS IN *BRADYRHIZOBIUM JAPONICUM*

TESIS DOCTORAL

NATHALIE BONNARD

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Fdo. Nathalie Bonnard
Licenciada en Biología
Universidad de Nantes

Los directores

Fdo. Dra. Maria Jesús Delgado Igeño
Científico titular CSIC
Estacion experimental del Zaidin

Fdo. Dr. Eulogio José Bedmar Gómez
Científico titular CSIC
Estacion experimental del Zaidin

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Pour mes parents

Pour mes grand-parents

Pour Pierre

Pour Jesus

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1. INTRODUCTION

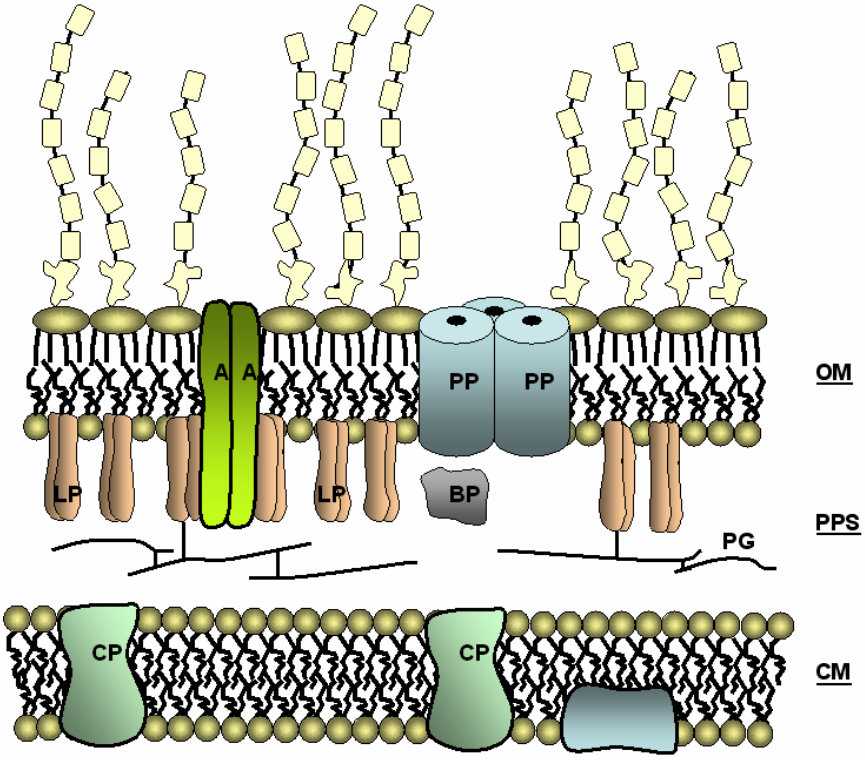
1. The cell envelope of bacteria

The cell envelope structure in prokaryotes is composed of different parts: the cytoplasmic membrane and the cell wall. The cytoplasmic membrane is a phospholipid bilayer acting as an essential barrier, separating the interior of the cell and the exterior, allowing the concentration of determined metabolites in the cytoplasm, and the selective export of waste substances. The cytoplasmic or inner membrane is similar in structure and composition to the eukaryotic plasma membrane and mitochondrial membrane, but it carries out many more functions than the eukaryotic plasma membrane such as molecules transport, electron transport and oxidative phosphorylation, energy production, motility and replication. The cell wall is a rigid structure surrounding the cell membrane, and it protects the cell from osmotic lysis, from external stresses, contributes to the virulence process and interactions between symbionts.

Bacteria can be distinguished by their staining characteristics and by the structural differences of their cell envelope in two groups: Gram positives and Gram negatives. The structure of the Gram positive bacteria envelope is composed by a cellular membrane and a thick cell wall of peptidoglycan which contains teichoic and lipoteichoic acids. The structure of the Gram negative bacteria envelope is characterized by the presence of an additional lipid bilayer known as the outer membrane which consists of phospholipids in the inner leaflet and lipopolysaccharides in the outer leaflet (Figure 1.1) (Madigan, Martinko, Parker; Brock 1999). The space located between the cellular membrane (inner membrane) and the outer membrane is known as the periplasm, and is the compartment of numerous metabolic reactions. A unique layer of peptidoglycan occupies the periplasmic space (Figure 1.1). Some specific proteins from the outer membrane (porines) allow the diffusion through this membrane (Figure 1.1). The outer face of the outer membrane contains a lipid compound, lipopolysaccaride (LPS), which is very important for example in pathogenic or symbiotic bacteria (Lugtenberg, 1998)

Figure 1.1. Molecular organization of the cell envelope of *Enterobacteriaceae* (adapted from Lugtenberg, 1998). CM, cytoplasmic membrane; CP, carrier proteins involved in transport of nutrient; PG, peptidoglycan consisting in amino sugar chains crosslinked to each other by peptide bridges; PPS, periplasmic space; BP, binding proteins involved in transport of

nutrients; OM, outer membrane; PP, pore proteins which are aqueous channels through which nutrients can diffuse; A, OmpA protein, a transmembrane molecule which links the outer membrane non-covalently to the PG; LP, Braun's lipoprotein inserted in the inner leaflet of the bilayer by three fatty acids and of which the protein part is located in the PPS. One third of the LP molecules is covalently linked to the PG.



2. Extracytoplasmic proteins

Among bacteria, extracytoplasmic proteins are expected to play important roles in cell functions like respiration, nutrient uptake, osmoregulation, motility, sensing of stimuli, protein transport, cell wall turnover, signal transduction, and virulence processes. Extracellular proteins are synthesised in the cytoplasm and have to be translocated to their site of action by specific targeting and transport mechanisms. In Gram-negative bacteria the secretion of extracellular or outer membrane proteins is more complicated because the protein has to cross the cytoplasmic or inner membrane and the outer membrane.

2.1. Transport of extracytoplasmic proteins through the inner membrane

Three major systems have been described for secretion of proteins across the bacterial cytoplasmic membrane: Sec (secretion pathway), SRP (signal-recognition particle), and Tat (twin-arginine translocation). Components of the Sec and SRP pathways are present in the majority of organisms (prokaryotes and eukaryotes) as substantiated by numerous completed genome sequencing projects. Homologues of the Tat family are not ubiquitous among bacteria, and are not found in yeast or animals.

The precursors of extracytoplasmic proteins are synthesised with an N-terminal end of extra 15-20 amino acids, named signal peptide. In the core region of this peptide, neutral or hydrophobic amino acids predominate which allow the protein to go through the membrane lipids. In numerous cases, the ribosomes synthesising the secretion proteins, are directly bound to the membranes so that the protein is cotranslationally translocated across the inner membrane. Once the C-terminal part of the protein has crossed the inner membrane, the signal peptide is eliminated by the signal peptidase. The signal sequence of an extracytoplasmic protein precursor can be either a signal peptide (cleavable) or a signal anchors (non-cleavable). Most cleavable signal peptides have a tripartite structure, a net positive charged 1-5 residues N-domain, a α-helical 7-15 residues hydrophobic core (H-domain), and a polar 3-7 residues C-domain. Due to the positive charge of the N-domain, the N-terminus is retained in the cytosol since the electrochemical potential of the cytoplasmic membrane is negative on the inner side. The H-domain has been proposed to be inserted into the lipid bilayer. The cleavage site is contained within the C-domain. Despite the common general structure of signal peptides, no conservation in amino acid sequence is observed (von Heijne, 2002). Signal peptides of the Tat pathway have an essentially invariant twin-arginine motif in the extended N-domain. The consecutive arginine residues are part of a consensus sequence within the signal peptide, S-R-R-x-F-L-K, where X is a polar amino acid or a glycine, and other residues are found at frequencies exceeding 50%.

In most cases, bacterial cytoplasmic membrane proteins do not have cleavable signal peptides but are instead recognised by the extended hydrophobic regions that are responsible of the transmembrane helices in the mature protein. Non-cleavable signal peptides can anchor proteins in the membrane, when they contain a sufficienlty hydrophobic H-domain (Martoglio and Dobberstein, 1998). Based on studies of *Escherichia coli*, cleavable signal peptides are

rarely found in bacterial cytoplasmic membrane proteins, but it is very difficult to distinguish between cleavable and non-cleavable signal peptides (Broome-Smith *et al.*, 1994).

2.1.1. Sec and SRP pathways

The Sec and SRP pathways merge at the Sec translocase. Although exceptions have been identified, the Sec pathway is responsible for a post-translational protein translocation, while the SRP pathway mainly mediates co-translational translocation. Chaperones, like SecB and SRP, keep the mature protein in an unfolded state. The post-translational pathway translocates mostly water-soluble proteins. Substrates with an N-terminal signal peptide are recognised by the SecA ATPase associated to the Sec core (SecYEG) in the cytoplasmic membrane through which the substrate protein is transported by threading, requiring energy input in the form of ATP and a proton motive force (PMF). Additional proteins, SecD, SecF, and YajC, are membrane proteins involved in translocation, although their exact functions have not yet been fully elucidated. In the post-translational pathway, insertion of integral membrane proteins into the cytoplasmic membrane normally also involves the SecYEG translocase via the SRP receptor FtsY associated with the translocase (Driessen and van der Does, 2002).

2.1.2. The Tat pathway

The twin-arginine transport (Tat) system is a protein-targeting pathway found in the cytoplasmic membranes of many prokaryotes and the thylakoid membranes of chloroplasts (Berks *et al.*, 2003). Tat-targeted substrate proteins are synthesized as precursors with N-terminal twin-arginine signal peptides that exhibit distinctive SRRxFLK amino acid motifs (Palmer *et al.*, 2005). In *E. coli*, the TatA/TatE, TatB, and TatC membrane proteins form the core components of the Tat translocase. The Tat proteins have been proposed to form two types of complexes in *E. coli*, the Tat(A)BC (indicating a minor proportion of Tat A protein) and TatA(B) (composed predominanlty of TatA protein) complexes. The TatA(B) complex is supposed to form the transport channel, and Tat(A)BC forms the substrate receptor complex (Berks *et al.*, 2003). Protein translocation is powered solely by the transmembrane proton gradient, and there is some evidence to suggest the Tat translocase acts as a genuine protein–proton antiporter (Hatzixanthis *et al.*, 2005). The majority of Tat-translocons are redox proteins containing cofactor molecules. The physiological role of the bacterial Tat pathway is to complete the maturation of periplasmic proteins in the cytoplasm before they are exported.

Tat pathway is also critical for proteins relevant to the initial stages of the symbiotic process (Meloni *et al.*, 2003).

2.2. Transport of extracytoplasmic proteins through the outer membrane

Gram-negative bacteria secrete a wide range of proteins whose functions include biogenesis of organelles, such as pili and flagella; nutrient acquisition; virulence; and efflux of drugs and other toxins. Export of these proteins to the bacterial surface involves transport across the inner membrane (IM), periplasm, and outer membrane (OM) of the cell envelope. Several pathways have evolved to fulfill the task of secretion (Thanassi and Hultgren, 2000; Kostakioti *et al.*, 2005). These pathways can be divided into two main groups: (i) Sec dependent and (ii) Sec independent. Proteins secreted via the Sec-dependent pathways utilize a common machinery, the Sec translocase, for transport across the IM and are mainly differentiated based on their mechanisms of secretion across the OM. Sec-dependent pathways include the type II secretion, the autotransporter, or type V secretion, the two-partner secretion, and the chaperone/usher secretion systems. The type IV secretion, or adapted-conjugation, pathway can be Sec dependent but is mostly considered Sec independent. Sec-independent pathways tend to allow direct export from the cytoplasm to the extracellular environment in one step and do not involve periplasmic intermediates. These pathways also include the type I secretion, or ABC (ATP-binding cassette), exporters and the type III secretion systems. Bacterial toxins, such as the a-hemolysin of *E. coli*, are released in the extracellular space via type I exporters, whereas type III secreted proteins are mostly pathogenicity determinants some of which are targeted to the eukaryotic cell cytosol.

2.3. Extracytoplasmic proteins in the *Rhizobiales* order

The *Rhizobiales* order includes bacterial species which are characterized by their capability to infect cortical root cells of leguminous plants and to induce the formation of nodules, typical plant organs of the plant-bacteria association, where the biological nitrogen fixation is carried out, for its reduction to ammonium (Figure 1.4). Whereas the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Allorhizobium* induce the formation of nodules on the root system of their leguminous host, *Azorhizobium* generates the nodules on the roots as well as on the stems of *Sesbania rostrata*, and *Phyllobacterium* generate them in the leaves of *Myrsinaceae* and *Rubiaceae* families. Finally, the genus *Agrobacterium* includes phytopathogenic species which induce plant tumor formation on

stems and roots of various plants, except *Agrobacterium radiobacter* (van Berkum and Eardly, 1998; Amarger, 2001).

The establishment of an effective symbiosis between legumes and rhizobia is a complex process, involving signaling and recognition by both partners from the outset. Flavonoids found in root exudates trigger the expression of the rhizobial genes (nod, nol, noe) involved in the synthesis and secretion of Nod factors, a family of complex lipo-chito-oligosaccharides required for nodulation (Perret *et al.*, 2000). Rhizobial attachment to root hairs, penetration of the epidermis, and invasion of cortical tissue via the infection thread is accompanied by initiation of merismatic activity in root cortical and pericycle cells and by suppresion of plant defense responses (Denarie *et al.*, 1996; Downie and Walker, 1999; Long, 2001). Once released into the host cytoplasm, rhizobia stop dividing and undergo differentiation into nitrogen-fixing bacteroids which synthesize the nitrogenase enzyme complex responsible for the catalysis of nitrogen to ammonium.

In addition to Nod factors, a variety of prokaryotic signals are known to control successive stages of the plant infection process. Among these are surface and extra-cellular polysaccharides (EPS), as well as several proteins exported via type I or type III secretion systems. Among these, the *nodO* product is required for nodulation and is a Ca2+ binding protein which forms cation-specific channels in membranes of leguminous plants (Economou *et al.*, 1990, Sutton *et al.*, 1994). The C-terminal domain of NodO is required for its secretion via the type I transport system (Sutton *et al.*, 1996). In addition to NodO, at least three other proteins are exported by type I transporter, two of which (PlyA and PlyB) are involved in processing bacterial EPS's (Finnie *et al.*, 1998). Recently, type III secretion systems were also shown to play a role in the host-specific nodulation of legumes by rhizobia (Viprey *et al.*, 1998; Suss *et al.*, 2006). Those proteins seem to represent another determinant of host-specifity, possibly by directing compatible or incompatible reactions between symbionts and host-plants, and/or avoiding host plant defense responses.

The rhizobial periplasmic proteins, outer membrane proteins, and secreted proteins have been estimated to constitute as much as 36% of the total amount of proteins (Lugtenberg, 1998). Most of them are expected to be involved in fully functional nodules and bacteroids .

In order to elucidate the putative functions of extracytoplasmic proteins in the *Rhizobiales* order, de Maagd and Lugtenberg (1986) performed some analysis to localyse these proteins. By sub-cellular fractionation of *Rhizobium leguminosarum* biovar *viciae* 248 cells, and further SDS-PAGE analysis, they could estimate the proportion of proteins present in each fraction: 45% were detected in the cytosol, 19% in the cytoplasmic membrane, 16% in

the periplasm and 19% in the outer membrane. 1% of the total proteins was expected to be secreted to the medium. Among the 6,667 proteins encoded by the *Sinorhizobium meliloti* genome, Galibert *et al.* (2001) calculated that around 10% have N-terminal signal peptides. Recently, the genome sequence of *Bradyrhizobium japonicum* strain USDA110 has been completed by the Kazusa Institute (http://www.kazusa.or.jp/rhizobase/). Its size is 9.1 Mb, and 8,317 potential proteins were described (Kaneko *et al.*, 2002). Analysis performed by Rosander *et al.*, (2003) predicted that at least 9 % of the open reading frames (ORFs) among 1,479 proteins randomly chosen from the *B. japonicum* genome database, presented N-terminal signal peptide.

The identification and characterization of new extracytoplasmic proteins and of the molecular architecture of cell surface in bacteria from the *Rhizobiales* order can help the understanding of the molecular basis involved in their interactions with the host plant at several stages of the nodulation.

2.3.1 The phage display strategy to identify new extracitoplasmic proteins

The strategy more commonly used to identify extracytoplasmic proteins is the construction of fused proteins with specific export “reporter” protein, as alkaline phosphatase (PhoA) (Manoil *et al.*, 1990) and TEM β-lactamase (BlaM) (Broome-Smith *et al.*, 1990) of *E. coli*. Nevertheless, it has been shown that the “phage display” technique can be used to identify genes coding for signal peptides (Rosander *et al.*, 2002). Phage display is usually used for identification of proteins and peptides with affinity to other molecules. Steiner *et al.* (2006) recently found that selection of signal sequences engaging the Signal Recognition Particle (SRP) pathway expand the range of proteins that can be efficiently displayed on filamentous bacteriophage.

Generally systems based on the bacteriophage M13 are used for phage display experiments (Smith and Petrenko, 1997). M13 is a filamentous phage containing single strand DNA. It infects *E. coli* and replicates without killing the host. The mature particles of M13 are released from the host cell by a budding process, and it is possible to obtain cultures to provide a continuous source of phage DNA. In order to use M13 for cloning, a double strand DNA is necessary, as the restriction enzymes only work on double strand DNA. Rosander *et al.* (2002 and 2003) have constructed hybrids vectors from a filamentous phage M13 and a plasmid. Such vectors are named phagemid and contain the origins of replication of the phage as well as the plasmid. Normally the replication depends on the origin of replication of the

plasmid but when a cell containing a phagemid is infected with a wild type phage, the phage origin of replication is responsible for the replication and single strand DNA copies are generated.

The phage is continuously assembled and released from the bacterial membrane (Russel *et al.*, 1997). The protein III first crosses the membrane where proteins are inserted in this membrane. The intracellular circular DNA is covered by dimers of another phage protein, these dimers are displaced by proteins of the envelope while the DNA crosses the cytoplasmic membrane. As the minor protein of the phage capside (protein III) is directed to the inner membrane by its signal peptide. A display library made in a phagemid vector lacking the signal peptide for protein III is an easy and efficient way for the selection of genes encoding signal peptides (Figure 1.2 and 1.3), as recombinant fusion proteins are displayed on the phage surface only if a functional signal peptide is provided by the inserted DNA sequence (Rosander *et al.*, 2002 and 2003). In addition, the N-terminal half of protein III has been replaced by a short linker region (the E-tag) that is recognized by a monoclonal antibody, which enables the isolation of the phage displaying a fusion protein (Figure 1.3). In the work of Rosander *et al.* (2003), this novel display method has been applied on the genome of *B. japonicum* and it has been shown that it can be used for an efficient analysis of signal peptide dependent secretion by the creation of selective gene library. Among the clones isolated from this library, it has been estimated that about 90% encoded extracytoplasmic proteins (Rosander *et al.*, 2003).

Figure 1.2. Schematic presentation of vector pG3DSS (adapted from Rosander *et al.*, 2003). The unique *Sna*BI restriction site was used for the insertion of *B. japonicum* DNA fragments. The promotor for expression of the insert is from the *spa* gene derived from *Staphylococcus aureus*.

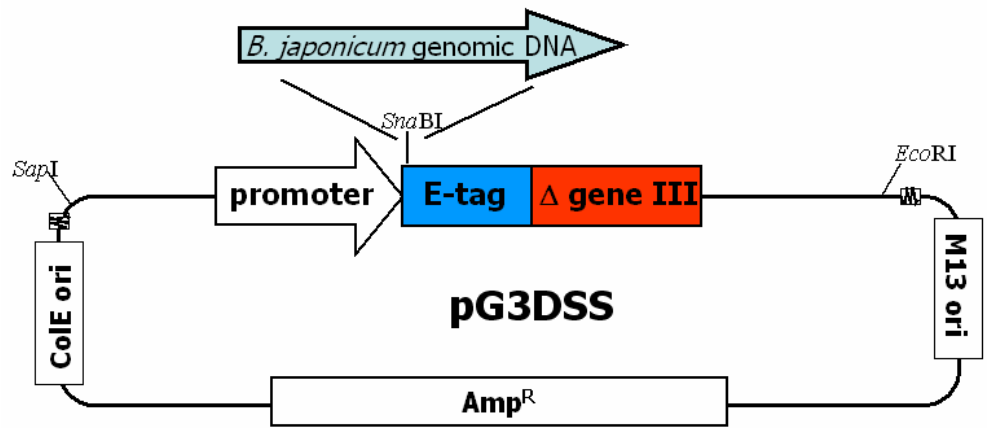
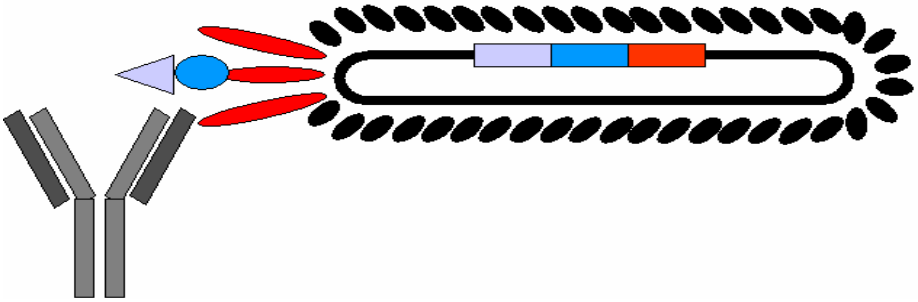


Figure 1.3. Schematic presentation of phage display (adapted from Rosander *et al.*, 2003). The truncated protein III is a part of the phage coat. The E-tag linker and partial *B. japonicum* protein will be displayed on the phage surface and recombinant phagemid particles can be isolated by affinity-selection against immobilized monoclonal anti-E-tag antibodies.



3. Periplasmic proteins

3.1. Periplasm

The periplasmic space is an important cellular compartment. This space is supposed to make around 10% of the bacterial volume, and it contains a substance similar to a gel solution of oligosaccharides, proteins and ions (Ferguson, 1992; Beveridge, 1995; Castillo *et al.*, 2000). By electronic microscopy techniques, cryofixation and cryosubstitution**,** it has been estimated that its thickness varies between 11 and 25 nm and that its composition of solutes is similar to the cytoplasm. In the periplasm a high quantity of proteins is present: proteins implicated in electron transport (cytochromes *c* and *d*, copperproteins (NirK, Nos, Pseudoazurins), formate dehydrogenases, methylamine reductases, nitrate reductases, etc.), proteins binding different substrates (sugars, amino acids, nitrate, molybdate, etc.) and some biosynthetic, degrading and detoxifying enzymes (Moreno-Vivian *et al.*, 2003). On the other hand, there are proteins integrated in the cytoplasmatic membrane with one or various structural domains exposed towards the periplasm. The periplasmic space is a specific prokaryotic cellular compartment where numerous metabolic and regulatory processes take place. Some of them are very important for the balance of gas emmissions to the biosphere, for energy transduction or for interconvertions of the different carbon, nitrogen, sulfure or metabolic compounds involved in the global biogeochemical cycles (Ferguson, 1992;

Beveridge, 1995; Castillo *et al.*, 2000). The importance of periplasm in biogeochemical cycles has been reviewed by Moreno-Vivian *et al*. (2003).

3.2. Periplasm and the biogeochemical nitrogen cycle

3.2.1. The biogeochemical nitrogen cycle

All living cells require nitrogen for the synthesis of many of their biomolecules. The assimilation of nitrogen occurs via the incorporation of the ammonium salt. In Nature, however, nitrogen is present in many more oxidation states. The biologically most important compounds are nitrate, nitrite, nitric oxide, nitrous oxide, dinitrogen and ammonium. The nitrogen cycle involves a number of redox processes which are shematically represented in figure 1.4. Together these processes drive the global nitrogen cycle and ensure a balanced recycling of the nitrogen compounds. The nitrogen cycle constitutes an oxidative phase, the conversion of ammonium to nitrate, and a reductive phase, the conversion of nitrate back to ammonium (Figure 1.4).

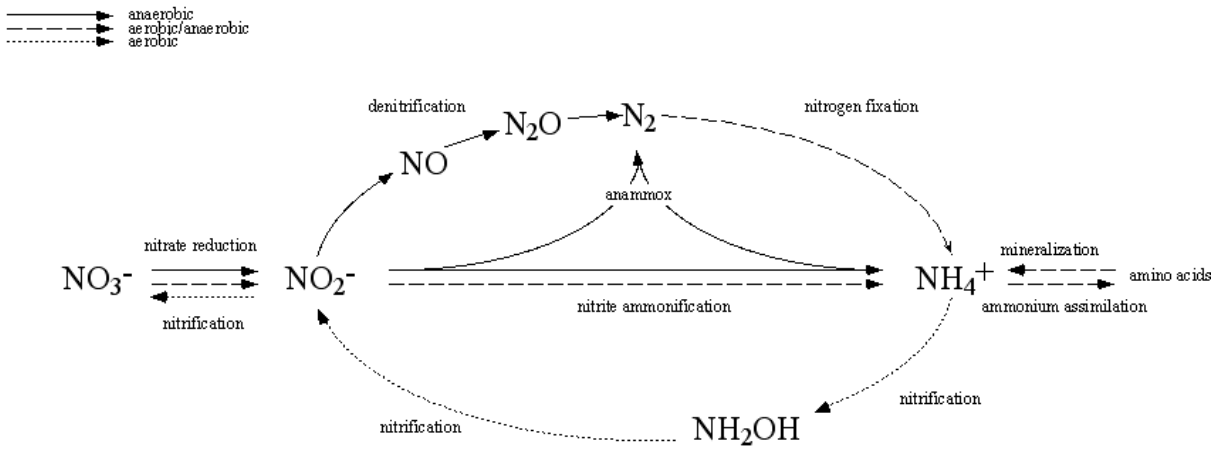
In the reductive assimilatory process, nitrate is reduced to ammonium which is incorporated into central metabolism through the combined action of glutamine synthetase and glutamate synthase or through glutamate dehydrogenase. The reductive dissimilatory processes involve the conversion of nitrate (NO_3^-) into nitrite (NO_2^-) (respiration) or into ammonia (NH_4^+) (respiration/ammonification). Both respiration and ammonification are energy conserving and can be used as an electron sink. In addition ammonification may play an important role in cell detoxification.

The oxidation of ammonium to nitrate is achieved by the sequential activities of ammonia oxidizers, the nitrosifiers, which oxidize ammonia to nitrite, and the nitrite oxidizers, the nitrifiers, which oxidize nitrite to nitrate. Together these processes make up the nitrification pathway (Figure 1.4). Ammonia and nitrite oxidizers have an important role in the conversion of nitrogenous compounds in their natural environment (e.g. soils, sediments, and lakes) and in wastewater treatment systems. These habitats are very dynamic especially with regard to the availability of oxygen. As a result of biological, climatological, or mechanical factors, the concentration of oxygen is constantly fluctuating and requires subtle adaptive responses of its inhabitants to survive in the numerous microhabitats present in these environments. The anaerobic oxidation of ammonium is catalyzed exclusively by some

members of the Planctomycetes like *Brocadia anammoxidans*. This process is called anammox (anoxic ammonia oxidation). The oxidation of ammonium in this process is coupled to the reduction of nitrite, and yields molecular dinitrogen and free energy for maintenance and growth. The biochemistry of the anammox process is still under investigation, but nitrite appears to be an electron acceptor, and hydrazine an intermediate (Strous *et al.*, 1999; Van de Graaf *et al.*, 1995). The anammox process is of great ecological importance and allows the removal of ammonium from anaerobic sites in natural environments and in wastewater treatment plants.

The fixation of atmospheric nitrogen is in part achieved by the chemical reaction of dinitrogen and oxygen induced by lightning, which gives rise to nitric oxide (NO). In the oxygen-rich atmosphere, nitric oxide is then oxidized to nitrogen dioxide and taken up in the oceans in the form of nitrate ions. Biological nitrogen fixation into ammonium as carried out by certain bacteria, however, is much more efficient and makes most of the nitrogen available to all living cells.

Figure 1.4. The biogeochemical nitrogen cycle (adapted from van Spanning *et al.*, 2003).



3.2.2. Denitrification

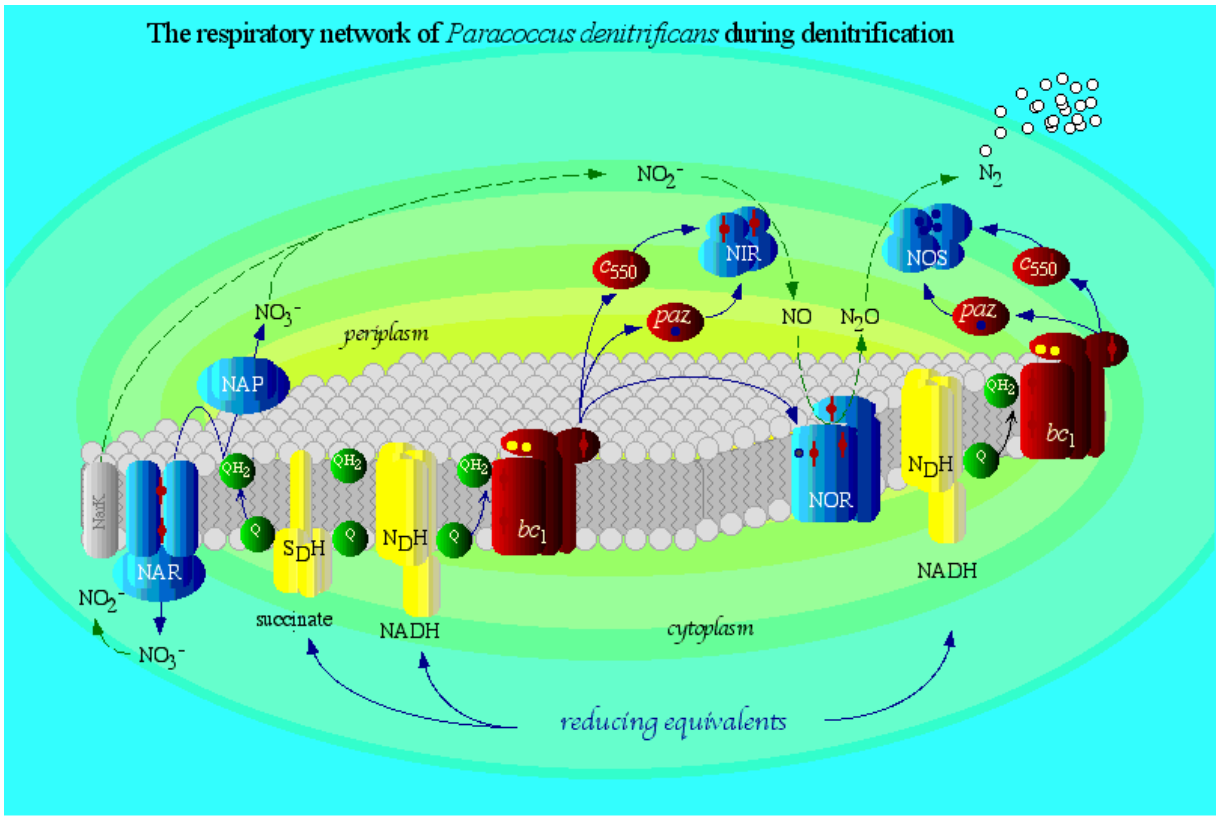
Denitrification, one of the main branches of the global nitrogen cycle, is an energy-yielding process in which microorganisms utilise nitrate as terminal respiratory electron acceptor under oxygen limited conditions. The overall reaction sequence employs the following intermediates in a pathway where nitrate is reduced via nitrite to gaseous products:

$$\text{Nitrate} \rightarrow \text{Nitrite} \rightarrow \text{Nitric oxide} \rightarrow \text{Nitrous oxide} \rightarrow \text{Dinitrogen}$$

Denitrification is also called nitrate respiration or dissimilatory nitrate reduction, where these terms stress different physiological roles of the process. For many years it was believed to be performed exclusively by eubacteria. However, there are indications that some fungi (e.g. the pathogenic species *Fusarium oxysporum*) and archaea are also able to denitrify. Bacteria of many different systematic groups can perform denitrification. However, some microorganisms can reduce nitrate only to nitrite and others only to nitrous oxide.

Products of denitrification have manifold, mainly adverse, effects on the atmosphere, soils and waters and thus have both agronomic and environmental impact. When nitrate is converted to gaseous nitrogen by denitrifying bacteria in agricultural soils, nitrogen is lost as an essential nutrient for the growth of plants. In contrast to ammonium, which is tightly bound in soil, nitrate is easily washed out and flows to the groundwater where it (and its reduction product nitrite) adversely affects water quality. In addition, nitrogenous oxides released from soils and waters are in part responsible for the depletion of the ozone layer above the Antartic, and in part for the initiation of acid rain and global warming. Thus the impact of products of denitrification, both in soils, waters and the atmosphere is of extreme relevance for human welfare and makes a detailed knowledge of this process essential.

Figure 1.5. Scheme of a full denitrification process in *Paracoccus denitrificans* (adapted from van Spanning *et al.*, 2003). Dashed arrows, N-oxide transport; straight arrows, electron transport. SDH, succinate dehydrogenase; NDH, NADH dehydrogenase; Q, quinone; bc_1 cytochrome bc_1 complex; c_{550}, cytochrome *c*; paz, pseudoazurin; NAR, membrane bound nitrate reductase; NAP, periplasmic nitrate reductase; NIR, cd_1-type nitrite reductase; NOR, *bc*-type NO reductase; NOS, nitrous oxide reductase.



The single steps of the denitrification process are catalysed by specific reductases which are located in the cytoplasmic membrane or in the periplasm (Figure 1.5). Comprehensive reviews covering the physiology, biochemistry and molecular genetics of denitrification have been published elsewhere (Baker *et al.*, 1998; Hendriks *et al.*, 2000; Richardson *et al.*, 2001; Richardson and Watmough, 1999; Watmough *et al.*, 1999; Zumft, 1997).

3.2.2.1. Respiratory nitrate reductases

The first reaction, the conversion of nitrate to nitrite, is catalysed by a Mo-containing nitrate reductase. Two types of dissimilatory nitrate reductases have been found in denitrifying bacteria. One is known as the respiratory membrane-bound nitrate reductase, and the other is the periplasmic nitrate reductase (Nap) (Moreno-Vivián and Ferguson, 1998; Moreno-Vivián *et al.*, 1999; Richardson and Watmough, 1999; Richardson *et al.*, 2001; Potter *et al.*, 2001). Membrane-bound nitrate reductase (Nar enzyme) employs a redox loop to couple quinol oxidation with proton translocation and energy conservation, which permits cell growth under oxygen limited conditions. Periplasmic nitrate reductase (Nap enzyme) also oxidizes quinol, but this enzyme is thought to participate indirectly in nitrate respiration by

functioning in an electron-transport chain with a proton-translocating enzyme, such as NADH dehydrogenase I (Berks *et al.*, 1995; Moreno-Vivián and Ferguson 1998; Richardson, 2000).

The *E. coli* and *Paracoccus* Nar enzymes have been the focus of the most biochemical and genetic studies (review in references). Nar is a 3-subunit enzyme composed of NarGHI. NarG, the alpha-subunit of about 140 kDa, contains the bis-MGD molybdopterin cofactor at its catalytic site and a [4Fe-4S] cluster. NarH, the beta-subunit of about 60 kDa contains four additional iron-sulphur centres: one [3Fe-4S] and three [4Fe-4S]. NarG and NarH are located in the cytoplasm and associate with NarI, the gamma subunit. NarI is an integral membrane protein of about 25 kDa with 5 transmembrane helices and the N-terminus facing the periplasm. Nar proteins are encoded by genes of a *narGHJI* operon. The organization of this operon is conserved in most species that express Nar. The *narGHI* genes encode the structural subunits, and *narJ* encodes a dedicated chaperone required for the proper maturation and membrane insertion of Nar. *E. coli* has a functional duplicate of the *narGHJI* operon, *narZYWV*. The subunits of the two enzymes are interexchangeable.

Nap is widespread in all classes of denitrifying and non-denitrifying proteobacteria but has not yet been found in other phyla of the bacterial superkingdom (Richardson, 2000). The best-studied Nap enzymes were isolated from *Pa. denitrificans* (Sears *et al.*, 1995), *Paracoccus pantotrophus* (Bell *et al.*, 1993; Berks *et al.*, 1994), *E. coli* (Grove *et al.*, 1996), *Rhodobacter sphaeroides* (Richardson *et al.*, 1990), *Ralstonia eutropha* (Siddiqui *et al.*, 1993) and *Pseudomonas putida* (Carter *et al.*, 1995). Nap is a 3-subunit enzyme composed of NapABC. The NapAB complex is located in the periplasm and associates with a transmembrane NapC component (see Section 3.2.3).

3.2.2.2. Nitrite reductases

The next step in the denitrification pathway is the reduction of nitrite to nitric oxide catalysed by nitrite reductase. There are two types of respiratory nitrite reductase characterized in denitrifying bacteria, a homodimeric enzyme with haems *c* and d_1 (NirS, cd_1-type; 120 kDa in its dimeric form), and a homotrimeric enzyme with copper atoms (NirK, copper-type; 3 x 36 kDa). Both are periplasmic proteins and receive electrons from cytochrome *c* and/or a blue copper protein, pseudoazurin (Koutny *et al.*, 1999; Moir and Ferguson, 1994). Neither of the enzymes is electrogenic since both take up the electrons and protons required for nitrite reduction at the same side of the membrane, i. e. the periplasm.

NirK and NirS are never found together in a single species. The structural and functional characteristics of both enzymes have recently been reviewed (Cutruzzola, 1999; Watmough *et al.*, 1999). In contrast to the complex organization of the genes encoding the cd_1-type Nir, e.g. *nirSTBMCFDLGH* in *Pseudomonas stutzeri*, a single gene, *nirK*, is responsible for the synthesis of Cu-Nir in other denitrifiers.

3.2.2.3. Nitric oxide reductases

Since nitric oxide is toxic and highly reactive, it is effectively utilized by Nor making sure that the free nitric oxide-concentration in denitrifying organisms is kept in the nanomolar range. Two types of Nor enzymes have been identified in denitrifying species: one type that receives electrons from cytochrome *c* or pseudoazurin, referred to as cNor, and another type that receives electrons from quinol, referred to as qNor (Zumft 1997, Wautmouth 1999, Hendriks *et al.*, 2000). Both types are structurally related integral membrane proteins, which catalyse the two-electron reduction of two nitric oxide molecules to nitrous oxide. Nitric oxide reductase of the cNor-type is a membrane bound enzyme which contains two subunits, a small one (17 kDa) containing haem *c* and a larger one (53 kDa) with haems *b* and a non-haem iron. The qNor-type nitric oxide reductase is encoded by a single *norB* gene, has 14 putative transmembrane helices and has been purified from *Ra. eutropha* where it has been shown that it consists of a single subunit of 75 kDa containing both high-spin and low-spin haem *b* and a ferric non-haem iron (Cramm *et al.*, 1997).

3.2.2.4. Nitrous oxide reductase

The last step in denitrification is the two-electron reduction of nitrous oxide to dinitrogen gas. This reaction is carried out by a nitrous oxide reductase (Nos), which is a homodimeric soluble protein located in the periplasmic space. The enzyme has been purified from a large number of denitrifying strains, including *Pa. denitrificans* (Snyder and Hollocher, 1987), *Pa. pantotrophus* (Berks *et al.*, 1993) and *Ps. stutzeri* (Coyle *et al.*, 1985). Nos is a homo-dimer of a 65 kDa copper-containing subunit that binds a mixed valence di-nuclear Cu_A electron entry site and a tetra-nuclear Cu_Z catalytic centre. Each monomer is made up of two domains the 'Cu_A domain' that has a cupredoxin fold and the 'Cu_z domain', which is a seven-bladed propeller of beta-sheets. Electron input into Cu_A is usually via mono-haem *c*-type cytochromes or cupredoxins. The enzyme was first isolated from *P. stutzeri* and

the corresponding structural gene was designated *nosZ*. Additional genes required for associated regulatory and electron transfer components, and information for metal processing and protein assembly or maturation are encoded by the *nosRZDFYL tatE* operon (Honisch and Zumft, 2003).

3.2.3. Periplasmic nitrate reductase

The *nap* genes have been identified in several prokaryotic organisms (Figure 1.6.B) (Moreno-Vivian *et al.*, 2003; Richardson *et al.*, 2001; Potter *et al.*, 2001; Gónzalez *et al.*, 2006). In contrast with *nar* operon, the *nap* operons present heterogeneity in gene composition as well as in ordering. Nine different genes have been identified as components for operons that code Naps in different organisms. Except for *Shewanella oneidensis*, *Wollinella succinogenes*, *Desulfobacterum hafniensi* and *Campylobacter jejuni*, which lack *napB* or *napC*, all operons studied thus far have the *napABCD* genes in common (Figure 1.6.B).

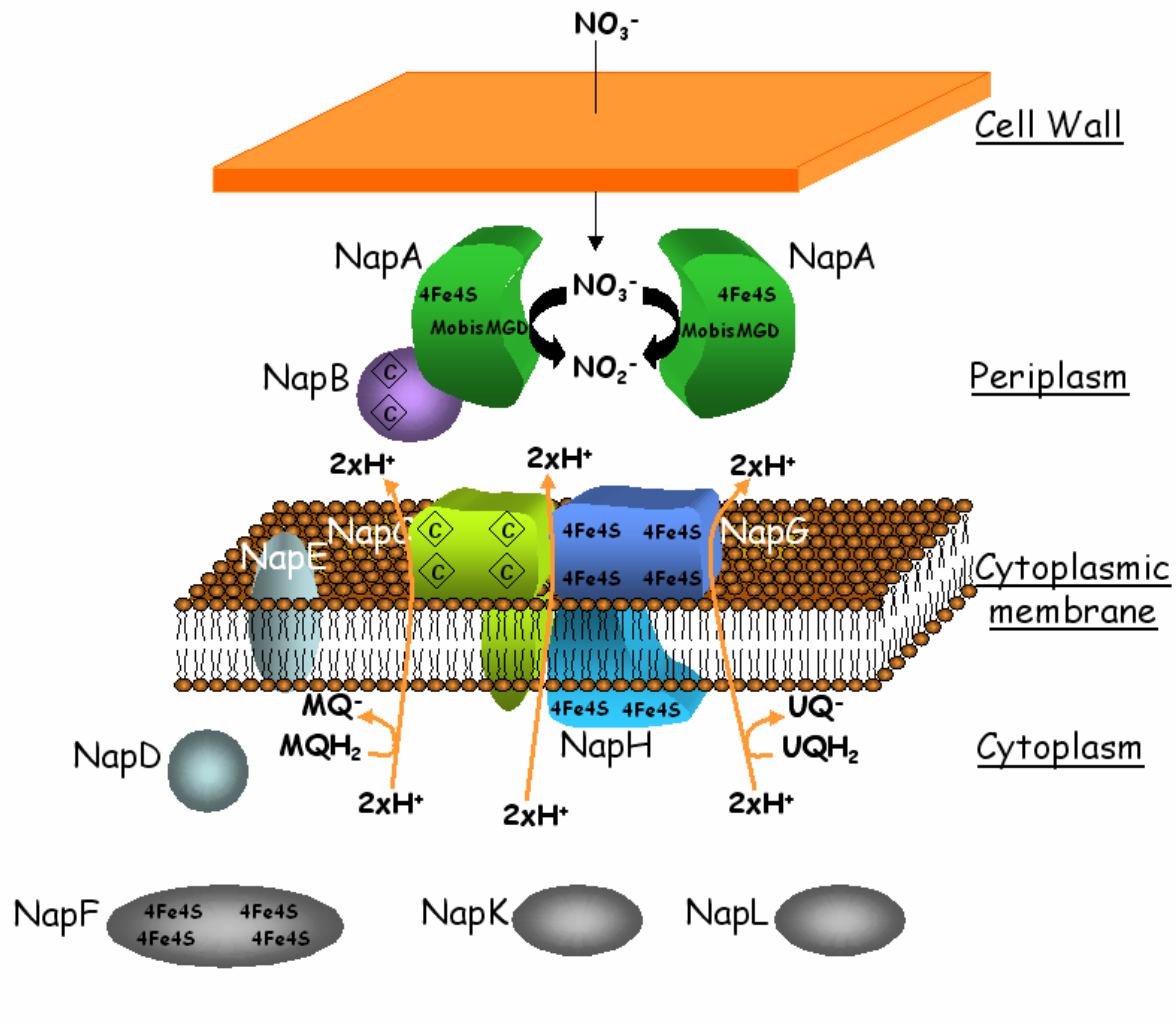
The *napA* gene codes the catalytic subunit NapA (about 90 kDa) that contains the Mo-*bis*MGD active site and a FeS center (Figure 1.6.A). The assembling of the apo-protein with the metal cofactors is carried out in the cytoplasm. Then, the folded holo-protein is transported to periplasm by the TAT (Twin Arginine Translocator) system by recognizing the signal-peptide present in NapA (Thomas *et al.*, 1999; Gonzalez *et al.*, 2006). However, this is not valid for all the catalytic subunits since although NapA from *Pseudomonas* G-179 is found in the periplasm, it lacks this signal peptide. Once in the periplasm, NapA and the product of *napB* gene are assembled to give the heterodimeric NapAB. NapB (16 kDa) is a dihaem cytochrome c_{552}, with both haems being bis-histidinyl ligated, this protein is secreted into periplasm by the general secretory pathway (Figure 1.6.A) (Pugsley,1993). Almost all the *nap* operons code the protein NapC (Figure 1.6.B), which is a *c*-type tetra-haem membrane-anchored protein (25 kDa) belonging to the NapC/NirT family (Roldan *et al.*, 1998; Cartron *et al.*, 2002; Zajicek *et al.*, 2004). This protein is involved in the electron transfer from the quinol pool to periplasmic reductase. When NapC is not coded in the *nap* operon, its function is substituted by another protein of the NapC/NirT family which is coded out of the operon and is expressed under denitrifying conditions. Furthermore, some operons code the ferredoxins NapG and NapH. NapG is a periplasmic protein having 4x [4Fe–4S] clusters. NapH is an integral membrane protein with 2 x [4Fe–4S] exposed to the cytoplasm (Brondijk *et al.*, 2002 and 2004) (Figure 1.6A).

The remaining genes, *napD*, *napE*, *napF*, *napK* and *napL* code for different proteins that are not directly involved in the nitrate reduction. NapD is a cytoplasmic protein that belongs to the TorD family, which act as chaperones and are always present in operons that encodes molybdo-enzymes (Ilbert *et al.*, 2004). NapF is a cytoplasmic iron–sulfur containing protein with four loosely bound [4Fe–4S] clusters, which is thought to participate in the assembling of the iron–sulfur cluster of NapA (Brondijk *et al.*, 2002; Olmo-Mira *et al.*, 2004). The other genes code for proteins with so far unknown functions.

The role of Nap and the regulation of *nap* gene expression, however, varies among bacteria. In *Haemophilus influenzae* (Brigé *et al.*, 2001), and the denitrifying bacteria *Rh. sphaeroides* f. sp. *denitrificans (*Liu *et al.*, 1999*),* and *Pseudomonas* sp. strain G-179 (Bedzyk *et al.*, 1999), Nap is the only nitrate reductase that has a role in supporting anaerobic growth in the presence of nitrate. *E. coli* possesses a Nap enzyme in addition to two Nar isozymes. Expression of the *nap* genes of *E. coli* is induced by anaerobiosis and only at low nitrate concentrations, suggesting the role of Nap in anaerobic respiration in nitrate-limited environments (Potter *et al.*, 1999; Stewart *et al.*, 2002; Brondijk *et al.*, 2004). On the other hand, Nap is synthesized during aerobic growth, in the denitrifying bacterium *Pa. pantotrophus* (formerly *Thiosphaera pantotropha*) (Richardson and Ferguson, 1992), irrespective of whether nitrate is added. *Pa. pantotrophus* contains the Nap enzyme in addition to Nar and Nas, as in *Ra. eutropha* (formerly *Alcaligenes eutrophus*) (Siddiqui *et al.*, 1993; Sears *et al.*, 1997), and is responsible for initiating aerobic denitrification (Bell *et al.*, 1990). Remarkably, the expression of the *nap* operon of *Pa. pantotrophus* is negatively regulated during anaerobiosis such that the expression is restricted to aerobic growth, but only when the organism is grown on a highly reduced carbon source such as butyrate (Ellington *et al.*, 2002). Thus, aerobic nitrate reduction by Nap is considered to function at least in part to dispose of excess reductant (Richardson, 2000; Sears *et al.*, 2000). An analogous role of Nap in redox balancing during both anaerobic photosynthesis and aerobiosis has been suggested in phototrophic bacteria such as *Rhodobacter capsulatus* and *Rh. sphaeroides* DSM158 (Richardson, 2000; Gavira *et al.*, 2002). They have the ability to express Nap in the presence of nitrate under either oxic or anoxic conditions.

Figure 1.6. (A) Dissimilatory nitrate reduction by Nap enzymes. (B) Gene organization of the *nap* gene clusters. Adapted from Gonzalez *et al.* (2006).

A



B

Escherichia coli K12	*napFDAGHBC*
Rhodobacter sphaeroides	*napKEFDABC*
Paracoccus pantotrophus	*napEDABC*
Wautersia eutropha	*napEDABC*
Bradyrhizobium japonicum	*napEDABC*
Pseudomonas G-179	*napEFDABC*
Campylobacter jejunii	*napAGHBLD*
Wollinella succinogenes	*napAGHBFLD*
Haemophylus influenzae	*napFDAGHBC*
Shewanella oneidensis MR1	*napDAGHB*
Desulfitobacterium hafniense	*napDGAH*

3.2.3.1. Regulation of *nap* expression by low oxygen, nitrate and nitrite.

The O_2 sensor involved in expression of *nap* genes under microaerobic conditions is Fnr (Fumarate nitrate reduction). Fnr is a single component, oxygen sensitive protein that belongs to an expanding family of Crp/FNR like transcriptional activators. All these regulators contain a signal sensing domain, a dimerization domain, a helix-turn-helix DNA-binding domain, and up to three sites that are involved in contacting RNA polymerase. One such site is a surface exposed loop that contacts the sigma factor of RNA-polymerase (Williams *et al.*, 1991; Williams *et al.*, 1996). Crp-type proteins have conserved residues in their sensing domain for reversible binding of cAMP. Once bound, the monomers dimerize and bind to conserved sequences of their target promoters. In type II promoters, this site is at a position directly upstream of the site where RNA-polymerase has its binding site (Busby and Ebright, 1997). Fnr of *E. coli* contains a sensory domain with 4 conserved cysteine residues that ligate an oxygen sensitive [4Fe-4S] cluster. This cluster disintegrates by direct interaction with oxygen making Fnr inactive. Once the oxygen concentration drops below threshold levels, the cluster is assembled. This event triggers a conformational change, resulting in dimerization of Fnr. The dimer binds to a conserved target sequence TTGAT-N4-ATCAA (Fnr-box) directly upstream of the RNA-polymerase binding site to facilitate contact with RNA-polymerase. Just like for Crp, this contact stabilizes the transcription initiation complex and promotes transcription of the target promoters (Green *et al.*, 2001; Kiley and Beinert, 1998). Under anaerobic conditions, nitric oxide reversibly inactivates FNR, which is the result of nitric oxide-binding to the [4Fe-4S] cluster and formation of a dinitrosyl-iron-cysteine complex (Cruz-Ramos *et al.*, 2002). Homologues of Fnr are widespread in Nature and have been encountered in a variety of prokaryotes that have to cope with changes in oxygen availability, including pathogens. Only some of these protein members have cysteine signatures diagnostic for binding an oxygen sensitive [4Fe-4S] cluster. Most of them lack the cluster, indicating that these Fnr-homologues respond to signals other than oxygen.

Nitrate/nitrite regulation of *nap* genes is mediated by proteins NarXL, NarQP and NarR. NarXL and NarQP are members of two component regulatory systems. The NarX and NarQ proteins are the signal sensors, which both respond to nitrate and nitrite although with different affinities. NarX is more specific for nitrate and NarQ for nitrite (Rabin and Stewart, 1993). The NarL and NarP proteins are their cognate response regulators, respectively. In *E. coli*, they bind DNA to control induction of the *nar* and *nap* operon and repression of genes

encoding alternate anaerobic respiratory enzymes (Stewart, 1993; Darwin *et al*, 1998). The NarX and NarQ proteins communicate with both the NarP and NarL proteins in *E. coli*. The *narXL* and *narQP* genes from this bacterium are found upstream the *narGHJI* and *narZYWV* operons, respectively. Denitrifiers from the beta and gamma proteobacteria have genes encoding orthologues of NarXL also located up- or downstream their *narGHJI* operons. Their products regulate a nitrate-induced expression of Nar. Expression of the *Neisseria nir* and *nor* genes are subjected to a nitrite response mediated by a NarQP couple, the genes of which are separate from the *nir* and *nor* genes (Lissenden *et al.*, 2000). NarR is a member of the FNR family of transcriptional activators, but it lacks the cysteines to incorporate a [4Fe-4S] cluster. NarR of *Pa. pantotrophus* and *Pa. denitrificans* is specifically required for transcription of the *narKGHJI* genes in response to increasing concentrations of nitrite. The mechanism of the nitrite response is not clear, but it is notable that NarR can also be activated by azide, which normally binds to metal centres, raising the possibility that NarR is a metalloprotein (Wood *et al.*, 2001; Wood, 2002). Genes encoding NarR are found in the alpha proteobacteria *Brucella suis*, *Brucella melitensis*, *Pa. denitrificans* and *Pa. pantotrophus* upstream their *narGHJI* gene clusters. There are no indications that they have counterparts of *narXL*. It therefore seems that NarR substitutes for the NarXL system in the alpha proteobacteria.

3.3. Denitrification in bacteria of the *Rhizobiales* order

In recent years it has emerged that many rhizobia species have genes for enzymes for some or all of the four reductases reactions for denitrification. In fact, denitrification can be readily observed in many rhizobia species, in their free-living form, in legume root nodules, or as isolated bacteroids (O'Hara *et al.*, 1985).

Although the ability to denitrify may enhance bacterial survival and growth capability in anaerobic soils, denitrification among rhizobia is rare, and only *B. japonicum* and *Azorhizobium caulinodans* have been shown to be true denitrifiers, this is, to reduce nitrate simultaneously both to ammonia (assimilation) and nitrogen (denitrification) when cultured microaerobically with nitrate as not only the terminal electron acceptor but also the sole source of nitrogen. Other soil microorganisms have been shown to possess higher rates of denitrification activity than rhizobia (Garcia-Plazaola *et al.*, 1993), but the vast area all over the world of cultivated legumes makes contribution of rhizobia to total denitrification highly significant.

Denitrification genes encoding either periplasmic nitrate reductase (Nap), Cu-containing nitrite reductase (Nir), nitric oxide reductase (Nor), or nitrous oxide reductase (Nos), have been identified in several rhizobia species such as *Rhizobium sullae* (formerly *Rhizobium hedysari*) (Toffanin *et al.*, 1996), *Rhizobium etli* (Bueno *et al.*, 2005), *S. meliloti* (Holloway *et al.*, 1996; Galibert *et al.*, 2001), and *B. japonicum* (Kaneko *et al.*, 2002; Bedmar *et al.*, 2005) (Table 1.1). None of the denitrification genes have been detected in *M. loti* strain MAFF 303090 (http://www.kazusa.or.jp/rhizobase).

Table 1.1. Denitrification genes in nitrogen-fixing endosymbiotic rhizobia

Species	**Genes**	**Reference**
R. sullae	*NirK*	Toffanin *et al.*, 1996
R. etli	*nirK, norCBQD*	Bueno *et al.*, 2005
S. meliloti	*napEFDABC, nirK, norCBQD, nosRZDFYLX*	Holloway *et al.*, 1996 Galibert *et al.*, 2001
B. japonicum	*napEDABC,* *nirK,* *norCBQD,* *nosRZDFYLX*	This work, Velasco *et al.*, 2001 Mesa *et al.*, 2002 Velasco *et al.*, 2004

R. sullae, induces nodule formation on *Hedysarum coronarium*. *R. sullae* strain HCNT1 has been shown to contain a Cu-containing Nir encoded by a *nirK* gene. Expression of *nirK* is atypical in that it does not require the presence of a nitrogen oxide, but only a decrease in oxygen concentration (Casella *et al.*, 1986). Reduction of nitrite by strain HCNT1 results in inhibition of growth due to the accumulation of nitric oxide to toxic levels, suggesting that *R. sullae* does not contain any Nor (Casella *et al.*, 1986). Nodulation, plant growth and rates of nitrogen fixation are similar between wild type and *nirK*-deficient strains.

R. etli forms N_2-fixing symbiosis with *Phaseolus vulgaris*. Studies of genome structure and sequence analyses in the type strain *R. etli* CFN42 permitted the identification of genes encoding proteins closely related to denitrification enzymes, among them the *norCBQD* genes coding for the cytochrome *c*-type Nor and the *nirK* gene encoding a Cu-containing Nir. Strain CFN42 lacks genes coding for Nap and Nos. In fact, *R. etli* CFN42 is unable to grow under

denitrifying conditions with nitrate and does not express nitrate reductase activity (Bueno *et al.*, 2005). However, *R. etli* CFN42 is capable of growth with nitrite as a terminal electron acceptor. Mutational analysis has demonstrated that a *nirK* deficient mutant is unable to grow under nitrite-respiring conditions, and that microaerobic growth of the mutant was inhibited in the presence of nitrite. Since Nir activity and nitrite-uptake were very low in the *nirK* mutant, it is possible that the Cu-containing Nir may have both a respiratory and a nitrite detoxifying role in *R. etli* (Bueno *et al.*, 2005).

DNA sequences showing homology with those published coding for the rhizobial *nap*, *nir*, *nor* and *nos* denitrification genes have been found in the symbiotic plasmid pSymA complete genome sequence of *S. meliloti* strain 1021 (http://www.kazusa.or.jp/rhizobase). Intriguingly, despite possessing the complete set of denitrification genes, *S. meliloti* 1021 does not grow under oxygen-limiting conditions with either nitrate or nitrite as terminal electron acceptors. None of the Tn*5* insertions in the *S. meliloti* strain JJ1c10 *nos* region affected nitrogen-fixing ability in symbiosis with alfalfa, which demonstrated that denitrification is not essential for nitrogen fixation (Holloway *et al.*, 1996).

3.3.1. Denitrification in *B. japonicum*

B. japonicum, the N_2-fixing microsymbiont of soybeans, is a slow growing, true denitrifying rhizobia that has been shown to reduce ^{15}nitrate simultaneously to ammonia and nitrogen when cultured under oxygen-limiting conditions. In this bacterium, denitrification *nirK* (Velasco *et al.*, 2001), *norCBQD* (Mesa *et al.*, 2002), and *nosRZDFYLX* (Velasco *et al.*, 2004) genes encoding, nitrite-, nitric oxide- and nitrous oxide-reductase, respectively have been identify and characterized.

3.3.1.1. *nirK* gene

In *B. japonicum*, a *nirK* gene was identified whose deduced primary sequence has greater than 68% identity with translated sequences of *nirK* genes from other denitrifiers (Velasco *et al.*, 2001). NirK is soluble and apparently exported to the periplasm via the Tat translocon, as judged by a twin-arginine motif present in its N terminus sequence. Implication of *B. japonicum nirK* in denitrification was obtained when *nirK* mutants were shown to be

unable to grow when cultured under microaerobic conditions in the presence of either nitrate or nitrite.

3.3.1.2. *norCBQD* genes

In *B. japonicum*, the *nor* genes are organized in the *norCBQDE* gene cluster, in which *norC* and *norB* encode the cytochrome *c*-containing subunit II and cytochrome *b*-containing subunit I of cNor, respectively; *norQ* encodes a protein with an ATP/GTP-binding motif, and the predicted *norD* gene product is of unknown fuction (Mesa *et al.*, 2002). Sequence analysis of the *norCBQD* genes revealed that *norB* has sufficient sequence similarity to terminal oxidases to include cNor within the family of haem-copper oxidases (Hendriks *et al.*, 2000 and references therein). Inspection of the complete genome sequence of *B. japonicum* shows the existence of an open reading frame, blr3212, whose sequence has more than 60% identity with *norE* genes from various denitrifiers. The NorE protein has also similarity to CoxIII, the third subunit of the aa_3-type cytochrome *c* oxidases.

Mutational analysis indicated that the two structural *norC* and *norB* genes are required for microaerobic growth under nitrate-respiring conditions. Each *norC* and *norB* insertional mutants accumulated nitric oxide during incubation under anaerobic conditions, and the *norC* mutant lacked the *c*-type cytochrome found in membranes from wild-type cells (Mesa *et al.*, 2002).

3.3.1.3. *nosRZDFYLX* genes

The *B. japonicum nos* genes were identified using a major internal portion of the *P. stutzeri nosZ* gene as a probe (Viebrock *et al.*, 1987), and found to be organized in the *nosRZDFYLX* gene cluster (Velasco *et al.*, 2004). *nosR* codes for an integral membrane regulatory protein with six transmembrane helices and a C-terminal cytoplasmic domain containing two cysteine clusters similar to the [4Fe-4S] binding motifs of several bacterial ferredoxins. *nosZ* encodes the monomers of NosZ, whose deduced primary sequence has between 50% and 77% identity with the translated sequences of the *nosZ* gene from other denitrifiers. In its N-terminus, a twin arginine motif indicates NosZ is exported via the Sec-independent Tat translocon. The NosZ sequence also contains the site signature believed to bind the copper metal ligand. The NosD, -F, -Y and L proteins show significant homology to the bacterial ATP-binding-cassette (ABC) transporter systems, which are tipically composed

of a cytoplasmic ATP/GTP binding protein (NosF), a transmembrane protein (NosY) and one or two periplasmic components (NosD and NosL).

B. japonicum strains carrying either a *nosZ* or a *nosR* mutation grew well when cultured microaerobically with nitrate as the final electron acceptor. Nevertheless, Nos was not an active enzyme because cells accumulated nitrous oxide during growth (Velasco *et al.,* 2004).

3.3.1.4. Regulation of denitrification genes in *B. japonicum*

Maximal expression of denitrification genes requires simultaneously both the absence of oxygen and the presence of nitrate or a derived N-oxide. Microaerobic induction of transcription from the *nir*, *nor* and *nos* promoter regions depends on the *fixLJ* and $fixK_2$ genes (Velasco *et al.*, 2001; Mesa *et al.*, 2002; Velasco *et al.*, 2004), whose products form the FixLJ/$FixK_2$ regulatory cascade (Nellen-Anthamatten *et al.*, 1998). FixLJ is a two-component regulatory system consisting of the heme-based sensor kinase FixL and the FixJ response regulator. The only known target of FixJ in *B. japonicum* is $fixK_2$ whose product encodes the Fnr- (fumarate and nitrate reductase regulator) and Crp- (cyclic AMP receptor) type transcriptional regulator $FixK_2$, which was also shown to activate genes involved in microaerobic and anaerobic metabolism (Nellen-Anthamatten *et al.*, 1998). Homologous sequences to the consensus DNA binding sites of the Fnr and FixK proteins (5´-TTGAT-N_4-ATCAA/GTCAA-3´, respectively (Zumft, 1997; Spiro, 1994; Fisher, 1994) are present in the promoter region of each denitrification gene.

In *B. japonicum*, the *nnrR* gene has been identified whose product, the NnrR protein, shares between 47% and 76% identity with other Fnr/FixK-type transcriptional activators (Mesa *et al.*, 2003). NnrR lacks in its N terminus the cysteine motif that is characteristic for redox-responsive Fnr-like proteins, and contains in the C terminus a predicted helix-turn-helix motif likely to be involved in DNA binding. In fact NnrR-like proteins, including *B.japonicum* NnrR, constitute a distinct cluster within the ID subgroup of the Fnr family of transcriptional regulators (Mesa *et al.*, 2003; Körner *et al.*, 2003). Mutant strains carrying an *nnrR* null mutation were unable to grow anaerobicolally in the presence of nitrate or nitrite, and they lacked both nitrate and nitrite reductase activities. Regulatory studies indicated that, in addition to microaerobiosis, nitrate, or an N-oxide derived from it, presumably either nitrous oxide or nitric oxide or both, are required for maximal induction of the *B. japonicum* denitrification genes. *nnrR* does not control its own expression and was not expressed in *fixLJ*

and *fixK*$_2$ mutants. A model compatible with these results has been proposed which places NnrR in the FixLJ-FixK$_2$ cascade downstream the FixK$_2$ (Mesa *et al.*, 2003).

3.3.1.5.. Denitrification in soybean nodules

Expression of *B. japonicum* wild-type strain USDA110 *nirK*, *norC* and *nosZ* denitrification genes in soybean root nodules has been reported by *in situ* histochemical detection of β-galactosidase activity due to transcriptional fusions of the *nirK*, *norC* and *nosZ* genes to the reporter gene *lacZ* (Mesa *et al.*, 2004). Similarly, isolated bacteroids also expressed the P$_{nirK}$-*lacZ*, P$_{norC}$-*lacZ*, and P$_{nosZ}$-*lacZ* fusions. Levels of β-galactosidase activity were similar in both bacteroids and nodule sections from plants that were solely N_2-dependent or grown in the presence of 4 mM KNO_3, which suggests that oxygen, and not nitrate, is the main factor controlling expression of denitrification genes in soybean nodules.

The symbiotic phenotype of *B. japonicum* strains carrying a mutation in either the *nirK*, *norC*, or *nosZ* strucural genes has also been reported in comparison with that of the wild-type strain. In soybean plants not amended with nitrate, *B. japonicum* mutants showed a wild-type phenotype with regard to nodule number and nodule dry weight as well as plant dry weight and nitrogen content. In the presence of 4 mM KNO_3, plants inoculated with either the *nirK* or the *norC* mutants showed fewer nodules, and lower plant dry weight and nitrogen content, relative to those of wild type and *nosZ* mutant strains. Thus, although not essential for nitrogen fixation, mutation of either the *nirK* or *norC* genes encoding respiratory Nir and Nor, respectively, confers on *B. japonicum* a reduced ability for nodulation in soybean plants grown with nitrate. Because nodules formed by the parental and mutant strains exhibited similar nitrogenase activities, it is possible that denitrification enzymes play a role in nodule formation rather than in nodule function (Mesa *et al.*, 2004).

OBJECTIVES

Members of the *Rhizobiales* order are known to interact with legume plants and to induce the formation of nodules. In addition to Nod factors, a variety of prokaryotic signals are known to control successive stages of the plant infection process. Amongst these, there are secreted proteins exported by type I or type III secretion systems. Although rhizobial extracitoplasmic proteins have been estimated to constitute around 36 % of the total amount of proteins, very little is known about their characterization and involvement in rhizobia-legume interaction.

In addition to participate in the symbiotic interaction with plants, the extracitoplasmic proteins can also be involved in other processes essential for free-living metabolism as respiratory electron transfer. Among these processes, denitrification, one of the main branches of the global nitrogen cycle, is an energy-yielding process in which microorganisms utilize nitrate as terminal respiratory electron acceptor under oxygen limited conditions. The overall reaction sequence employs the following intermediates in a pathway where nitrate is reduced via nitrite to gaseous products:

$$\text{Nitrate} \rightarrow \text{Nitrite} \rightarrow \text{Nitric oxide} \rightarrow \text{Nitrous oxide} \rightarrow \text{Dinitrogen}$$

The single steps of the denitrification process are catalysed by specific reductases which are bound to the membrane with their catalytic sites facing the periplasm or are located in the periplasm. *Bradyrhizobium japonicum,* the nitrogen-fixing microsymbiont of soybeans, is a slow growing, true denitrifying rhizobia that has been shown to reduce nitrate simultaneously to ammonia and nitrogen when cultured under oxygen-limiting conditions (Vairinhos *et al.*, 1989). In Eulogio Bedmar's Group it has been demonstrated that denitrification in *B. japonicum* depends on the *nirK* (Velasco *et al.* 2001), *norCBQD* (Mesa *et al.* 2002), and *nosRZDFYLX* (Velasco *et al.*, 2004) genes encoding nitrite-, nitric oxide- and nitrous oxide-reductase, respectively. However, the identification of the nitrate reductase involved in the first step of the denitrification process was unknown. In a random cloning and sequencing project from Dr. Peter Müller's Group, destined to identify genes coding preproteins with a N-terminal signal peptide, *B. japonicum napA* gene which encodes the catalytic subunit of the periplasmic nitrate reductase was identified. This extracytoplasmic, which has not been previously

described in *B. japonicum*, may be involved in nitrate respiration such it as been described in other bacteria. In order to establish the role of the *B. japonicum nap* genes in the denitrification process, one of the research topics of Bedmar's Group, in this Thesis and in collaboration with Dr. Peter Müller, the cloning, sequencing and characterization of the *B. japonicum nap* genes has been carried out.

A novel phage display method has been applied on the genome of *B. japonicum* by Dr. Müller´s Group to identify signal-peptide dependent secretion proteins (Rosander *et al.*, 2002, 2003). A phage display expression library of about 132 E-tag fusion clones was obtained and it provides a powerful means to select genes encoding extracytoplasmic proteins. In order to identify new extracytoplasmic proteins with important roles in rhizobia-legume interaction, in this Thesis some of the E-tag fusion clones obtained by Rosander *et al.* (2003) were selected and used to construct *B. japonicum* gene disruption mutants which were inoculated on soybean seedlings to test their symbiotic properties.

In summary, the main aim of this work is to identify new extracytoplasmic proteins in *B. japonicum* with potential role in free-living metabolism and in the symbiotic interaction with soybean plants. More specifically, the objectives of the research work carried out during this PhD have been:

1. Identification and sequencing of *nap* genes encoding the periplasmic nitrate reductase of *B. japonicum.*
2. Functional analysis of *nap* genes under free-living and symbiotic conditions.
3. Transcription analysis and regulation of *nap* genes.
4. Identification of new genes encoding extracytoplasmic proteins by using a phage display library, construction of *B. japonicum* mutants and analysis of their symbiotic properties.

The results described in this work have been presented in 7 international meetings, 3 national meetings and have been published in the following manuscripts:

Bedmar, E.J., Mesa, S., Velasco, L., Robles, E., Bonnard, N., Delgado, M.J.
Desnitrificación en *Bradyrhizobium japonicum*: un misterio resuelto.

In “Avances en el Metabolismo del Nitrógeno: de los Microorganismos a las Plantas”. Ed. Universidad de Huelva. 2006. Page 35 to 44.

Robles, E.F., Sanchez, C., Bonnard, N., Delgado, M.J. and Bedmar, E.J.
The *Bradyrhizobium japonicum napEDABC* genes are controlled by the FixLJ-FixK2-NnrR regulatory cascade.
Biochemical Society transactions. 2006. Volume 34. Page 108 to 110.

Müller, P., Bonnard, N.
Studying extracytoplasmic proteins of *Bradyrhizobium japonicum* by E-tag fusion clones obtained from phage display.
Endocytobiosis and Cell Research. 2004. Volume 15 (2). Page 527 to 537. (http://ecb.thulb.uni-jena.de/ecb/content/below/index.xml)

Delgado, M.J., Bonnard, N., Tresierra-Ayala, A., Bedmar, E. J. and Muller, P.
The *Bradyrhizobiun japonicum napEDABC* genes encoding the periplasmic nitrate reductase are essential for nitrate respiration.
Microbiology. 2003. Volume 149. Page 3395 to 3403.

2. MATERIAL AND METHODS

1. Microbiological techniques

1.1. Bacterial strains

The bacterial strains of *Escherichia coli* and *Bradyrhizobium japonicum*, and the plasmids used in this work together with their relevant characteristics are listed in Tables 2.1 and 2.2.

Table 2.1. Bacterial strains used.

Strain	**Relevant characteristics**	**Reference**
	E. coli	
$DH_{5\alpha}$	Host strain in standard cloning experiments; *supE44*, *ΔlacU169*, f80, *lacZΔM*, 5hsdR17, *recA1*, *endA1*, *gyrA96*, *thi1*, *relA1*	Stratagene, BRL®
S17-1	Donor strain in conjugative plasmid transfert experiments *thi*, *pro*, recA, *hsdR*, *hsdM*, RP4Tc::Mu, Km::Tn*7*; Tp^r, Sm^r, Spc^r	Simon *et al.*, (1983)
TG1	Construction of the phage display library and production of phage stocks	Sambrook *et al.*, (2001)
	B. japonicum	
USDA110	Parental strain; Cm^r	Maier and Brill (1978)
USDA110*spc*4	Parental strain; Cm^r; Spc^r	Regensburger and Hennecke (1983)
GRPA1	USDA110*napA*::Ω; Cm^r; Spc^r; Sm^r	This work
GRPA1C	USDA110*napA*::Ω, with pPM0611; Cm^r; Spc^r; Sm^r; Gm^r	"
0609	USDA110*napC*::Tn*KPK*$_2$; Gm^s; Km^r	"
0610	USDA110::pTn*KPK2*-6 ; Gm^r; Km^r	"
BJ-S148	USDA110Spc4::pJQS148; Spc^r; Gm^r	"
BJ-FK106	USDA110Spc4::pJQFK106; Spc^r; Gm^r	"
BJ-MR021	USDA110Spc4::pJQMR021; Spc^r; Gm^r	"
BJ-MR042	USDA110Spc4::pJQMR042; Spc^r; Gm^r	"
BJ-MR045	USDA110Spc4::pJQMR045; Spc^r; Gm^r	"
BJ-MR048	USDA110Spc4::pJQMR048; Spc^r; Gm^r	"
BJ-MR055	USDA110Spc4::pJQMR055; Spc^r; Gm^r	"
BJ-FK243	USDA110Spc4::pJQFK243; Spc^r; Gm^r	"
BJ-FK333	USDA110Spc4::pJQFK333; Spc^r; Gm^r	"

Table 2.2. Plasmids used in this work.

Plasmid	**Relevant charachteristics**	**Reference**
pBS II KS(+)	Routine cloning vector; Ap^r, *lacZ*, *ori f1*	Stratagene®
pK18*mobsacB*	Cloning and mobilizable vector, Km^r	Schäfer *et al.* (1994)
pHP45Ω	Plasmid carrying the Sm/Spc resistance genes (Ω interposon), Sm^r Spc^r	Prentki and Kirsch (1984)
pTOPO-TA	Cloning vector specific for PCR products, Ap^r, Km^r	TOPO TA Cloning® kit, from Invitrogen, Living Science
pJQ200SK	Mobilizable vector, Gm^r, *lacZ*	Quandt and Hynes (1993)
pTn*KPK2*	In vitro mutagenesis plasmid with transposable element and truncated version of '*phoA* gene; Km^r, Neo^r	Müller (2004)
Nap work from random sequencing experiment		
pPM200P9134	pJQ200SK with a *Pst*I (6.3 kb) fragment containing *napE*, *napD* and part of *napA*; Gm^r	This work
pPM0610	pJQ200SK with a *Apa*I-*Pst*I fragment (1.96 kb) from pPM200P9134; Gm^r	"
pBG602	pK18*mobsacB* with a *Bam*HI-*Pst*I fragment (1.2 kb) from pPM200P9134; Km^r	"
pBG602Ω	pBG602 containing the Ω Spc/Sm interposon (2 kb) of pHP45 Ω; Spc^r, Sm^r, Km^r	"
pBG605	pBS II KS(+) with a *Apa*I fragment (14 kb) from GRPA1 containing the whole *nap* operon with the Ω interposon; Spc^r, Km^r, Ap^r	"
pPM606-1/-3	pJQ200SK with a *Pst*I fragment (3.7 kb) from pBG605 cloned in both directions (-1/-3); Gm^r	"
pPM606-1Δ	pJQ200SK with a *Pst*I-*Bam*HI fragment (3.14 kb) from pPM606-1; Gm^r	"
pPM0611	pJQ200SK *ApaI-BamHI-BamHI* fragment (5.1 kb) carrying *napEDABC* ; Gm^r	"
E-tag fusions cloned in pJQ200SK		
PJQS148	pJQ200SK with the E-tag fusion in *S148 ORF*; Spc^r; Gm^r	This work
PJQFK106	pJQ200SK with the E-tag fusion in *FK106 ORF*; Spc^r; Gm^r	"
PJQMR021	pJQ200SK with the E-tag fusion in *MR021 ORF*; Spc^r; Gm^r	"
PJQMR042	pJQ200SK with the E-tag fusion in *MR042 ORF*; Spc^r; Gm^r	"
PJQMR045	pJQ200SK with the E-tag fusion in *MR045 ORF*; Spc^r; Gm^r	"

PJQMR048	pJQ200SK with the E-tag fusion in *MR048 ORF*; Spc^r; Gm^r	“
PJQMR055	pJQ200SK with the E-tag fusion in *MR055 ORF*; Spc^r; Gm^r	“
PJQFK243	pJQ200SK with the E-tag fusion in *FK243 ORF*; Spc^r; Gm^r	“
PJQFK333	pJQ200SK with the E-tag fusion in *FK333 ORF*; Spc^r; Gm^r	“
PJQMR037	pJQ200SK with the E-tag fusion in *MR037 ORF*; Spc^r; Gm^r	“
PJQS101b	pJQ200SK with the E-tag fusion in *S101b ORF*; Spc^r; Gm^r	“
PJQS209	pJQ200SK with the E-tag fusion in *S209 ORF*; Spc^r; Gm^r	“
PJQMR074	pJQ200SK with the E-tag fusion in *MR074 ORF*; Spc^r; Gm^r	“
PJQFK225	pJQ200SK with the E-tag fusion in *FK225 ORF*; Spc^r; Gm^r	“
PJQFK248	pJQ200SK with the E-tag fusion in *FK248 ORF*; Spc^r; Gm^r	“
E-tag fusions cloned in pTOPO-TA vector		
TS148	pTOPO-TA with the E-tag fusion in *S148 ORF*; Km^r	This work
TFK106	pTOPO-TA with the E-tag fusion in *FK106 ORF*; Km^r	“
TMR021	pTOPO-TA with the E-tag fusion in *MR021 ORF*; Km^r	“
TMR042	pTOPO-TA with the E-tag fusion in *MR042 ORF*; Km^r	“
TMR045	pTOPO-TA with the E-tag fusion in *MR045 ORF*; Km^r	“
TMR048	pTOPO-TA with the E-tag fusion in *MR048 ORF*; Km^r	“
TMR055	pTOPO-TA with the E-tag fusion in *MR055 ORF*; Km^r	“
TFK243	pTOPO-TA with the E-tag fusion in *FK243 ORF*; Km^r	“
TFK333	pTOPO-TA with the E-tag fusion in *FK333 ORF*; Km^r	“
TMR037	pTOPO-TA with the E-tag fusion in *MR037 ORF*; Km^r	“
TS101b	pTOPO-TA with the E-tag fusion in *S101b ORF*; Km^r	“
TS209	pTOPO-TA with the E-tag fusion in *S209 ORF*; Km^r	“
TMR074	pTOPO-TA with the E-tag fusion in *MR074 ORF*; Km^r	“
TFK225	pTOPO-TA with the E-tag fusion in *FK225 ORF*; Km^r	“
TFK248	pTOPO-TA with the E-tag fusion in *FK248 ORF*; Km^r	“
TMR089	pTOPO-TA with the E-tag fusion in *MR089 ORF*; Km^r	“
TS129b	pTOPO-TA with the E-tag fusion in *S129b ORF*; Km^r	“
TS204	pTOPO-TA with the E-tag fusion in *S204 ORF*; Km^r	“
TL108	pTOPO-TA with the E-tag fusion in *L108 ORF*; Km^r	“
TS112b	pTOPO-TA with the E-tag fusion in *S112b ORF*; Km^r	“
TMR117	pTOPO-TA with the E-tag fusion in *MR117 ORF*; Km^r	“

1.2. Culture conditions

1.2.1. Culture media

The culture media, as well as the description of their composition are listed in Table 2.3.

Table 2.3. Culture media used.

Bacteria	Growth media	Composition	Reference
E. coli			
Routine culture	Luria Bertani (LB)	Tryptone 10 g; yeast extract 5 g; NaCl 5 g; water 1 l.	Miller (1972)
Liquid culture for TOPO-transformation	SOC	2% tryptone ; 0.5% yeast extract ; 10mM NaCl ; 2.5 mM KCl ; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose.	TOPO TA cloning kit® (Invitrogen)
B. japonicum			
Routine culture	PSY	KH_2PO_4 0,3 g; K_2HPO_4 0,3 g; $CaCl_2.2H_2O$ 0,005 g; $MgSO_4.7H_2O$ 0,1 g; peptone 3 g; yeast extract 1 g; [1]mineral salts (100×) 10 ml; water 1 l.	Regensburger and Hennecke (1983)
Anaerobic culture	YEM	NaCl 0,1 g; K_2HPO_4 0,5 g; $MgSO_4.7H_2O$ 0,2 g; mannitol 5 g; yeast extract 1 g, water 1 l	Vincent (1970)
	YEMN	Same as YEM supplemented with 10 mM KNO_3	
Solid culture for transconjugants selection	Bergensen	K_2HPO_4 0,23 g; $MgSO_4.7H_2O$ 0,1 g; glycerol 5 g; Na-glutamate 1,1 g; [1]mineral salts (100×) 10 ml; water 1 l. After autoclaving: [2]vitamines (1000×) 1 ml.	Bergensen (1977)

[1]**Mineral salts stock solution (100×):** 1 g/l of H_3BO_3; 0.2 g/l of $ZnSO_4.7H_2O$; 0.05 g/l of $CuSO_4.5H_2O$; 0.05 g/l of $MnCl_2.4H_2O$; 0.01 g/l of $Na_2MoO_4.2H_2O$; 0.1 g/l of $FeCl_3$; water. Stelirization by autoclaving. Storage at 4°C.

[2]**Vitamine stock solution (1000×):** thiamine 0.1 g/l; biotine 0.1 g/l; sodium pantotenate 0.1 g/l; water. Sterilization by passing through 0.2 µm steril filter. Storage at 4°C.

All media were prepared with deionized water and high-quality chemical products and at a pH 7. Sterilization of the media was performed by autoclaving at 120°C during 20 minutes. Solid media were prepared by the addition of agar 15 g/l before sterilization.

1.2.2. Antibiotics

Antibiotics were added to culture medium using concentrated stock solutions prepared by dilution of them in distilled water and sterilization with 0.2 µm pore filters (Minisart® NML; Sartorius). Final concentrations of the different antibiotics are indicated in Table 2.4.

Table 2.4. Antibiotics added to the media when necessary.

Antibiotics	***E. coli***	***B. japonicum***
	Concentration (µg/ml)	
Ampicillin (Ap)	200	-
Chloramphenicol* (Cm)	50	20
Kanamycin (Km)	20	180
Streptomycin (Sm)	25	200
Spectinomycin (Spc)	25	200
Gentamycin (Gm)	10	110

*The concentrated stock solution of chloramphenicol was prepared in ethanol.

1.2.3. Conservation of bacterial strains

The storage of the bacterial strains was achieved in eppendorf tubes containing aliquots of cultures in logarithmic phase with 20 % of sterile glycerol kept at -20°C or -80°C.

1.3. Growth conditions

Liquid and solid aerobic cultures of *E. coli* and *B. japonicum* strains were kept at 37°C and 28ºC, respectively**.** Optical densities of the cultures were measured once a day at 600 nm wavelength using an UVICON 860 spectrophotometer, until the stationary growth phase was reached. As a reference, steril culture medium was used. For each strain two cultures were analysed in parallel and average of both measurements was used for the elaboration of a growth curve.

1.3.1. Culture of *B. japonicum* cells under anaerobic conditions

Liquid and solid aerobic cultures of *E. coli* and *B. japonicum* strains were kept at 37°C and 28ºC, respectively**.** Optical densities of the cultures were measured once a day at 600 nm wavelength using an UVICON 860 spectrophotometer, until the stationary growth phase was reached. As a reference, steril culture medium was used. For each strain two cultures were analysed in parallel and average of both measurements was used for the elaboration of a growth curve.

1.3.2. Incubation of *B. japonicum* cells under anaerobic conditions

Since some mutations in *B. japonicum* affected the growth capacity under anaerobic conditions with nitrate, incubation of those cells was necessary for further analysis. In these cases, cells were grown aerobically during 4 days either in 10 ml or 500 ml PSY medium until the optical density of the culture raised to 0.7-0.8. Cells grown aerobically were harvested by centrifugation (8,000 r.p.m. for 10 minutes at 4ºC), washed twice with PSY, resuspended either in 17 ml or in 1 l rubber-stoppered serum tubes or bottles, respectively and completely filled of YEMN medium. Finally, cells were incubated under anaerobic conditions for 96 hours.

1.4. Isolation of periplasmic, cytosolic and membrane fractions of *B. japonicum*

Cells of *B. japonicum* grown aerobically in PSY medium (500 ml) were harvested by centrifugation as above, washed twice with PSY, resuspended in 1 l of YEMN, and finally incubated under anaerobic conditions for 96 hours.

1.4.1. Isolation of periplasmic fraction

Preparation of periplasmic proteins was carried out as described previously (McEwan *et al*., 1984);

1. After incubation, centrifuge the cells at 8,000 g for 15 minutes at 4°C. The weight of the pellet should be around 0.5-0.7 g.
2. Resuspend the cells in 50 ml of 0.9 % NaCl to wash the cells, and repeat the centrifugation step. Discard the supernatant.

4. Resuspend the pellet in 2ml of SET buffer containing 60 µl of lysozyme (50 mg/ml). Incubate two hours at 30°C under shaking.
5. Add again 1 ml of SET buffer and 80 µl of lysozyme, and incubate two hours more at 30°C under shaking.
6. Centrifuge 10 minutes at 12,000 *g*. The resulting supernatant, containing periplasmic proteins, was concentrated to about 200 µl by using Amicon Centriprep 3 and Centricon 3 filters (Millipore). The concentrated periplasmic fractions were stored at -20ºC until use. The pellet corresponding to the spheroplasts can be frozen at -20°C until its use for isolation of membrane and cytosol fractions.

SET buffer: 100 mM TrisHCl pH 8; 0.5 M sucrose; 3 mM EDTA.
Lysozyme stock solution: 50 mg/ml of lysozyme in water; the solution has to be prepared before use.

1.4.2. Separation of membrane and cytosolic fractions of *B. japonicum*

Membrane and soluble preparations were performed as described earlier (Mesa *et al*. 2002). Pellet containing the spheroplasts obtained in step 6 of Section 1.4.1. or obtained from anaerobically incubated cells was processed as following;
1. Wash two times the cells or spheroplasts with 2-3 ml of washing buffer.
2. Resuspend the pellet in 3ml of fractionation buffer.
3. Break the cells by three passages through an ice-cold French pressure cell (SLM-Aminco) at a pressure of about 120 MPa. Repeat this 2-3 times.
4. Remove unbroken cells by centrifugation (10,000 *g* for 10 minutes at 4ºC).
5. Recover the supernatant and ultracentrifuge it at 140,000 *g* for 2 hours at 4ºC. The resulting supernatant corresponds to the cytosolic fraction (in case of spheroplasts) or the soluble fraction (cytosol and periplasm in case of cells) and the pellet corresponds to the membrane fraction.
6. The supernatant containing the cytosolic or the soluble fractions is concentrated to about 200 µl by using Amicon Centriprep 3 and Centricon 3 filters (Millipore) and is stored at -20ºC until use.
7. The pellet containing the membrane fraction is resuspended in washing buffer and centrifuged again at 140,000 *g*. The pellet is resuspended in 100 µl of fractionation buffer, and stored at -20°C.

Washing buffer: 50 mM of PO_4NaH_2/PO_4Na_2H, pH 6.8; 1 mM $MgCl_2$; 0.1 mM $CaCl_2$ and 0.9 % NaCl.

Fractionation buffer: washing buffer with 1 mM of *p*-Amidinophenylmethylsulfonylfluoride (p-APMSF), 20 µg/ml DNase, 20 µg/ml RNase.

2. Molecular biology techniques

2.1. Isolation of plasmid DNA of *E. coli*

Plasmid DNA of *E. coli* cells was isolated after alkaline lysis as described by Sambrook *et al.* (1989):

1. Centrifuge 3 ml of LB cultures at 13,000 r.p.m. for 3 minutes. Discard the supernatant.
2. Resuspend the pellet in 100 µl of solution A. Incubate 5 minutes at room temperature.
3. Add 200 µl of solution B for the lysis of the cells. Mix well and incubate on ice 5 minutes.
4. Add 150 µl of solution C for the precipitation of genomic DNA and proteins. Mix well manually and incubate on ice at least 5 minutes.
5. Centrifuge at 13,000 r.p.m. for 8 minutes and transfer the supernatant into new tubes.
6. Add 1 volume (400-450 µl) of phenol:chlorophorm:isoamylalcohol (25:24:1), and mix strongly for 2 minutes.
7. Centrifuge at 13,000 r.p.m. for 5 minutes. Transfer the upper phase to new tubes.
8. Add 1.5 volumes (0.75-1 ml) of 100 % cold ethanol 100 % to precipitate the DNA and mix well. Incubate 15 minutes on ice.
9. Centrifuge at 13,000 r.p.m. for 15 minutes. Discard carefully the supernatant.
10. Add 200 µl of 70 % cold ethanol. Centrifuge at 13,000 r.p.m. for 3 minutes and discard carefully the supernatant.
11. Dry the pellet of DNA by using dry vacuum during 10-15 minutes at room temperature.
12. Resuspend with 20 µl of pure water or TE buffer.

Solution A: 50 mM TrisHCl; 10 mM EDTA; 100 µg/ml RNAse A; pH 8.

Solution B: 0.2 M NaOH; 1 % SDS.

Solution C: 3 M potassium acetate; pH 5.5.

TE buffer: 10 mM Tris; 1 mM EDTA; pH 8.

Phenol:chlorophorm:isoamylalcohol (v/v) (25:24:1): this mixture is prepared using 100% phenol containing 0.1% 8-hydroxyquinoline in 0.1 M TrisHCl (pH 8), which is shaken overnight. Then the mixture of phenol:chlorophorm:isoamylalcohol is prepared in the corresponding proportion (25:24:1), and is strongly shaken and then decanted overnight. Use the lower phase of the mixture.

2.2. Isolation of genomic DNA from *B. japonicum*

Genomic DNA from *B. japonicum* was obtained as described (Sambrook *et al.*, 1989):

1. Grow each *B. japonicum* strain in 5 ml PSY liquid culture medium at 28°C until it raises an optical density at 600 nm of 0.8.
2. Centrifuge 2 ml of each culture at 13,000 r.p.m. for 5 minutes. Discard the supernatant and wash the pellet once with 0.5 ml 0.9 % NaCl. Centrifuge again as described before and discard well the supernatant. In this step, the pellet can be kept at -20°C until its use.
3. Resuspend the pellet with 756 µl of TE buffer, and mix gently with vortex.
4. Add 40 µl of 10 % SDS and 5 µl of 20 mg/ml proteinase K. Mix well without vortexing.
5. Incubate 1 hour at 37° C for lysis and protein denaturing.
6. Add 133 µl of 5 M NaCl, and mix well.
7. Add 106 µl of CTAB preheated at 65° C and incubate 15 minutes at 65° C, for precipitation of proteins and cells.
8. Add 850 µl of chlorophorm:isoamylalcohol (24:1), mix well.
9. Centrifuge at 14,000 r.p.m for 5 minutes.
10. Transfer the upper phase to a new eppendorf tube.
11. Add 850 µl of phenol:chlorophorm (1:1) and mix.
12. Centrifuge at 14,000 r.p.m. for 5 minutes.
13. Transfer the upper phase to a new eppendorf tube.
14. Add 470 µl of 100 % isopropanol, and mix gently.
15. Centrifuge at 14,000 r.p.m. for 10-15 minutes, discard the supernatant.
16. Wash the pellet DNA with 470 µl of 70 % ethanol, without mixing.
17. Centrifuge at 14,000 r.p.m. for 5 minutes, and discard the supernatant.
18. Dry the pellet at room temperature for at least 2 hours.
19. The pellet is then resuspended in 50 µl TE at 4°C overnight.

TE buffer: 10 mM Tris; 1 mM EDTA; pH 8.

Proteinase K stock solution: 20 mg/ml of proteinase K in water. Stored at -20°C.

CTAB (Cetyl Trimethyl Ammonium Bromide): 10 g of CTAB in 90 ml of water. Stir and warm up to 60°C until completely dissolved.

Phenol: the phenol used is provided by Rotiphenol®. After removing the upper phase, add one equal volume of chlorophorm. Wait until the two phases separate before use. The preparation has to be stored in dark and at 4°C.

2.3. Isolation of total RNA from *B. japonicum*

Total RNA of *B. japonicum* was isolated as described by Cabanes *et al*. (2000):

1. Incubate the *B. japonicum* strain in 50 ml of YEM or YEMN medium until it raises an optical density of 0.6 to 0.8.
2. Cool quickly the culture by introducing the flask in liquid nitrogen. Centrifuge the culture at 8,000 r.p.m. for 15 minutes at 4°C.
3. Discard the supernatant. When necessary the pellet can be inmediately freezed in liquid nitrogen and kept at -80°C until its use.
4. Resuspend the pellet with vortex in 600 µl of 65°C preheated lysis solution. Inmediately share the resuspension in two microfuge tubes (300 µl/tube).
5. Incubate at 65°C during 10 minutes, shaking occasionally with vortex (around 3 times).
6. Keep the tubes on ice and add 150 µl of 5 M precooled NaCl at 4°C, to precipitate the proteins. Incubate on ice during 10 minutes.
7. Centrifuge at 4°C and 13,000 r.p.m. during 15 minutes.
8. Transfer the supernatant to a new microfuge tube and precipitate the nucleic acids by adding 0.8-1 ml of 100% precooled ethanol at -20°C. Vortex and maintain at least one hour at -80°C. When necessary the tubes can be stored for several days.
9. Centrifuge at 13,000 r.p.m. and 4°C, during 30 minutes.
10. Carefully discard the supernatant with micropipette.
11. Resuspend the pellet in 42.5 µl of H_2O_{DEPC} and then mix together both tubes, having in total 85 µl.
12. Eliminate the contaminating DNA by treatment with DNAse in the following reaction: 85 µl of RNA, 10 µl of DNAse buffer (10x), 2 µl of (RNAse 40 U/µl), RNAse inhibitor and 5 µl of DNase I. Incubate at 37°C during 30 minutes.

13. Add one volume of cool phenol:chlorophorm:isoamyl alcohol (25:24:1).
14. Mix vigourously with vortex and centrifuge at 13,000 r.p.m. and 4°C, during 5 minutes.
15. Transfer the upper aqueous phase to a new tube and add one volume of cold chlorophorm:isoamylalcohol (24:1).
16. Mix vigourously with vortex and centrifuge at 13,000 r.p.m. and 4°C during 5 minutes.
17. Transfer the upper phase to a new tube and add 20 µl of 3 M sodium acetate pH 5.2 and 600 µl of 100% precooled ethanol (at -20°C). Mix by inversion and keep at least one hour at -80°C. When necessary tubes can be stored several days under these conditions.
18. Centrifuge at 13,000 r.p.m. and 4°C during 30 minutes.
19. Discard the supernatant with micropipette and add 1 ml of 70 % precooled ethanol (-20°C) and centrifuge at 13,000 r.p.m. and 4°C during 10 minutes.
20. Carefully discard the supernatant with a micropipette and dry the sample at room temperature or by vacuum dry.
21. Resuspend the RNA in 20 µl of H_2O_{DEPC} and measure its purity and concentration in a spectrophotometer by determination of absorbance at 260 and 280 nm (GeneQuantII photometer from Pharmacia Biotech).
22. Run an electrophoresis in 1 % agarose gel, loading 1 µl of the RNA solution to check if there is any degradation.

For RNA isolation, it is very important to prepare or treat all solutions, material and equipment employed with H_2O_{DEPC} (diethylpyrocarbonate, DEPC), a potent inhibitor of RNAses. Because of its toxicity, the DEPC has to be handled safely with gloves.

H_2O_{DEPC}: add 1 µl of DEPC per ml of water. Mix at least several hours (overnight) and autoclave.

Agarose gel: TBE buffer containing 0.0001% SDS has to be prepared with H_2O_{DEPC}.

Lysis solution: 1.4 % SDS; 4 mM EDTA; 25 µl (5 mg/ml) of proteinase K.

DNAse buffer (10×): 500 mM Tris-HCl pH 7.5; 10 mM $MgCl_2$.

TBE buffer (1×): 50 mM Tris; 2.5 mM EDTA-Na_2; 50 mM BO_3H_2; pH 8.2.

2.4. Measurement of DNA and RNA concentrations

DNA and RNA concentration was mesured as described by Sambrook *et al.* (1989). The optical densities (OD) at 260 and 280 nm for DNA or RNA solution in water or TE were measured in a GeneQuantII photometer (Pharmacia Biotech). An absorption value at 260 nm

(Abs_{260}) of 1 is equivalent to a double stranded DNA concentration of 50 µg/ml, or to a total RNA concentration of 40 µg/ml. Values of the Abs_{260}:Abs_{280} ratio lower than 1.8 is indication of protein or phenol contamination. For contaminated or low concentrated DNA samples, the concentration was estimated comparatively using the fluorescence in agarose gel under UV light, with DNA markers of known concentration (Smart Ladder; 200 bp- 10,000 bp; Eurogentec).

TE buffer: 10 mM Tris; 1 mM EDTA; pH 8.

2.5. DNA restriction with endonucleases

Digestion of DNA with restriction enzymes was performed in the optimal conditions for each enzyme, as temperature and buffer, as indicated by the suppliers (Roche®, Germany; Amersham Biosciences, Germany).

2.6. Identification of DNA fragments in agarose gel electrophoresis

2.6.1. Electrophoresis of DNA or RNA

Agarose gels were prepared at 0.8 % or 2 % for DNA or RNA, respectively in TBE (1×) or TA (0.5×). In case of longer fragments of DNA, the concentration decreased to 0.7%, and in case of smaller fragments of DNA, the concentration increased to 1%. Electrophoresis was developped in TBE (1×) or TA (0.5×) buffer at 50-100 V. The samples were loaded into the gel diluted in 1/5 volume of loading buffer.

TBE buffer (1×): 50 mM Tris; 2.5 mM EDTA-Na_2; 50 mM BO_3H_3; pH 8.2.
TA buffer (10×): 48.4 g Tris-HCl, 11.42 ml glacial acetic acid, 0.5 M EDTA, 1 l water; pH 8.
Loading buffer (5×): 40 % sucrose; 0.25 % bromophenol blue.

2.6.2. Visualization of DNA

DNA or RNA samples present in the agarose gel were visualized after incubation of the gel into an ethidium bromide solution (1 µg/ml) for 15-20 minutes and exposition of the gel

to a UV light (302 nm). The gel images were either digitized with a CCD camera (INTAS, Göttingen, Germany) and stored in a disk as a tiff file for further image processing and analysis, or with the equipment of image analysis (BIO-RAD) and the "Quantity One" software.

2.7. Purification of DNA fragments from agarose gel

Purification of DNA fragments from agarose gel was performed by using the comercial "Qiaex-II® Gel Extraction Kit" (Qiagen Inc., Chatsworth, California, USA) according to the suppliers instructions.

2.8. Ligation of DNA fragments

Cloning of DNA fragments which have been amplified by PCR reaction can be achieved by insertion into a Topovector from a TOPO TA Cloning kit (version K2, from Invitrogen™, living science) which allows a five-minute cloning of *Taq* polymerase-amplified PCR products. The reaction was performed as described by the provider:

Prepare the reaction mix:

- fresh PCR product 0.5 to 4 µl depending on its concentration
- sterile water add to a final volume of 5 µl
- TOPO vector 1 µl

Mix gently and incubate 5 minutes at room temperature. The length of the reaction can be varied from 30 seconds to 30 minutes. When reaction time is over add 1 µl of salt solution. The reaction can be directly used for transformation in *E. coli* competent cells as described in Section 2.9 with the following modification: add 2 µl of the Topo cloning reaction into a vial of competent cells and mix gently. Incubate on ice for 5 to 30 minutes. Heat-shock the cells at 42°C for 30 seconds and immediately place the tube on ice. Add 250 µl of room temperature SOC medium and incubate at 37°C for one hour before plating on the required medium for selection of the transformed cells.

In other cases, the amplified or purified DNA fragment was digested with suitable restriction enzymes, as well as the vector, and inserted into the vector using T4 phage ligase. The ligation was performed with the following reaction mix:

- x µl of vector*

- y µl of insert*
- 1 µl of DNA ligase (from T4 phage) (1 unit/µl)
- 2 µl of ligase buffer (10 × concentrated).
- complete to 20 µl with bidistilled H_2O.

The reaction mixture is incubated at 16°C overnight, and it can be kept at 4°C until it is transformed into competent cells.

*The proportion of vector:insert has to be kept in 1:3 ratio in moles, and the total quantity of insert plus vector DNA has to be around 200 ng.

2.9. Transformation of plasmid DNA into *E. coli*

2.9.1. Preparation of *E.coli* competent cells

The preparation of competent cells of *E. coli* DH5α or S17.1 has been performed as described previously by Hanahan (1983):

1. Grow *E. coli* cells in 5 ml of LB medium at 37°C overnight.
2. With this preculture, inoculate 200 ml of LB medium supplemented with 10mM $MgSO_4$ and incubated at 37°C with shacking until the optical density raises 0.5-0.6 at 600 nm. Chill the culture on ice 5 minutes, in two falcon tubes containing 100 ml of the culture.
3. Pellet the cells by centrifugation at 3,000 r.p.m. and 4°C during 5 minutes. Remove the supernatant.
4. Resuspend each pellet in 25 ml of ice-cold 0.1 M $CaCl_2$. Chill on ice 20 minutes.
5. Pellet the cells by centrifugation at 3,000 r.p.m. for 10 minutes at 4°C. Remove the supernatant.
6. Resuspend each pellet in 10 ml of ice-cold 0.1 M $CaCl_2$. Incubate on ice 1-4 hour.
7. Add 10 ml of 40 % sterile glycerol to each tube and mix gently.
8. Aliquot in 1.5 ml eppendorf tubes (100µl).
9. Freeze at –70°C.

2.9.2. Transformation of plasmid DNA into *E. coli*

To transform competent cells with plasmid DNA, the technique described by Rodríguez and Tait (1983) has been modified as following:

1. Defreeze aliquots of 100 µl of competent cells kept at -70°C maintaining them on ice during 15-20 minutes.
2. Add 50-100 ng of the DNA which has to be transformed. Mix gently and incubate on ice during 30 minutes.
3. Heat shock at 42°C during 90-120 seconds. Directly cool on ice for 2-5 minutes.
4. Add 1ml LB and incubate at 37°C during 1 hour while shaking. At the same time, prepare Petri plates with LB containing the corresponding antibiotics for selection of the transformed strains. In order to achieve a colorimetric sreening by using the β-galactosidase assay, 40 µl of X-gal stock solution and 4 µl of IPTG stock solution were added to the medium.
5. Plate the cells on the plates and incubate at 37°C overnight.

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside): stock solution of 20 mg/l of X-gal in dimetylformamide. Keep at -20°C in aliquots.
IPTG (1-isopropyl-β-D-1-tiogalactopyranoside): 200 mg/l of IPTG in water.

2.10. In vitro transposon insertions using Tn*KPK2*

For mutagenesis of DNA fragments by insertions of transposable element using Tn*KPK2* (Müller, 2004), the following steps were achieved:

Transposon Tn*KPK2* was amplified by a standard PCR protocol in a Biometra PCR temperature-gradient cycler, using pTn*KPK2* as the template, the oligonucleotides Mod-FP-1 (5'-ATTCAGGCTGCGCAACTGTTGGG-3') and Mod-RP-1(5'-GTCAGTGAGCGAGGAA GCGGAAG-3') as forward and reverse primers (MWG Biotech), and the Pwo proof reading polymerase (Hybaid), in a total reaction volume of 25 µl. The 2.8 kb amplification product was checked for purity and for size by agarose gel electrophoresis in TRIS-acetate buffer, and the desired DNA sample was isolated from the gel and purified using the Concert Rapid Gel extraction and purification system (Gibco/BRL, EZ::TN<Tet-1>) according to the recommendations of the manufacturer (Epicentre Technologies). The plasmid containing the DNA fragment to be mutated was isolated and purified using the Nucleospin Multi-8 plasmid preparation kit (Macherey-Nagel) and added at equimolar concentrations to the purified Tn*KPK2* DNA in a 200-µl test tube. The buffered reaction mix was adjusted to a final volume of 10 µl by the addition of 1 U of transposase (Epicentre Technologies) and then incubated for 2 hours at 37°C. The reaction was stopped by adding 1.1 µl of stop buffer. After desalting, the

reaction mix was resuspended in the same volume of sterile distilled water, and 1 µl of the mixture was added to electrocompetent *E. coli* DH10B cells. Electroporation was carried out in cuvettes in a BioRad Gene Pulser (the distance between the poles was 2 mm) at 25 lF, 200 W and 2.0 V, resulting in a pulse duration of 4.0 ms. Immediately after the pulse, the cells were incubated in 1 ml of SOC medium (Table 2.3) for 1 hour at 37°C. Aliquots were then spread on selective LB medium, containing Km, Gm, and X-phosphate (40 mg/l). After 2 days of incubation, colonies were purified by transferring them onto fresh plates of the same medium. The cells were then grown in liquid LB medium for plasmid DNA preparation. Recombinant plasmids were digested with *Pst*I, and analysed by agarose gel electrophoresis. Recombinant plasmids with different size of inserted DNA, but similar size of the vector band (5.6 kb), were selected for further sequencing in order to determine where the insertion took place.

2. 11. Conjugative transfer of plasmid DNA to *B. japonicum*

1. Grow the receptor *B. japonicum* strain in 5 ml PSY medium at 28°C with shaking, until its optical density raises 0.8 at 600 nm.
2. Inoculate 5 ml of LB medium with the donor *E. coli* S17.1 strain from an overnight preculture and incubate at 37°C with shaking until the optical density raises 0.6-0.7 at 600 nm.
3. In a 2 ml eppendorf tube, mix 1.8 ml of the receptor strain culture, and 0.2 ml of the donor strain culture. Mix gently.
4. Centrifuge 3 minutes at 9,000 r.p.m. and discard the supernatant.
5. Resuspend the pellet in 100 µl of steril liquid PSY medium and drop this volume on a PSY medium agar plate. Incubate the plate at 28°C during two days.
6. The third day, recover the bacteria with an inoculating loop and resuspend it in an eppendorf tube with 1 ml of sterile 0.9 % NaCl solution.
7. Centrifuge 3 minutes at 9,000 r.p.m. and discard the supernatant.
8. Resuspend the pellet in 500 µl of sterile PSY medium and plate different volumes (25-50-100-150 µl) of it on PSY plates containing the correct antibiotics for the selection of the transconjugant *B. japonicum* strains.

2.12. Hybridization DNA-DNA

2.12.1. Preparation of the Dig-labelled DNA probe

In order to determine if the correct recombination event occurred in the genome of *B. japonicum*, a DNA fragment was choose to synthesise the probe which would hybridize to the genomic DNA. Two specifique probes were synthesised to check the *napA* and *napC* mutant strains according to the sequence of the Ω interposon inserted in pBG602Ω and the sequence inserted in pPM606/-1, respectively. A general probe was synthesised according to the E-tag sequence inserted into pJQ200SK to check the mutant strains obtained in Chapter 2. The inserted fragment enabled the probe to bind to the fragment of the genomic DNA where the integration of the plasmid has taken place. The DNA marker used for Southern blots was the Smart Ladder (200 bp- 10,000 bp; Eurogentec). The probe was amplified using the PCR DIG Probe Synthesis Kit (Roche®) for the generation of highly-sensitive probes labelled with DIG-dUTP (alkali-labile) in the polymerase chain reaction, following the instructions provided by the manufacturer. After establishing the conditions for the amplification of non-labelled DNA probe, the reaction was repeated performing the standard DIG-labelling PCR mix as follows:

- bidistilled water 33.2 µl
- 10x PCR buffer (with 15 mM $MgCl_2$) 5 µl
- DMSO 2.5 µl
- 10x dNTP labelling mix 5 µl
- sense primer (10 pmol/µl) 1.5 µl
- antisense primer (10 pmol/µl) 1.5 µl
- template DNA 1 µl

(50 ng genomic DNA, 1-10 ng plasmid DNA, or 1/100 diluted PCR product)

- *Taq* polymerase (10 U/µl) 0.3 µl

The universal M13 forward and reverse primers (see Section 2.13.2) specific for the multicloning sites of the vectors in which the selected sequences are inserted were used for this reaction.

The success of DIG-UTP incorporation into the PCR product could be easily checked on an agarose gel. DIG-labelled PCR-products have a lower relative mobility than the unlabelled ones due to the steric hinderance imposed by the digoxigenin molecules linked to the amplification products. An apparent size increase of about 100 bp is usually observed for efficiently labelled fragments having a real size greater than 400 bp.

2.12.2. Separation of DNA and transfer to a nylon membrane

Before transfering the genomic DNA onto a nylon membrane, it was restricted with appropriate enzymes for the further analysis of the fragments detected by hybridization. The restricted DNA was then separated in an agrose gel as described before (Section 2.6). The DNA transfer to the nylon membrane was then performed as described in Sambrook (1989) as following;

1. The agarose gel is placed in close contact on the top of the nylon membrane which was previously placed on the blotter and surrounded by an adequate plastic foil to ensure that vacuum can be generated with an appropriate pump (Vacuum blotting system Vacu Gene XL; Pharmacia, Germany).
2. The DNA is then denatured in the gel by covering it with denaturation buffer and by the application of 35 mbar vacuum for 15 minutes.
3. Denaturing is followed by neutralization with neutralization buffer at the same negative pressure and incubation time.
4. The transfer is achieved by increasing the vacuum to 45 mbar for about 20 minutes using the same solution as in neutralization step.
5. After the transfer has been completed the DNA is covalently linked to the membrane by UV cross-linker from Stratagene (automatic program).
6. The membranes are kept dry at room temperature in a dark and dust-free environment or at -20°C covered by a plastic film.

Denaturation buffer: 1.5 M NaCl; 0.5 M NaOH.
Neutralization buffer: 1 M ammonium acetate.

2.12.3. Prehybridization, DNA-DNA hybridization, and detection.

Prehybridization, hybridization, stringency washes, incubation of probed filters with antidigoxigenin activity, and reprobing of filters were done following the detailed protocols found in the DIG System "User's Guide for Filter Hybridization" from Boehringer-Mannheim (Germany) as indicated;

1. The membrane is placed into a hybridization tube, providing that its surface carrying the DNA face the inside of the tube. Add 6 ml of "hybridization solution" with 500 µl of salmon sperm DNA preheated at 95°C during 5 minutes. Incubate at 65°C during at least 30 minutes.

This allows the salmon sperm DNA to bind to the spaces of the membrane free of DNA after the transfer.

2. Discard the prehybridization solution from the tube. Add 6 ml of hybridization solution containing 20 µl of the probe preheated at 95°C during 5 minutes for its denaturation. Incubate at 65°C overnight, the temperature of hybridization is defined by the probe (around 65°C for a probe with more than 100 bases). After use, the hybridization solution containing the probe can be stored at -20°C, and reused at least 3 times.

3. Wash the excess of probe two times at room temperature during 5 minutes with 2×SSC+0.1%SDS buffer.

4. Wash the membrane two times at 65°C during 15 minutes with 0.5×SSC+0.1%SDS buffer.

5. Wash the membrane one minute with washing buffer.

6. Discard the washing solution, and add 6 ml of blocking buffer. Incubate at least 30 minutes at room temperature with gentle agitation.

7. Discard the blocking solution, and add 6-10 ml of antibody solution (anti-digoxigenin, Fab, fragments conjugated to alkaline phosphatase, diluted 1/10,000 (75 mU/ml) in blocking solution (1×). Incubate at least 30 minutes at room temperature with gentle agitation.

8. Discard the antibody solution, and wash the membrane two times 15 minutes with washing buffer at room temperature.

9. Discard the washing buffer and add 6 ml of detection buffer to preincubate the membrane for 2 minutes at room temperature.

10. Take the membrane out from the tube and place it in a plastic bag. Add 10 ml of detection buffer with 35 µl of X-phosphate solution and 45 µl of NBT solution. Seal the bag and incubate in dark at least one hour without moving or shaking, until appearance of bands. Then stop the reaction by washing with water.

11. Discard the detection buffer from the bag and wash with distilled water. Add 2 ml of TE (1×) and close again the bag hermetically. Store at 4°C.

12. Alternatively, the detection was performed by the addition to the membrane of 10 ml CSPD® (1:100 in Tris-HCl 100 mM, pH 9.5; NaCl 100 mM) (step 10) and further incubation during 5 minutes in dark. The solution is then thrown away, and the membrane exposed to an autoradiography film (Kodak X-Omat) during 30 minutes to 6 hours, depending on the concentration of DNA and the sensivity of the probe. The film was then revealed in developer and fixer TETENAL®, in the recommended dilutions ans times.

20 × SSC: 3 M NaCl; 300 mM sodium citrate; pH 7.

Standard (pre)hybridization solution : 5 × SSC; 1 % (w/v) blocking reagent; 0.1 % N-laurylsarcosine; 0.02 % SDS.

Salmon sperm DNA solution: 10 mg/ml of salmon sperm DNA dissolved in bidistilled water at room temperature by stirring during one hour at least. Shear the DNA by passing it several times through an 18-gauge hypodermic needle and boil it during 10 minutes before storage in aliquots at –20°C.

Maleic acid buffer: 150 mM NaCl; 100 mM maleic acid; adjust pH to 7.5 with solid or 10 N NaOH.

Washing buffer: 0.3 % Tween 20 in maleic acid buffer.

10 × blocking reagent stock solution: 10 % (w/v) blocking reagent in maleic acid buffer; dissolve by stirring and heating but not boiling.

Detection buffer: 0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl; 0.05 M $MgCl_2$.

NBT Nitroblue Tetrazolium Chlorid: 70-75 mg/ml in dimethylformamide, store at –20°C.

X-phosphate: 50 mg/ml in dimethylformamide, store at –20°C.

CSPD®: 3-(4-metoxipiro(1,2-dioxiethan-3,2'-(5'-cloro)triciclo(3.3.1.1)decan)-4-il) fenil fosfato disódico.

2.13. Sequencing of DNA

2.13.1. DNA purification for sequencing

Purification of DNA for sequencing was achieved by using the Qiaprep® Spin Plasmid kit (Qiagen Inc.) as following;

1. Bacteria are grown in 3 ml of LB medium supplemented with the corresponding antibiotics.
2. Cells are pelleted by centrifugation at 13,000 r.p.m. during 2 minutes. The supernatant is discarded.
3. Resuspend the cells in 250 µl of solution P1.
4. Add 250 µl of solution P2 and mix gently. Let 5 minutes at room temperature.
5. Add 350 µl of solution N3. Mix gently several times and immediatly centrifuge at 13,000 r.p.m. during 15 minutes.

6. Recover the supernatant and place it in a Qiaprep column. Centrifuge 1 minute to retain the plasmid DNA and discard the eluate.
7. Wash the DNA in the column by adding 750 µl of solution PB and centrifuge during 1 minute. Discard the eluate.
8. Add 750 µl of solution PE and centrifuge 1 minute at 14,000 r.p.m. Repeat this step once more to eliminate the excess of solution PE.
9. Finally place the column in a new eppendorf tube of 1.5 ml and add 50 µl of sterile bidistilled water in the column to dissolve the DNA. After 1 minute, centrifuge at 13,000 r.p.m. during 1 minute to recover the eluate (around 25 µg of DNA).

Solution P1: 50 mM Tris-HCl; 10 mM EDTA; 100 µg/ml RNAse A; pH 8.0.
Solution P2: 0.2 M NaOH; SDS 1%.
Solution N3: 3 M Acetato potásico; pH 5.5.

2.13.2. Authomatic sequencing

The authomatic sequencing was performed by using the sequencing services of the "Instituto de Parasitología y Biomedicina López-Neyra" (CSIC, Granada) where a Perkin Elmer 9,600 thermocycler, the Abi Prism kit (Perkin-Elmer) and the Amplitaq FS polymerase are employed. The sequence determination was realized by the system using fluorochrome-labelled termination bases. The specific primer (1.6-3.2 picomoles) and bidistilled water were added to the DNA to be sequenced (300-600 ng) until a final volume of 6 µl.

In all the cases, both strands of DNA were sequenced. The universal forward and reverse primers used correspond to the sequences of the M13 phage (-40) forward, and (-20) reverse present in the pBS vector (KS+).

Forward M13F	5'-GTTTTCCCAGTCACGAC-3'
Reverse M13R	5'-AACAGCTATGACCATG-3'

2.13.3. Manual sequencing

For the preparation of the sequencing gel, the glass plates of the equipment need to be totally free from dust and cleaned with 70 % and 96 % ethanol. The spacers allow the preparation of 0.25 mm thick gels. For the preparation of polyacrylamide gels, 24 ml of solution I and 6 ml of solution II (Sequagel solutions, Biozym) were mixed by stirring. From

the resulting mix, a small volume (3 ml) was taken apart and used for the gel base after adding 30 µl of 20 % APS solution. When the gel base was polymerised, 270 µl of APS solution was added to the rest of the mix for the preparation of the complete gel. A minimum of two hours was necessary for the polymerisation of the sequencing gel, protected from the light, but it could also be stored overnight at 4°C before its use.

The sequencing reaction was performed as described earlier by Sanger *et al.* (1975,1977) using the DYE-namic Thermosequenase cycle sequencing kit and the IRD-800 labelled primers (at 5'-end): universal forward or reverse primers from MWG Biotech, Germany, corresponding to the vector pJQ200SK and TOPO-TA (see sequences in Section 2.13.2); and G3delrev primer corresponding to the vector pG3DSS used for the template in the case of the phage display library project (G3delrev: 5'-CCAGCATTGACAGGAG GTTGAGGC-3'). Cycle sequencing reactions were carried out as described earlier (Becker *et al.*, 1998), using a LI-COR 4000 sequencing device which allows visualization of the IRD 800 dye which is bound to the 5'-end of the primer. The dye is infrared detectable at 790 nm after excitation with a laser beam.

Reaction mix in a microfuge tube for 1 sample:

DMSO	1 µl
Template	14 µl
IRD800 labelled primer solution	1.3 µl

Mix gently and share the raction mix in 4 tubes (4 µl per tube) containing each one 1 µl of one of the termination mix. The sequencing reaction is performed in the conditions of the following program:

Step	Temperature	Time
1	94°C	2'
2	95°C	30''
3	62°C	30''
4	72°C	1'
5	29 cycles starting again from step 2	
6	4°C	until end

After the the program is finished, the reaction is stopped by the addition of 3 µl of loading buffer, and the samples can be stored at –20°C, or are loaded onto the sequencing gel.

4 different termination mix: Tris-HCl buffer, pH 9.5; $MgCl_2$; the 4 normal dNTPs and each termination mix has one analogue ddNTP; thermolabile pyrophosphatase; Thermo Sequenase DNA Polymerase.

2.13.4. Sequences analysis

Nucleotide sequences were analysed using the GeneBase software, purchased from Applied Maths, Belgium. The Signal PV.2.0 algorithm was used to evaluate deduced amino acid sequences for extracellularity and for the existence of N-terminal signal peptides. Sequence analysis was carried out using the computer programs Gene Base (Applied Maths, 1.0) and vector suite NTI (Informax). Homology searches were performed by using the National Center for Biotechnology Information BLAST network server (http://www.ncbi.nlm.nih.gov/blast/). Data bases used were EMBL, Genbank, Swissprot and Pir from EMBL and NCBI servers (http://www.ncbi.nlm.nih.gov/; http://www.ebi.ac.uk/). Transmembrane predictions and signal sequence analysis were performed using the TMHMM and on-line Signal P v1.1www programs of Center of Biological Services (http://www.cbs.dtu.dk/services).

The analysis of DNA and protein sequences, the restriction site search, the searching and location of putative open reading frames (ORFs), etc., have been performed with the following programs of "Centre de ressources INFOBIOGEN" (http://www.infobiogen.fr/deambulum/index.php): Readseq, Convseq, Restriction Analysis, Multiple Translation, Dialign2. Alignment of amino acid sequences were performed with the multalin program of "Laboratoire de génétique cellulaire" of INRA in Toulouse (http://www.toulouse.inra.fr/lgc/lgc.htm).

2.14. Amplification of DNA fragments

In the context of E-tag library clones study, the identical primer as for sequencing G3delrev (see above, but without the 5'-modification) was used as an universal primer for the amplification of specific fragments. The other primer was designed specifically (Table 2.5) according to the individual nucleotide sequence within the putative coding region of the signal peptides of the different clones.

Table 2.5. Specific primer sequences used for each E-tag candidate clone. In the sequence, the small letters represent modifications of the sequence used by the design of the primers in order to create additional restriction sites.

PS148	CGGGGGCCCAGGCCCACGCCACCAACG
PMR021	CGTCGACGACGGCGGCCTTTGCTGCCG
PMR042	cgtcGACGGCGGCCTTTGCTGCCG
PMR045	CGTCGACGCCATTCCGGCCCAGC
PMR048	CGTCGACGATTTTCCCTCCGCGATCTC
PMR055	GGTCGACGCGGGCGTCGCCAATGACG
PFK106	TTTATTCGTGCTGCAATTCTGGCGC
PFK333	GGAGGTCGCATCCGCCCAGA
PFK225	AAGAAGATTCTGTTTGCGACCGTGGC
PL108	GgcTGCAGTGTCGGCGGTAGCGCTGTC
PS105	GGGTCGACGGCATTGCCTATCTCGCCG
PFK209	CAGGCGGGTCGTCATCTGAAACAAG
PFK243	TTTCTAGACGCGTTCAGATCCTGGTG
PMR089	cgtcGACTCGCAATTCTTGCCACGATC
PMR117	tgtcGACACATTTTCGCTCGCAATCGCC
PMR074 o PS106	AAGATCTCGCTGACCTTGACCTTTG
PS112b	GGGATTCCAGGAGCATGCCGCGAAG
PMR037	cgaaTTCGCGGGTGAAGCCGCGTA
PS204	cgtcGACGAGGCGCAGCCGCTCCGCCTC
PS209 o FK112 o FK331	agaTCGTCGACGCCGTGAAGAAGG
PS101b o MR021§	CGTCGACGACGGCGGCCTTTGCTGCCG
PS129b	cgctcGAGCCGACGACCTCAAGGTCGCGCTG
PMR039	ctctAGACTGATCGCGGTCCTTCTCTG
PS123	ctcGAGCAGAGTAGGTATTGCGTCGAAG
PS126b	caagCTTGCTCTCGCCGCCGGCCTGACTG
PMR034 = mr 177	CATcTAGACGTCGAGAAGCAGCATCGG

2.15. Primer extension

Primer extension was performed as previously described by Marques *et al.* (1993). In a microfuge tube, the hybridization mix is prepared as follows:

20 µg of total RNA in a volume (with H_2O_{DEPC}) of	7 µl or *n*×7 µl
hybridization buffer	2 µl or *n*×2 µl
primer PEX-nap	l µl or *n*×1 µl

Mix gently and apply the specific temperature regime using a Biometra gradient cycler as follows: 30 seconds RNA denaturing at 78°C, cooling quickly to 62.3°C, and more slowly to 57.3°C with a plateau (30 seconds) at the optimal primer annealing temperature, then

gradually cooling down to 52.3°C and then to 42°C. Stop the cycler and immediately add the extension mix (preheated to 42°C during 1 minute) to the previous microfuge tube containing the preparation:

Extension mix (prepare extension mix for *n* + 1 samples):

H_2O_{DEPC}	26,6 µl
AMV-RT buffer (5 ×)	10,0 µl
dNTPs (c/u 100 mM)	0,5 µl
Actinomicine D (5 µg/ml)	0,6 µl
RNase inhibitor (40 U/µl)	0,5 µl
Reverse transcriptase (AMV-RT) (7 U)	0.3 µl

Primer extension is carried out with avian myeloblastosis virus reverse transcriptase (Roche) for 1 hour at 42°C. Give a pulse to the microfuge tube and stop the reaction by addition of 5 µl of 3 M sodium acetate, pH 4.8, and 150 µl of absolute ethanol (both volumes for each *n*). In this step, the preparation can be stored at -80ºC overnight.

Before loading the samples, they have to be centrifuged during 20 minutes at 13,000 r.p.m. and the supernatant is discarded. The pellet is dried in thermoblock and finally resuspended in 4 µl of TE buffer + 2 µl of loading buffer and kept on ice. Sequagel Complete and Sequagel XR solutions (Biozym) were used for the electrophoretic separation of cDNA products. The results of the separation were obtained and processed on the LI-COR DNA sequencer, model 4000 (MWG Biotech).

Hybridization buffer: 2M NaCl; 50 mM piperacine-N,N´-bis (2-ethanosulfonate)(PIPES); pH 7.

Primer PEX-nap: (5'-GGAACAGGAAGGCGAAAATC-3') PEX-nap is complementary to position 51-70 downstream of the ATG start codon of *napE*. Five picomoles of IRD-800-labelled primer was allowed to anneal 20 µg total RNA.

3. Biochemical techniques

3.1. Determination of protein concentration

The protein concentration in whole cells and periplasmic, cytosolic and membrane cell fractions was estimated using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as the standard reference (BSA, Sigma) as following:

1. Prepare dilutions of 5-25-50µl of sample in 500 µl of bidistilled water, and a reference with only water. In the case of whole cells, a previous treatment was necessary to achieve cell lysis by dilution in an equivalent volume of NaOH 1N, vortex, and by boiling them during 20 minutes. After that, these samples were centrifuged at 12,000 r.p.m. during 5 minutes to eliminate the cells debris, and the supernatant was used for protein determination.
2. Take aliquots of each sample and complete to 800 µl final volume with bidistilled water.
3. Add 200 µl of Bradford (*BIO-RAD*) reagent.
4. Mix with vortex and incubate 20 to 60 minutes.
5. The protein concentration was determined spectrophotometrically by measuring absorption at a 595 nm wavelenght using an UVICON 860 spectrophotometer.

3.2. Enzymatic activity assays

3.2.1. Determination of nitrate reductase activity

The determination of nitrate reductase activity using artificial electron donors has been performed by using the method described by Delgado *et al.* (2003):

1. Reaction mixture:
   250 µl of NR mixture.
   200 µl of 50 mM Tris-HCl.
   50 µl of the cell suspension (0.3 – 0.5 mg protein).
2. The reaction is initiated by adding 50 µl of sodium dithionite solution. Mix gently and immediately incubate the tubes at 30°C during 15 to 30 minutes.
3. As control mix vigorously the reaction when sodium dithionite is added until the blue color disappear.

NR mix: (for 100 ml) 40 ml of 125 mM Tris-HCl, pH 7.5; 20 ml of 100 mM KNO_3; 20 ml of 2 mM methyl viologen or benzyl viologen; 20 ml of bidistilled water.

The methyl viologen is an artificial electron donor which action is limited to the periplasmic space when used in intact cells, whereas the bencyl viologen is an artificial electron donor active in periplasm as well as in cytosol when used in intact cells, because of its capacity to pass through the inner membrane.

Sodium dithionite solution: 46 mM of sodium dithionite in 50 mM TrisHCl, pH 7.5 (8 mg/ml).

The determination of nitrate reductase activity using physiological electron donors has been performed by using the methodology described above and modified as followed;

1. Reaction mixture:

250 µl of NR mixture.

250 µl of cell suspension (1.5 – 2.5 mg protein).

2. The reaction iniciate immediately when cell suspension is added to the NR mixture. Mix gently and immediately incubate the tubes at 30°C during 15 to 60 minutes.

NR mixture: (for 30 ml) 10 ml of 300 mM Tris-HCl; 10 ml of 60 mM KNO_3; 10 ml of 180 mM sodium succinate. The sodium succinate is a physiological electron donor for NapC.

3.2.2. Determination of nitrite

For measuring nitrite concentration, the diazotation assay of Griess-Yllosway has been used as described previously (Hageman and Hucklesby, 1971; Nicholas and Nason,1957);

1. For 0.5 ml of sample, add equal volume of sulfanilamide and N.N.E.D.A. Mix and incubate at room temperature during 20 minutes.
2. Measure the absortion at 540 nm wavelength. The standard curve of nitrite has been prepared with known concentrations of a KNO_2 solution.

Sulfanilamide: dissolve 5 g of sulfanilamide in 100 ml of concentrated chlorhydric acid (12 N). Dilute to 500 ml with distilled water. Store in dark at 4°C.

N.N.E.D.A. (N-naphthyl ethylene diamine acid): dissolve 100 mg of N.N.E.D.A. in 500 ml of distilled water. Store in dark at 4°C.

KNO_2 solution: different concentrations of KNO_2 in 50 mM Tris-HCl, pH 7.5.

3.2.3. Determination of alkaline phosphatase activity

Determination of alkaline phosphatase activity has been performed as described by Brickman and Beckwith (1975) and adapted as followed;

1. Prepare liquid culture of the *B. japonicum* strains of interest in the required conditions until they raise the stationary growth phase (0.6-0.8 at OD_{600}).
2. Mix:

    300 µl culture

    500 µl 1 M TrisHCl, pH 8

    100 µl chlorophorm

    50 µl 0.1% SDS in 1 M TrisHCl
3. Vortex.
4. Preincubate at 30°C during 5-10 minutes.
5. Add 200 µl of O.N.P.P. to initiate the reaction. Mix and incubate at 30°C.
6. Measure the time until seeing yellow color appearing, and stop the reaction by the addition of 200 µl of 1M K_2HPO_4.
7. Centrifuge 5 minutes at 13,000 r.p.m.to pellet the rest of cells.
8. Recover the supernatant and mesure the absortion at 420 nm.
9. Units of alkaline phosphatase are calculated by the following formula:

$$U = \{1000 \times O.D._{420}\} / \{t_{(min)} \times V_{(ml)} \times O.D._{600}\}$$

$O.D._{420}$ and $O.D._{600}$ are the optical densities at 420 and 600 nm respectively, t is the incubation time and V is the volume of cell culture.

O.N.P.P. (orthonitrophenylphosphate): 4 % in 1 M TrisHCl, pH 8.

3.3. Separation of protein samples in polyacrylamide gel (PAGE-SDS)

3.3.1. Preparation of polyacrylamide gel

Polyacrylamide gels were prepared as described by Laemmli (1970), using a acrylamide:bisacrylamide mixture. Acrylamide:biscrylamide final concentration of stacking

gels was usually 5 %, final concentration of resolving gels was between 12 % and 15 %, according to the expected size of the proteins (bigger or smaller, respectively) (Table 2.6). Denaturing gels were prepared with SDS for denaturation of the proteins, and β-mercaptoethanol for denaturation of covalent disulfure bonds when necessary. For non-denaturing gels, SDS was replaced with water.

Table 2.6. Composition of resolving and stacking gels.

RESOLVING GEL (12/15 %)		**STACKING GEL (5 %)**	
Water	6.6/4.6 ml	Water	6.8 ml
1.5 M TrisHCl, pH 8.8	5 ml	1 M TrisHCl, pH 6.8	1.25 ml
0.1 M EDTA, pH 7.5	0.4 ml	0.1 M EDTA pH 7.5	0.2 ml
10 % SDS	0.2 ml	10 % SDS	0.1 ml
Acrylamide:Bisacrylamide (29.2:0.8), 30%	8/10 ml	Acrylamide:Bisacrylamide (29.2:0.8), 30%	1.7 ml
10 % ammonium persulfate	200 µl	10% ammonium persulfate	100 µl
TEMED	10 µl	TEMED	10 µl

After polymerization, the gels are ready to use or they can be stored at 4°C overnight, covered with a wet filter paper and a plastic film.

3.3.2. Preparation of the samples and separation of proteins

Protein samples (10 to 50 µg protein) were dissolved into loading buffer (final concentration 1×) and were heated at 100°C during 1-2 minutes. For non-denaturing gels, the samples were not heated, and the loading buffer did not contain SDS and β-mercaptoethanol.

The electrophoresis was performed in a Miniprotean II (Bio-RAD), in which the gels (around 7 × 10 cm) were prepared with a 0.75 mm thickness. Electrophoresis was performed at 20 mA and 4°C in running buffer during 3 hours approximately.

Loading buffer (2×): 60 mM Tris-HCl, pH 6.8; 20 % glycerol; 2 % SDS; 2 mM EDTA; 10 % β-mercaptoethanol; 0.2 % bromophenol blue.

Running buffer (5×): 15 g Tris; 72 g glycine, 1 l distilled water. To prepare 1 × running buffer, dilute 200 ml of 5 × running buffer in 800 ml of distilled water and then add 10 ml of 10 % SDS.

3. 4. Protein transfer from PAGE gel to blotting membranes

After separation of the proteins by electrophoresis, they were transfered to nylon or PVDF membranes (Immobilon-P, Millipore) as described by Bradley *et al*. (1988);

1. Previously to its use, the membrane has to be equilibrated by immersion in methanol during 15 seconds, in milli-Q water during 2 minutes, and in at least 5 minutes in transfer buffer.
2. Some Whatman 3MM paper with the same size as the membrane have to be prepared and wet in transfer buffer as the sponges.
3. A sandwich is prepared with the following layers: sponge, Whatman paper, polyacrylamide gel, membrane, Whatman paper, sponge. This preparation is placed in the transfer system (Miniprotean II, *BIO-RAD*) and submitted to 10 mA during 3 hours at 4ºC.
4. After transfer, the membrane is kept in PBS buffer.
5. To check if transfer was correctly performed, the piece of membrane where the protein weight marker has been transferred is cuted, and stained with Ponceau red during 2-3 minutes before beeing stored in PBS buffer. If a prestained protein weight marker is used, this step is not necessary.
6. The rest of the membrane can be processed in different ways according to the wished detection (Section 3.5).

Ponceau red solution: 0.4 % (p/v) of Ponceau S in 3 % (v/v) trichloroacetic acid.

Transfer buffer: 25 mM Tris (18 g); 192 mM glycine (86.4 g); 20 % v/v methanol (1,200 ml); distilled water 4,800 ml; pH 8.3.

PBS (Sodium phosphate buffer): 80 mM $Na_2HPO_4.2H_2O$ (14.24 g); 20mM $NaH_2PO_4.H_2O$ (2.76 g); 100 mM NaCl (5.84 g); distilled water 1 l; pH 7.5.

3.5. Detection of proteins in the membrane

3.5.1. Detection of cytochromes *c* by heme-staining

The membrane was directly incubated in quimioluminescence solution for 2-3 minutes with shacking. For developing peroxidase activity, after removing the liquid, the membrane is packed in a plastic film before being exposed to the autoradiography film (Hyperfilm β-max, Amersham) for some seconds (to 10 minutes) which was immediately incubated in developer and fixer (TETENAL®) by following the recommended dilutions and times. As the film was drying, the membrane was rinsed in PBS buffer before being stained with Ponceau red to check the concentration of proteins in each lane.

Chemoluminiscence solution: 8 mg of luminol (Roche®) in 20 ml of [50 mM Tris-HCl pH 8.6 and 150 mM NaCl]; 200 µl of 1 % 4-iodophenol in dimethyl sulfoxide; 3.2 µl of 30 % H_2O_2. 4-iodophenol and H_2O_2 are added to the solution just before use.

3.5.2. Detection of the NapA protein by inmuno-staining

The membrane was incubated in bloking buffer, with shaking during 2 hours at room temperature or overnight at 4°C. The membrane was then quickly washed with PBST buffer, before being incubated in 10 ml of PBSTA containing 1/1000 (v/v) antibody dilution (anti-NapA of *Paracoccus pantotrophus*, kindly provided by Dr. David Richardson, University of East Anglia, Norwich, UK). The membrane was incubated by shaking gently during two hours at room temperature. After the incubation with the first antibody, the membrane was washed with PBST, three times during 10 minutes, always with shaking.

Incubation with the second antibody was performed in a dilution 1/1000 (v/v) solution of rabbit anti-IgG (conjugated with peroxidase) in PBSTA, during two hours at room temperature and with shaking. The membrane was then washed three times with PBST and two times with PBS, always six minutes and with shaking.

Finally, the detection was performed using the reaction of chemoluminiscence. For this purpose, the membrane was incubated 2-3 minutes with shaking in the solution. After removing the liquid, the membrane was packed in a plastic film before being exposed to the autoradiography film (Hyperfilm β-max, Amersham) for a maximun of 15 minutes depending

on the intensity of the luminiscence) which was immediately revealed in developer and fixer TETENAL®, in the recommended dilutions and times. As the film was drying, the membrane was rinsed in PBS buffer before beeing stained with Ponceau red to check the concentration of proteins in each lane.

Blocking buffer: PBS buffer with 5 % low fat dried milk.
PBST buffer: PBS buffer with 0.1 % Tween 20.
PBSTA buffer: PBSTA buffer with 0.1 % (p/v) bovine albumin serum (Sigma).

4. Inoculation with *B. japonicum* and physiological analysis in soybean plants

4.1. Seeds sterilization and germination

1. Immersion of the soybean seeds (*Glycine max* L. *Merr*., cv. Williams) in 96 % (v/v) alcohol during 30 seconds. Discard the alcohol.
2. Submerge the seeds in 15 % hydrogen peroxide during 8 minutes.
3. Wash 5-6 times with abundant sterile distilled water and place them in Petri plates containing sterile filter paper wet with sterile water or 15 % solid agar (around 12 seeds per plate).
4. Let the seeds germinating in dark at 28ºC during 48 hours.
5. Control humidity every day.

4.2. Nutrient solution for soybean plants growth

A mineral solution has been employed, derived from Rigaud and Puppo's recipe (1975) and modified as described in the following protocol;

- Macroelements (for 1 l solution): 68 mg of KH_2PO_4; 44 mg of K_2HPO_4; 123 mg of $SO_4Mg.7H_2O$; 174 mg of K_2SO_4; 173 mg of SO_4Ca; 25 mg of ferric-EDTA.
- Microelements (for 1 l solution): 0.11 mg of $MoO_4Na_2.2H_2O$; 2.85 mg of BO_3H_3; 3.07 mg of $SO_4Mn.4H_2O$; 0.55 mg of $SO_4Zn.7H_2O$; 0.2 mg of $SO_4Cu.5H_2O$.
- The pH of the solution was between 7.0 ± 0.1 and sterilization was performed in an autoclave at 121°C during 15 minutes.

4.3. Soybean plants growth conditions

Once germinated, soybean seeds having an uniform development grade were planted in Leonard jars (Leonard, 1943). The jars are composed by two elements, a upper component filled with vermiculite and a lower component containing the nutritient solution described above (Section 4.2). In each jar three seedlings were inoculated with 1 ml of liquid culture of *B. japonicum* (10^8 cells/ml). Then, the seedlings were covered with a layer of perlite which avoid a possible contamination.

The jars were incubated in a growth chamber with the following conditions: 500 $\mu Einstein.m^{-2}.s^{-1}$ (wavelenght: 400-700 nm) light intensity, photoperiod 16/8 hours light/darkness, temperature of 23/17°C day/night and 50 % relative humidity. Plants were harvested 42 days after inoculation, when they presented around 10 % flowering.

4.4. Measurement of nitrogen fixation capacity

The specific nitrogenase activity of nodules from plants inoculated with selected strains was routinely determined by measuring the Acetylene Reduction Activity (ARA) of the nodules attached to the root system.

1. Take the plants out from the jars and wash gently the roots with water.
2. Separate the root system from the rest of the plant and introduce it in 16 or 300 ml capacity flasks closed with perforable rubber tapes.
3. Extract from the frask 5 % of its gaseous atmosphere and inject 0.8 or 15 ml of acetylene.
4. Incubate at room temperature (for several minutes (2.5 to 7.5) to one hour according to the references and the equipment used for gas chromatography).
5. Extract 100 or 500 µl of the gaseous atmosphere and fractionate it by injection into a gas chromatograph (Hewlett Packard modelo 5890 with a flame ionization detector, a 1.80 m x 3.2 mm column covered with 80-100 mesh Porapak Q; or into a 1 m long Poropack® R 80/100 column in a Perkin Elmer gas chromatograph provided with a flame ionization detector).The temperature of hood, injector and detector were 65ºC or 60°C, 120ºC and 105ºC respectively. Nitrogen was used as the carrier gas at a flow rate of 1 ml/sec or 0.5 ml/sec. The flame was prepared with a mixture of hydrogen and air. The peak of ethylene was identified by comparison with a standard. The specific nitrogenase activity per hour and gram of fresh nodular tissue was calculated from the peak area of ethylene calibrated with an external C_2H_2 standard. Results were expressed as nmol ethylene/ gr × hr.

4.5. Analysis of physiological parameters of soybean plants inoculated with *B. japonicum*

After 35 days growing, plants were harvested and roots were washed with water and dried on paper. Nodules were counted and weighted to determine the nodule number (NN) and the nodules fresh weight (NFW). Nodules and plants were then dried at 80° C during 48 hours in order to determine the nodules dry weight (NDW), the plant dry weight (PDW). All the values obtained were analyzed stadistically with the programm Statgraphics Plus 5.1.

4.6. Determination of nitrogen content

After drying and weighting the plants, they were grinded. Samples were then submitted to mineralization as described by Lachica *et al.* (1965, 1973). The nitrogen was measured by the Kjedhal method using the Bouat and Crouzet system (1965), based on colorimetric determination of ammonia (NH_3) formed by the reaction of nitrogen with sodium hydroxide (NaOH). Measurements were realized with a Beckman-25 spectrophotometer. Values are expressed in mg of nitrogen / plant, and they have been analyzed stadistically with the programm Statgraphics Plus 5.1.

4.7. Bacteroids isolation

Nodules were harvested from 35 days old plants and bacteroids prepared as previously described by Arrese-Igor *et al.* (1998);

1. 1.5 g of fresh nodules are ground in 7 ml of cold extraction buffer supplemented with 300 mM sucrose just before use.
2. The homogenate is filtered through 4 layers of cheesecloth to eliminate plant cell rests and centrifuged at 1,500 r.p.m. and 4°C for 2 minutes to remove nodules debris.
3. The resulting supernatant is re-centrifuged at 8,000 r.p.m. and 4ºC for 10 minutes to pellet the bacteroids. The bacteroids are washed twice with 50 mM Tris-HCl (pH 7.5) and resuspended in a final volume of 2 ml of this buffer.

The determinations of protein concentration and nitrate reductase activity were achieved as described in Sections 3.1 and 3.2.1 respectively.

Extraction buffer: 100 mM Tris-HCl, pH 7.5. Sterilization by autoclaving. Storage at 4°C.

4.8. Electron microscopy technique

4.8.1. Fixation and embedding of nodules for electron microscopy

The fixation and embedding of nodules in L.R. White resin (The London Resin Company, Basingstoke, UK) were achieved as described by Müller *et al.* (1995);

1. The nodules harvested are washed to remove residual vermiculite, cutted in half with a rasor blade and placed in eppendorf tubes.
2. The nodules are rinsed with phosphate buffer.
3. Fixation of the nodules is achieved by incubation in ice-cold glutaraldehyde solution under vacuum during 4 hours or until nodules precipitate to the bottom of the recipient.
4. Wash 3 times with phosphate buffer during 20 minutes.
5. A post-fixation step is realized by rinsing the nodules with osmiumtetraoxide (2% in phosphate buffer) during 2 hours.
6. Wash 2 times with phosphate buffer during 20 minutes.
7. Dehydratation is achieved by several washes in ethanol. The first one in 30% ethanol during 30 minutes.
8. Wash then the nodules with 50% ethanol during 1 hour.
9. Wash the nodules overnight with 70% ethanol.
10. Wash the nodules with 80% ethanol during 1 hour.
11. Rinse the nodules in 90% ethanol.
12. Rinse the nodules 3 times in ethanol 100% during 20 minutes.
13. Nodules are then incubated overnight in L.R. White resin : ethanol (1:1) and finally infiltrated in L.R. White resin.
14. Polymerisation is carried out in gelatine capsules at 60°C during 24 hours.

Phosphate buffer: 0.2 M KH_2PO_4, 0.2 M Na_2HPO_4, pH 7.0

Glutaraldehyde solution: 4% glutaraldehyde in phosphate buffer

4.8.2. Microtomy and electron-microscopical analyses

Microtomy and electron-microscopical analyses were carried out as described earlier by Werner and Mörschel (1978). Ultrathin sections of ~70-90 nm are cutted with a diamond knife and placed on a copper grid. The sections are stained with uranyl acetate or lead citrate.

3. RESULTS

Chapter 3.1. Identification and characterization of the periplasmic nitrate reductase from *B. japonicum*.

ABSTRACT

The *napEDABC* gene cluster that encodes the periplasmic nitrate reductase from *Bradyrhizobium japonicum* USDA110 has been isolated and characterized. *napA* encodes the catalytic subunit, and the *napB* and *napC* gene products are predicted to be a soluble dihaem *c* and a membrane-anchored tetrahaem *c*-type cytochrome, respectively. *napE* encodes a transmembrane protein of unknown function, and the *napD* gene product is a soluble protein which is assumed to play a role in the maturation of NapA. Western blots of the periplasmic fraction from wild-type cells grown anaerobically with nitrate revealed the presence of a protein band with a molecular size of about 90 kDa corresponding to NapA. Two *B. japonicum* mutants carrying an insertion in the *napA* and in the *napC* genes respectively, were unable to grow under nitrate-respiring conditions and lacked nitrate reductase activity when succinate was used as physiological electron donor. The *napA* mutant lacked also nitrate reductase activity when methyl-viologen (MV^+) or bencyl-viologen (BV^+) were used as artificial electron donors, and did not show the 90 kDa protein band. However, the *napC* mutant presented similar MV^+ dependent or BV^+ dependent nitrate reductase activities as the wild type strain, and a 90 kDa protein band corresponding to NapA. Complementation of the *napA* mutant with a plasmid bearing the *napEDABC* genes restored both nitrate dependent anaerobic growth of the cells and nitrate reductase activity. A membrane-bound and a periplasmic *c*-type cytochrome, with molecular masses of 25 kDa and 15 kDa, respectively, were not detected in the *napA* mutant strain incubated anaerobically with nitrate, which identifies those proteins as the NapC and the NapB components of the *B. japonicum* periplasmic nitrate reductase enzyme. These results suggest that the periplasmic nitrate reductase is the enzyme responsible for anaerobic growth of *B. japonicum* under nitrate-respiring conditions. The promotor region of *napEDABC* genes has been characterized by primer extension. A major transcript initiates 66.5 bp downstream of the centre of putative FNR-like binding site. Primer extension experiments and expression analysis of a translational fusion of the NapC protein to the reporter gene phoA showed that maximal expression of nap genes requires low oxygen conditions and the presence of nitrate.

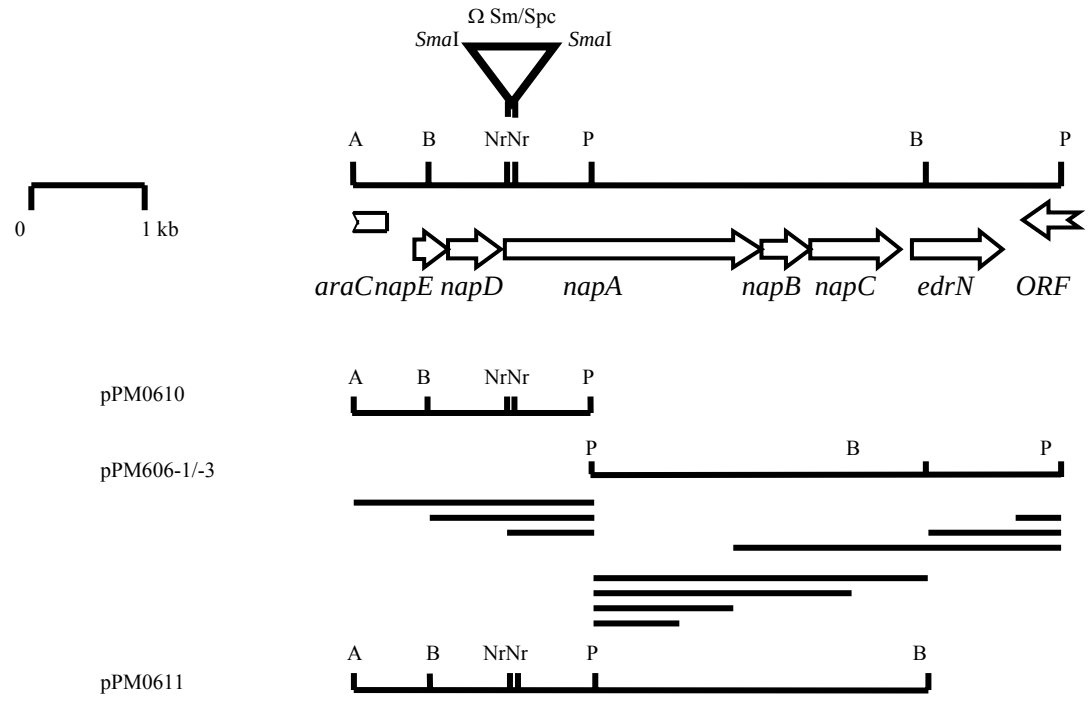
A shotgun cloning experiment in *B. japonicum* USDA110 was previously performed by Dr. Peter Müller from Marburg University. As result of this cloning experiment, DNA fragments were obtained after DNA restriction with *Pst*I and they were preselected for their size within a range of 6-15 kb. These fragments were ligated into the mobilizable vector pJQ200SK (Quandt and Hynes, 1993). Plasmid pPM200P9134 was thus isolated containing a 6.3 kb *Pst*I fragment of cloned *B. japonicum* DNA (data not shown). Partial sequencing of pPM200P9134 revealed the presence of one ORF showing homology with that of *napA* genes from other bacteria (data not shown) encoding the catalytic subunit of the periplasmic nitrate reductase. Because this extracytoplasmic protein was not previously studied in *B. japonicum*, in this chapter the sequence and characterization of the *B. japonicum* genes encoding the periplasmic nitrate reductase have been undertaken.

1. Isolation and sequencing of the *napEDABC* genes

After restriction analysis of pPM200P9134, a 1.96 kb *Apa*I-*Pst*I internal fragment of pPM200P9134 was subcloned into pJQ200SK, yielding plasmid pPM0610 (Figure 3.1). This plasmid was used to construct a *napA* mutant named GRPA1 (Figure 3.1) as described in Section 3.1. Genomic DNA of strain GRPA1 was restricted with *ApaI* and the resulting fragments were ligated to pBluescript KS+ (pBSKS, Stratagene). Plasmid pBG0605 was thus obtained, harbouring a 14 kb fragment of GRPA1(data not shown). An internal 4.7 kb *Pst*I fragment of pBG0605 was subcloned into pJQ200SK in both directions yielding plasmids pPM606-1 and pPM606-3, respectively. Plasmids pPM0610, pPM606-1 and pPM606-3 were used to create a series of deletion derivatives (Figure 3.1) which were truncated from both ends, and which were used for sequencing.

The 5'-end of the 1.96kb *Apa*I-*Pst*I fragment exhibited sequence similarity to the N-terminal part of an *araC*-like transcriptional regulator, which was encoded on the opposite strand. Therefore the promoters for two divergent operons were expected to be located within the intergenic region between this regulatory gene and *napE* (Figure 3.1). At the 5'-end, the central and C-terminal part of *napA* was identified, followed by *napB, napC* and an ORF (*edrN)* encoding an exodeoxyribonuclease (Figure 3.1).

Figure 3. 1. Organization of the *B. japonicum* USDA110 *napEDABC* genes. Arrows indicate the location and orientation of the deduced open reading frames.The insertion containing the streptomycin/spectinomycin resistence gene (Ω, Sm/Spc) is marked by a triangle. Deletion derivatives created from plasmids pPM0610 and pPM606-1/-3 used for sequencing are represented by lines of various lengths below them. A, *Apa*I; B, *Bam*HI; Nr, *Nru*I; P, *Pst*I.



2. Analysis of *napEDABC* sequences

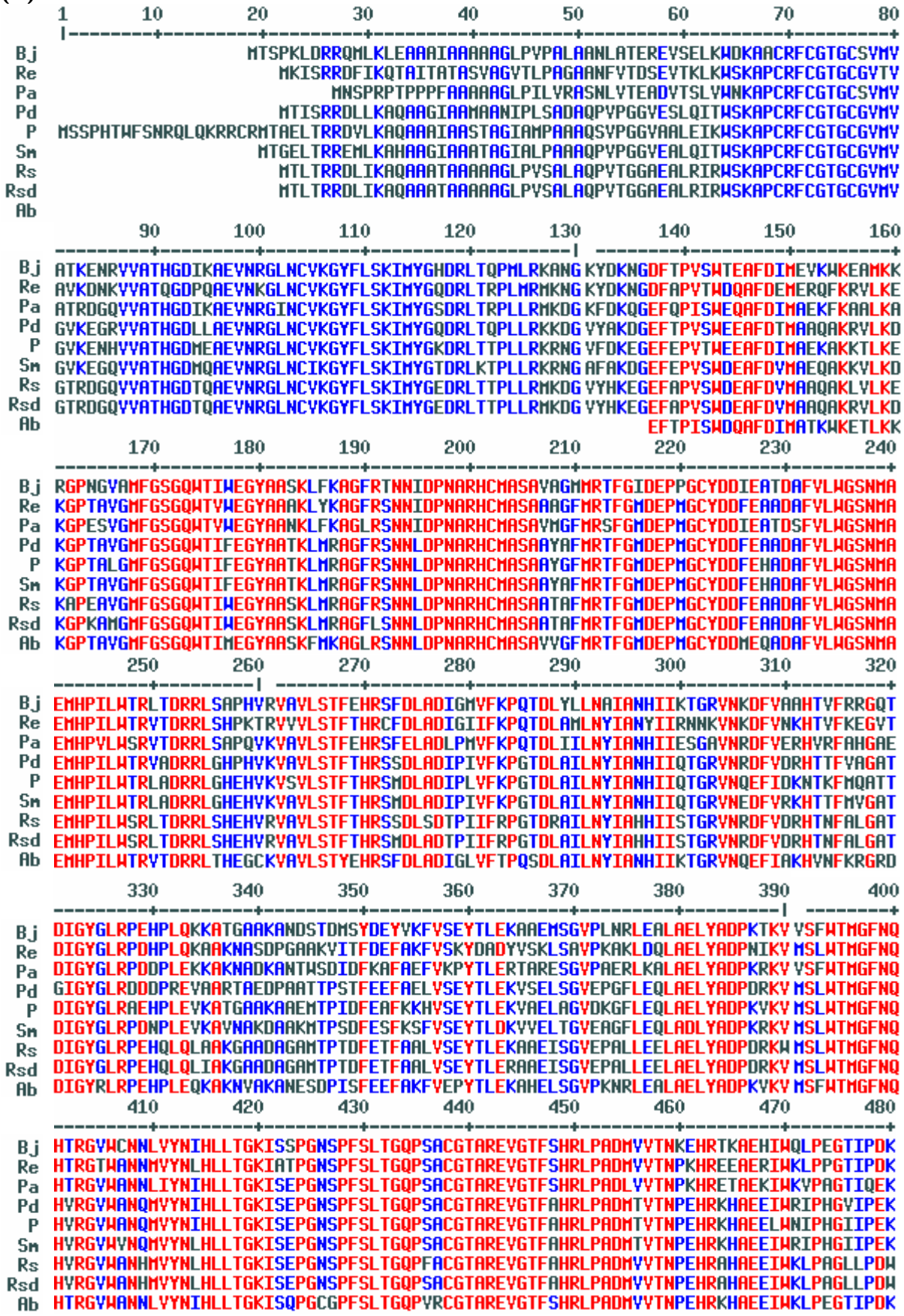
Sequence analysis of the DNA cloned in plasmids pPM0610, pPM606-1 and pPM606-3 described above (Section 1) showed that it contained five ORFs with a G+C content of 65 mol %. The *napE*, *napD*, *napA*, *napB* and *napC* ORFs are 186, 333, 2517, 471, and 642 bases in length, respectively, and encode proteins of 61 (6.6 kDa), 110 (11.8 kDa), 838 (94.5 kDa), 156 (16.9 kDa) and 213 (23.9 kDa) aa residues, respectively. The deduced primary sequences of NapA, NapB, and NapC have between 46% and 76% identity with the translated sequences of the *napA*, *napB*, and *napC* genes from *Ralstonia eutropha* H16 (Siddiqui *et al*., 1993), *Thiosphera pantotropha* (proposed for reclassification as a strain of *Paracoccus denitrificans*) (Berks *et al*., 1995), *Rhodobacter sphaeroides* DMS158 (Reyes *et al*., 1996, 1998), *Rhodobacter sphaeroides* sp. *denitrificans* (Liu *et al*., 1999), *Pseudomonas* sp. G-179

(Bedzyk *et al*., 1999), *Pseudomonas aeruginosa* PAO1 (Stover *et al*., 2000), *Azotobacter brasilense* (Steenhoudt *et al*., 2001), and *Sinorhizobium meliloti* (Galibert *et al*., 2001) (see alignments in Figure 3.2). The deduced primary sequences of NapE and NapD have between 31 % and 61 % identity with the translated sequences of the *napE*, and *napD* genes of *Ra. eutropha* H16 (Siddiqui *et al*., 1993), *Pa. denitrificans* (Berks *et al*., 1995), *Rh. sphaeroides* DMS158 (Reyes *et al*., 1996, 1998), *Rh. sphaeroides* sp. *denitrificans* (Liu *et al*., 1999), *Pseudomonas* sp. G-179 (Bedzyk *et al*., 1999), *Ps. aeruginosa* PAO1 (Stover *et al*., 2000), and *S. meliloti* (Galibert *et al*., 2001) (See alignments in Figure 3.3).

In the N-terminal region of NapA, the sequence ^{7}DRRQMLK^{14} (Figure 3.2) corresponds closely to the twin arginine motif identified in a large number of periplasmic metalloproteins that are exported via the Sec-independent TAT translocon (Berks, 2000). A conserved [4Fe-4S] binding motif is also present in the N-terminal end of NapA. In contrast, the amino acid sequence ^{4}RFGIALLAVAIAAG^{17} at the N-terminal end of NapB (Figure 3.2) indicates that the protein is translocated by the general secretory pathway (Pugsley, 1993). In the NapB sequence two CXXCH motifs are identified (^{82}CLSCH^{86} and ^{122}CTECH^{126}) (Figure 3.2), representing di-haem cytochrome *c* binding sites, whereas in NapC there are four such motifs (^{69}CTGCH^{73}, ^{99}CPDCH^{103}, ^{159}CRNCH^{163}, and ^{192}CIDCH^{196}) (Figure 3.2)**.** Localized after a putative transmembrane segment of 27 amino acids. There are 6 amino acids positively charged in the N-terminal end of NapC, which could interact with negatively charged phosphate residues, in the cytoplasmic membrane inner layer, anchoring the protein in the membrane, whereas the C-terminal end would be located in the periplasmic space. Sequence analysis predicts NapE to be a membrane protein of no currently known function. The postulated role for NapD is that of a private chaperone involved in maturation of NapA prior to export to the periplasm (Berks *et al*., 1995; Potter & Cole, 1999). The sequence of *napEDABC* genes described in this work has been deposited in the GenBank. The accession number for these genes is AF314590.

Figure 3.2. Alignment of amino acid sequences of NapA **(A),** NapB **(B)** and NapC **(C)** of *B. japonicum* (Bj) with NapA, NapB, and NapC proteins of other bacteria: *Ra. eutropha* H16 (Re), *Pa. denitrificans* (Pd), *Rh. sphaeroides* DMS158 (Rs), *Rh. sphaeroides* sp. *denitrificans* (Rsd), *Pseudomonas* sp. G-179 (P), *Ps. aeruginosa* PAO1 (Pa), *Az. brasilense* (Ab), and *S. meliloti* (Sm). In red, the amino acids in all the sequences present at least 90 % of identity, in blue the identity is between 50 and 90 %, in grey the identity of amino acids is less than 50 %.

(A)


1 10 20 30 40 50 60 70 80
Bj MTSPKLDRRQMLKLEAAAIAAAAAGLPVPALAANLATEREVSELKWDKAACRFCGTGCSVMV
Re MKISRRDFIKQTAITATASVAGVTLPAGAANFVTDSEVTKLKWSKAPCRFCGTGCGVTV
Pa MNSPRPTPPPFAAAAAGLPILVRASNLVTEADVTSLVWNKAPCRFCGTGCSVMV
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Ab
90 100 110 120 130 140 150 160
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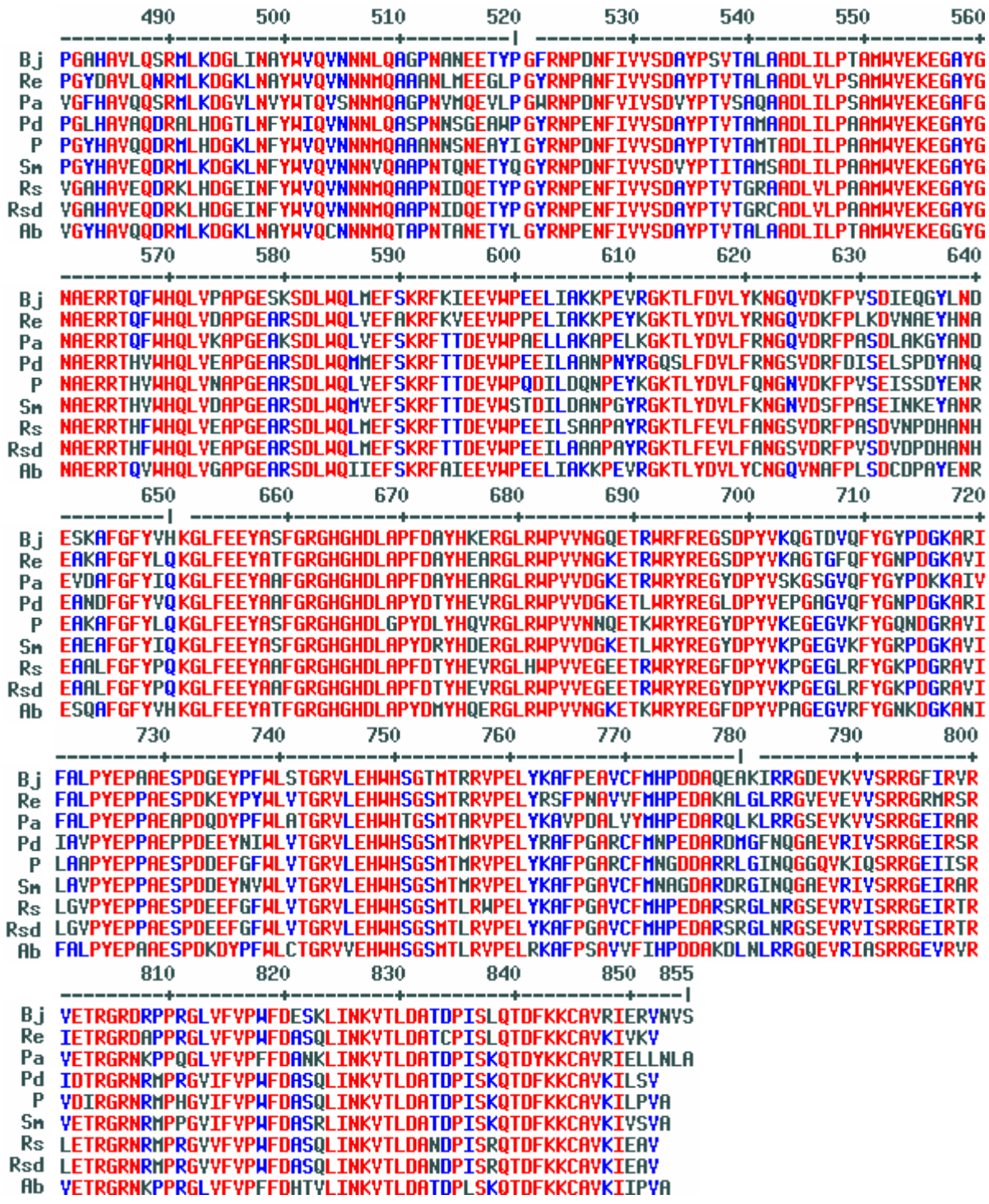
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Pd EANDFGFYVQKGLFEEYAAFGRGHGHDLAPYDTYHEVRGLRWPVVDGKETLWRYREGLDPYVEPGAGVQFYGNPDGKARI
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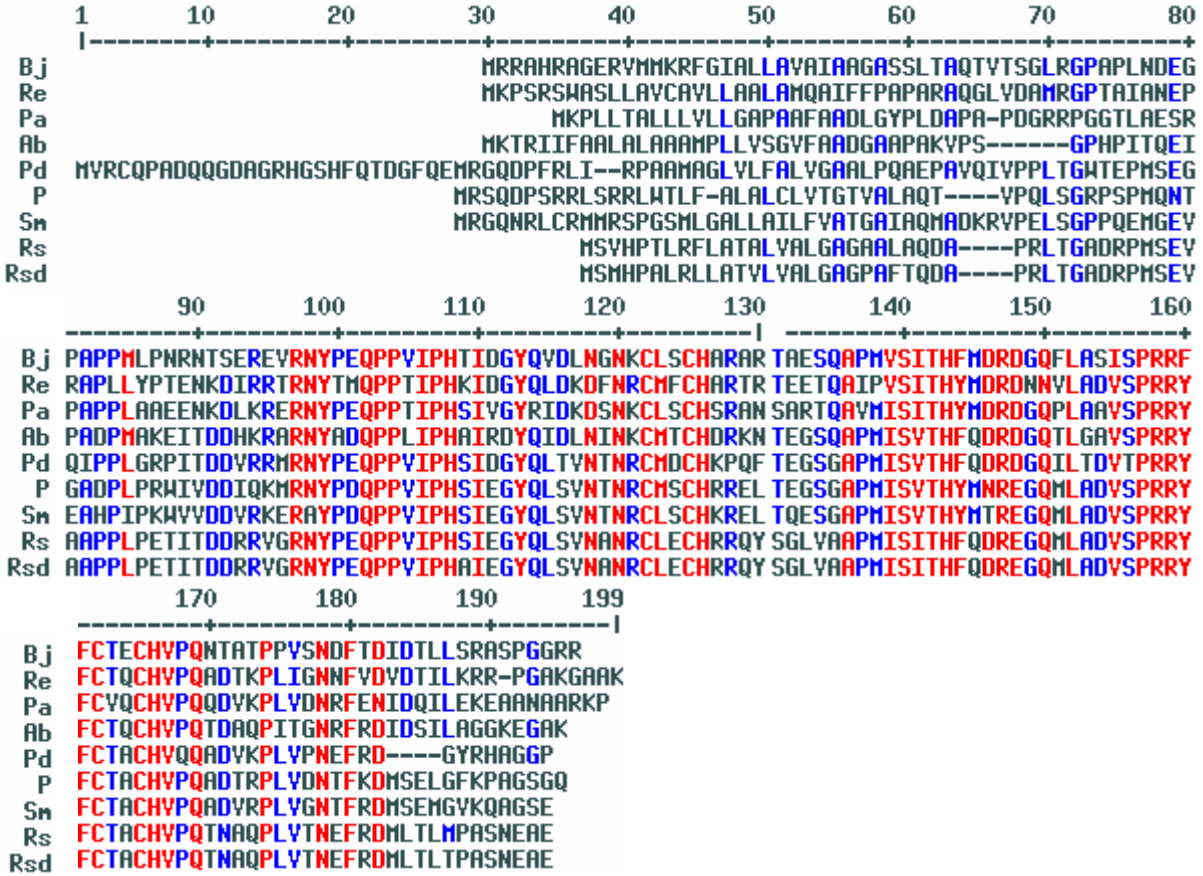
(B)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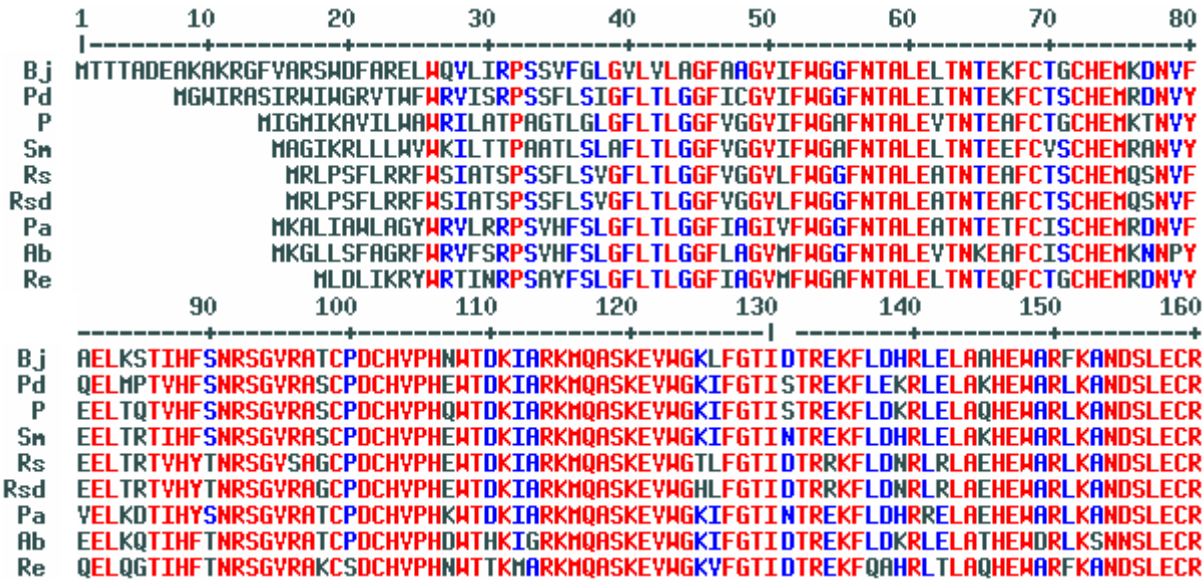
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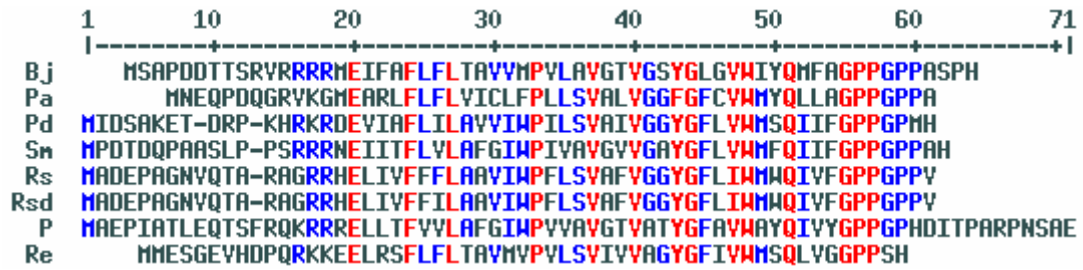
Figure 3.3. Alignment of amino acid sequences of NapE **(E)** and NapD **(D)** of *B. japonicum* (Bj) with NapE and NapD proteins of other bacteria: *Ra. eutropha* H16 (Re), *Pa. denitrificans* (Pd), *Rh. sphaeroides* DMS158 (Rs), *Rh. sphaeroides* sp. *denitrificans* (Rsd), *Pseudomonas* sp. G-179 (P), *Ps. aeruginosa* PAO1 (Pa), and *S. meliloti* (Sm). In red, the amino acids in all the sequences present at least 90 % of identity, in blue, the identity is between 50 and 90 %, in grey, the identity of amino acids is less than 50 %.

(E)



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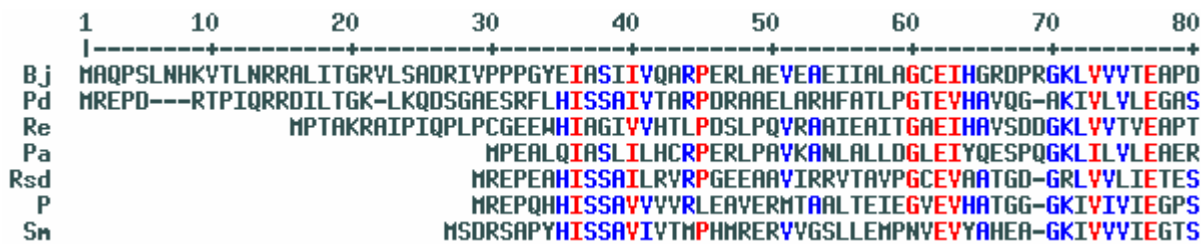
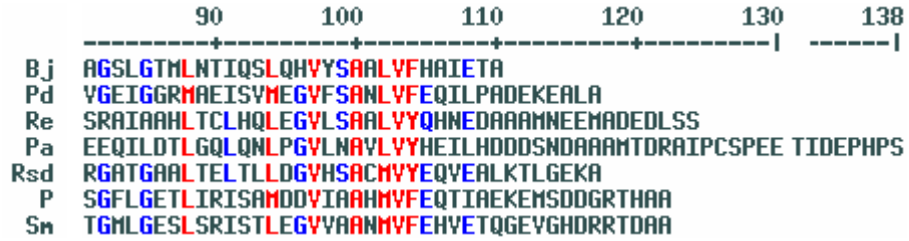




3. Isolation and characterization of *B. japonicum napA* and *napC* mutant strains

To investigate the function of the *B. japonicum nap* gene products in nitrate metabolism, the *napA* and *napC* genes were mutated by marker exchange and insertional mutagenesis, respectively.

3.1. Construction of the *napA* mutant strain GRPA1

The *napA* gene was mutated by performing gene-directed mutagenesis by marker exchange. A 1.2 kb *Bam*HI-*Pst*I fragment from pPM200P9134 was subcloned into pK18*mobsacB* (Schäfer *et al.*, 1994) to obtain plasmid pBG0602 (Section 1, Figure 3.1). Finally, the 2 kb *SmaI* fragment (Ω *Spc/Sm* interposon) of pH45Ω (Prentki and Krisch, 1984) was inserted to replace a central 24 bp *Nru*I fragment within pBG0602. The resulting plasmid pBG602Ω was transfered via conjugation to *B. japonicum* USDA110 using *Escherichia coli* S17-1 as donor strain. Double recombination events were favoured by growth on agar plates containing sucrose. Mutant strains resistant against spectinomycin/streptomycin but sensitive towards kanamycin were checked by Southern hybridization experiments (data not shown) for correct replacement of wild-type fragment by the Ω interposon. The mutant derivative GRPA1 that has been obtained was used in this study.

3.2. Genetic complementation of the *napA* mutant strain GRPA1.

To obtain a recombinant plasmid containing the complete *napEDABC* region, the *Pst*I-*Bam*HI deletion derivative from pPM606-1 was cloned in pJQ200SK to construct plasmid pPM606-1Δ. pPM606-1Δ was then linearized by double digestion with *Apa*I and *Pst*I and ligated with the 1.96 kb fragment of pPM0610, which was excised by the identical enzymes (Figure 3.1). The resulting recombinant plasmid (5.1 kb *Apa*I-*Bam*HI-*Bam*HI fragment

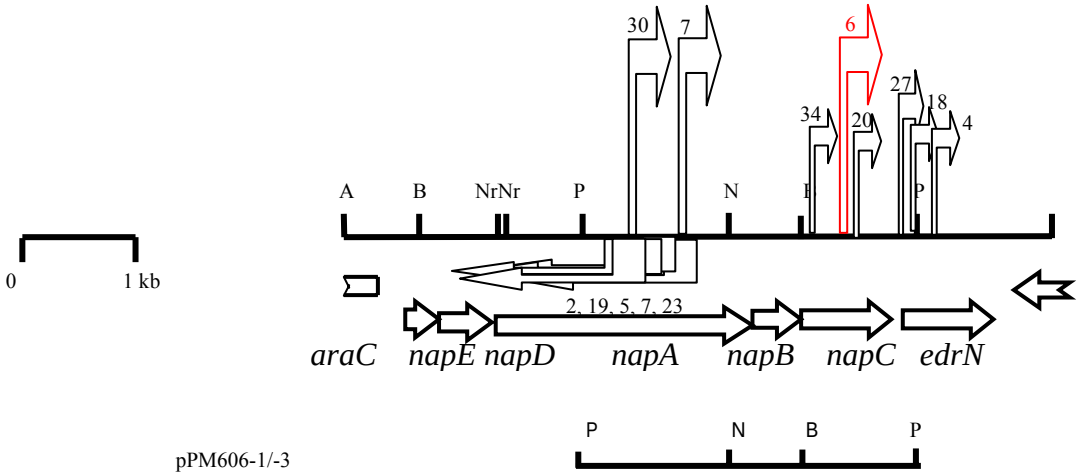
carrying the *napEDABC* cloned in pJQ200SK) was designated pPM0611 (Figure 3.1). Plasmid pPM0611 was integrated by homologous recombination into the chromosome of *B. japonicum* GRPA1 using *E. coli* S17-1 as a donor resulting in strain GRPA1C. Recombinant strains were selected for gentamycin resistance and their correct genomic structure was confirmed by Southern blot analysis of genomic DNA preparations (data not shown).

3.3. In vitro transposon insertions and construction of the *napC* mutant strains 0609, and strain 0610

Transposon Tn*KPK2* constructed by Müller (2004) was used for in vitro mutagenesis to saturate the 4.4 kb *Pst*I fragment of *B. japonicum* containing *napABC* genes and previously cloned in plasmids pPM606-1/3 (Figure 3.1).

After in vitro Tn*KPK2* transposition onto pPM606-1/3, plasmids were subsequently transformed in electrocompetent DH10B *E. coli* cells as indicated in Section 2.10 of Material and Methods. Recombinant plasmids were digested with *Pst*I and analyzed by using agarose gel electrophoresis. Plasmids differing in the fragment size of their DNA insert but not in the size of the vector band (5.6 kb), were selected for further sequencing using the insertions as starting points and the *phoA*-START (5'-GGGTGCAGTAATATCGCCCTGAGC-3') or Kan-STOP (5'- ATCCTCCAGCGCGGGGATCTCATG-3') primers, in order to determine the location and orientation of the insertions (Figure 3.4). One Tn*KPK2* insertion in *napC* named as number 6 (Figure 3.4) was detected. The plasmid pTn*KPK2*-6 containing this insertion was first transformed into *E. coli* S17-1 donor strain for its introduction into the *B. japonicum* wild-type genome via conjugation. Double recombination events were selected by resistance to kanamycin but sensitivity towards gentamycin which resulted in mutant strain 0609. The strain 0610 was obtained by single recombination event and was selected by resistance to kanamycin and gentamycin. The resulting mutants were subsequently tested genetically by Southern blot analysis of genomic DNA preparations (data not shown).

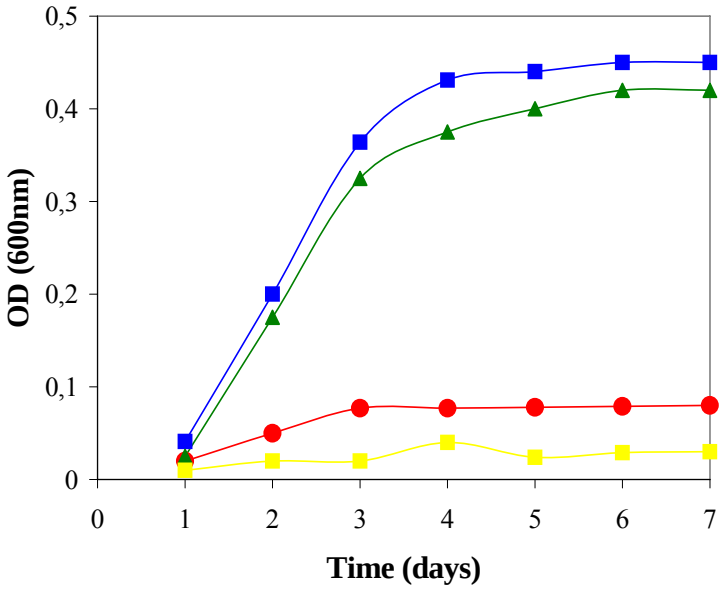
Figure 3.4. Organization of *napEDABC* genes in *B. japonicum*. The horizontal arrows indicate the location and the orientation of the predicted open reading frames. The different insertions of Tn*KPK2* containing the kanamycin resistance gene are marked by vertical arrows. The insertion in *napC* used in this work to obtain strains 0609 and 0610 is indicated in red and with number 6. Plasmids pPM606-1/-3 used for the construction of the mutants are also represented. A: *Apa*I, B: *Bam*HI, N: NotI, Nr: *Nru*I, P: *Pst*I.



3.4. Nitrate-dependent anaerobic growth of strains GRPA1, GRPA1C and 0609

Cells of *B. japonicum* USDA110, GRPA1, GRPA1C and 0609 were inoculated in YEM medium containing nitrate and they were grown under anaerobic conditions (see Section 1.2.1 of Material and Methods). Growth rate was checked every day by measuring the optical density of the culture at 600 nm. In contrast to *B. japonicum* USDA110, cells of the mutant strains GRPA1 and 0609 were unable to grow anaerobically with nitrate as the final electron acceptor (Figure 3.5). Both strains, however, grew well when the YEM medium was amended with 0.5 mM nitrite (data not shown). Complementation of strain GRPA1 with the chromosomally integrated plasmid pPM0611 containing the wild type *napEDABC* genes restored the ability of the cells to grow on nitrate as the alternative electron acceptor (Figure 3.5).

Figure 3.5. Nitrate-dependent anaerobic growth of wild-type *B. japonicum* USDA110 (■), *napA* mutant derivative GRPA1 (●), *napA* mutant complemented with plasmid pPM0611 (▲) and *napC* mutant derivative 0609 (■) strains cultivated anaerobically in YEM medium with nitrate. Growth of the cells under anaerobic conditions was measured by monitoring the optical density at 600 nm.



3.5. Nitrate reductase activity of strains GRPA1, GRPA1C and 0609

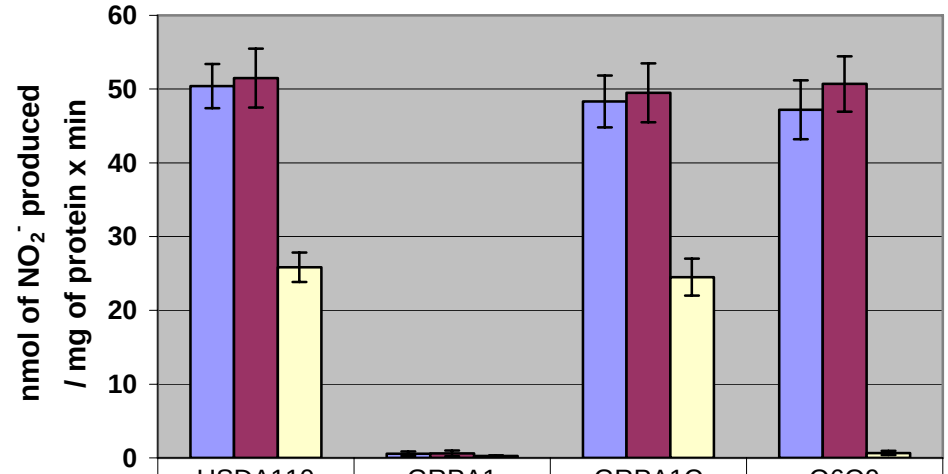
Since GRPA1 and 0609 mutants did not grow anaerobically with nitrate, cells were grown first aerobically in PSY medium, collected by centrifugation, and further incubated anaerobically in the same medium supplemented with 10 mM nitrate or 1mM nitrite. For determination of nitrate reductase activity methyl viologen (MV^+) or benzyl viologen (BV^+) were used as artificial electron donors and sodium succinate as physiological electron donor.

No differences in nitrate reductase activity were found in cells of USDA110 when either MV^+ or BV^+ was used as electron donors (Figure 3.6). Because BV^+ is permeable to the cell membrane (Bell *et al.*, 1990), the presence of membrane-bound nitrate reductase would result in higher activity when BV^+ is used as an electron donor as compared to that obtained with MV^+. Whereas no BV^+-, MV^+- or succinate-dependent activity was detected in the *napA* mutant, levels of nitrate reductase activity in cells of strain GRPA1C containing plasmid

pPM0611 were similar to those detected in the parental strain (Figure 3.6). Cells of strain 0609 showed similar values of BV^+- or MV^+-dependent nitrate reductase activity as those found in the parental strain (Figure 3.6). However, in contrast to the wild type strain USDA110, cells of the 0609 mutant incubated anaerobically with nitrate did not show nitrate reductase activity when succinate was used as physiological electron donor in the assay (Figure 3.6).

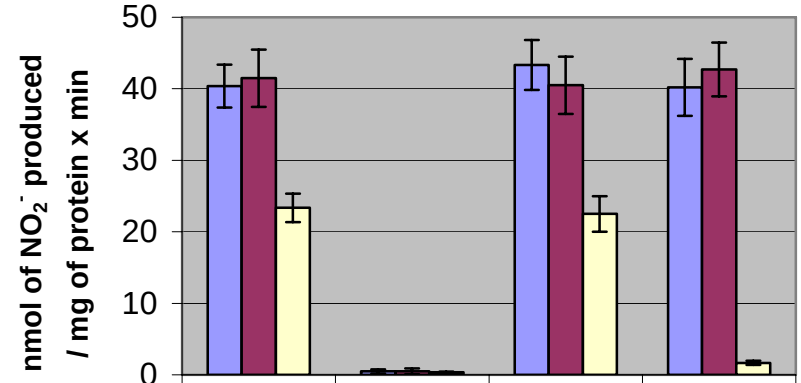
When cells of USDA110, GRPA1, GPRA1C and 0609 were cultivated with nitrite, levels of nitrate reductase activity regardless of the electron donor used were very similar as compared with those detected in cells grown with nitrate as electron acceptor (Figures 3.6 and 3.7).

Figure 3.6. Nitrate reductase activity in the parental strain USDA110, the *napA* mutant derivative GRPA1, *napA* mutant containing plasmid pPM0611, and *napC* mutant derivative 0609 of *B. japonicum.* Cells were incubated anaerobically in YEM medium supplemented with 10 mM KNO_3. Data, in nmol NO_2^- produced / mg of protein × min, are shown as means and standard error of the mean for at least three cultures which were assayed in duplicate.



	USDA110	GRPA1	GRPA1C	O6O9
MV+	50,39	0,58	48,32	47,2
BV+	51,48	0,6	49,5	50,7
succinate	25,85	0,25	24,5	0,67

Figure 3.7. Nitrate reductase activity in the parental strain USDA110, the *napA* mutant derivative GRPA1, *napA* mutant containing plasmid pPM0611, and *napC* mutant derivative 0609 of *B. japonicum.* Cells were incubated anaerobically in YEM medium supplemented with 1mM $NaNO_2$. Data, in nmol NO_2^- produced / mg of protein x min, are shown as means and standard error of the mean for at least three cultures which were assayed in duplicate.



	USDA110	GRPA1	GRPA1C	O6O9
MV+	40,39	0,48	43,32	40,2
BV+	41,48	0,5	40,5	42,7
Succinate	23,35	0,35	22,5	1,67

3.6. Detection of NapB and NapC proteins in strain GRPA1

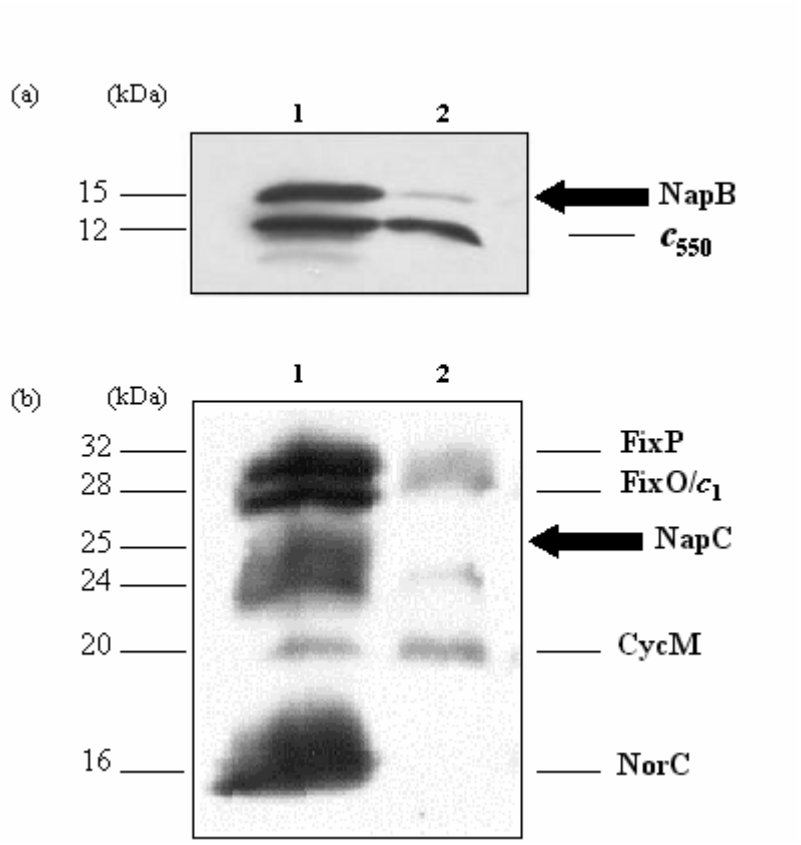
Cells of *B. japonicum* USDA110 and GRPA1 were grown aerobically in PSY medium and centrifuged at 8,000 r.p.m. and 4 °C during 10 minutes, washed with YEM medium and finally resuspended in YEM medium + 10 mM KNO_3 and incubated anaerobically for 96 hours at 28 °C. When proteins from the periplasmic fraction were stained for covalently-bound haem proteins, two stained bands of 15 and 12 kDa, respectively, were detected in the periplasmic fraction of *B. japonicum* USDA110 (Figure 3.8a, Lane 1). Whereas the 12-kDa protein was clearly visible in the *napA* mutant, the concentration of the 15 kDa protein was lower than in the parental strain (Figure 3.8a, Lane 2). The 12-kDa *c*-type cytochrome has been identified previously as the cytochrome c_{550} encoded by *cycA* and required for nitrate respiration (Bott *et al*., 1995). It is possible that the weak 15-kDa protein band still present in the periplasmic fraction of the *napA* mutant might correspond to another soluble *c*-type cytochrome co-migrating with NapB which is absent in such mutant. In fact, the presence of a soluble cytochrome c_{555} of about 15 kDa has been demonstrated in soluble crude-extracts

from *B. japonicum* (Bott *et al*., 1995), which might also be present in anaerobically incubated cells.

To establish the presence of the NapC cytochrome, proteins from the membrane fractions of the wild-type and mutant strain GRPA1 were separated by SDS-PAGE and stained for covalently bound haem proteins. Six stained bands of 32, 28, 25, 24, 20 and 16 kDa were detected (Figure 3.8b, Lane 1). The proteins of 28, 20 and 16 kDa have been identified previously as the *B. japonicum* cytochrome c_1 (Thöny-Meyer *et al*., 1989), CycM (Bott *et al*., 1991), and NorC subunit of the nitric oxide reductase enzyme (Mesa *et al*., 2002), respectively. As described by Preisig *et al*. (1993), there is even a seventh protein of 28 kDa co-migrating with cytochrome c_1. This 28 kDa protein and the 32 kDa *c*-type cytochrome have been identified as the *B. japonicum* FixP and FixO proteins, respectively, of the cbb_3-type, high-affinity cytochrome oxidase encoded by the *fixNOQP* operon (Preisig *et al*., 1993 and 1996). A haem-stainable band of approximately 25 kDa, which is the predicted size for NapC, is very prominent in anaerobic wild-type membranes and absent in the *napA* mutant GRPA1 (Figure 3.8b, Lanes 1 and 2 respectively), which identifies this protein as the NapC component of the *B. japonicum* USDA110 periplasmic nitrate reductase. The concentration of FixP and FixO/c_1 *c*-type cytochromes in the *napA* mutant was lower than in the parental strain (Figure 3.8b, Lanes 1 and 2). FixP and FixO are not synthesized under aerobic conditions and, because of the inability of the mutant strain to grow anaerobically with nitrate, the expression of those proteins during the anaerobic incubation period might be reduced. Membranes from strain GRPA1 also lacked the 16 kDa NorC protein (Figure 3.8b, Lane 2), which suggests that nitrate reduction by NapA is required for NorC expression.

The fact that in the *napA* mutant, NapB and NapC proteins were not detected indicates the existence of a polar effect of the *napA* mutation on *napBC,* suggesting that *napEDABC* are transcribed in one transcription unit as deduced from their sequence.

Figure 3.8. Detection of *c*-type cytochromes in the periplasmic fraction (a) and in membranes (b) of *B. japonicum* USDA110 (Lane 1) and *napA* mutant derivative GRPA1 (Lane 2). Periplasmic and membrane fractions were prepared from cells incubated anaerobically in YEM medium supplemented with 10 mM KNO_3. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and stained for covalently bound haem proteins. Each lane contains about 30μg protein. Haem-stained *c*-type cytochromes identified previously are specified at the right margin. Apparent molecular mass of the proteins (in kDa) are shown at the left margin.

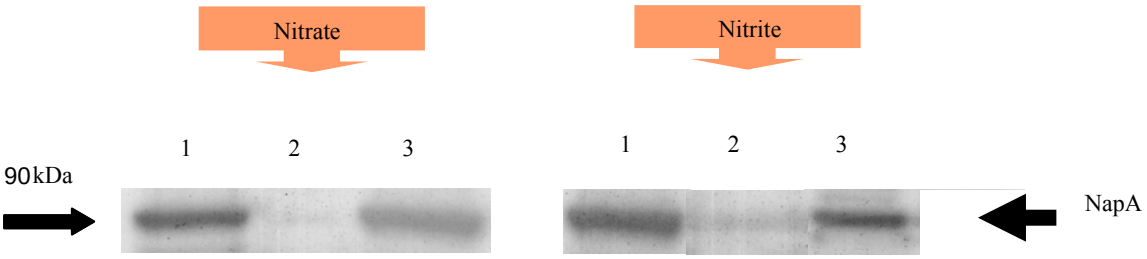


3.7. Detection of NapA in strains GRPA1 and 0609.

Periplasmic proteins of *napA* and *napC* mutant strains GRPA1 and 0609, respectively were obtained as described in Section 1.4.1 (Material and Methods), from 1 l of cells incubated under nitrate-respiring conditions. Proteins were separated at 4° C in 12 % SDS-PAGE, transfered to a nitrocellulose membrane and hybridized against *Paracoccus pantotrophus* NapA polyclonal antibodies (gift of D. Richardson). Whereas a protein band with a molecular size of about 90 kDa was detected in cells of USDA110, a similar band was not observed in cells of the mutant strain GRPA1 (Figure 3.9, Lanes 1 and 2 respectively).

These results demonstrate that the *B. japonicum* NapA protein is located in the periplasmic fraction, consistent with its sequence deduced properties. Similarly as in the wild type strain, NapA was detected in the *napC* mutant strain 0609 (Figure 3.9, Lanes 1 and 3). Since nitrate reduction does not occurs in the *napC* mutant, the presence of NapA in the *napC* mutant strain incubated in the presence of nitrate suggests that nitrate alone induces *nap* expression and the production of an intermediate from nitrate reduction such as nitrite, nitric oxide or nitrous oxide is not required for *nap* genes induction.

Figure 3.9. Detection of the NapA protein in *B. japonicum* USDA110 (Lane 1), *napA* mutant derivative GRPA1 (Lane 2), and *napC* mutant derivative 0609 (Lane 3) by specific staining with anti-NapA from *Pa. pantotrophus*. Periplasmic fractions were prepared from cells incubated anaerobically in YEM medium supplemented either with 10 mM KNO_3 or 1 mM $NaNO_2$. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immuno-stained. Each lane contains about 30 µg protein. Apparent molecular mass of the NapA protein (in kDa) is shown at the left margin.



4. Regulation of *napEDABC* expression

4.1. Analysis of the promoter region

Primer extension experiments were performed to analyze *nap* transripts in cells of *B. japonicum* USDA110 grown under different conditions (Figure 3.10). No transcript was detected when RNA from aerobically grown cells was used, regardless of the presence or the absence of nitrate in the medium (Figure 3.10, Lanes 1 and 2). In contrast, reverse transcription with RNA isolated from anaerobically grown *B. japonicum* USDA110 revealed the presence of a transcriptional start site that initiates at an A, 26 nt upstream of the putative

translational start codon (Figure 3.10). Levels of cDNA obtained after extension with the primer PEX-nap were higher in nitrate-respiring cells than in those that were incubated anaerobically in the absence of nitrate (Figure 3.10, Lanes 3 and 4 respectively).

Inspection of the DNA sequence (Figure 3.11) revealed a purine-rich Shine-Dalgarno-like sequence (AGAGAGA) 13 bases upstream of the putative translational start codon of *napE*. Located 101 bp upstream of the putative initiation codon of *napE,* there is the sequence 5′-TTGAT-N_4-ATCAA-3′, which has 10 out of 10 matches with the FNR consensus sequence 5′-TTGAT-N_4-ATCAA-3′ (Spiro, 1994), and 8 out of 10 matches with the FixK consensus sequence 5′-TTGAT-N_4-GTCAA-3′ (Fischer 1994; Zumft 1997) (Figure 3.11). Computer searches revealed no other totally conserved recognition motifs in the *nap* promoter region.

Figure 3.10. Mapping of the transcription start site of *B. japonicum napE* by primer extesion. The sequence ladder shown was generated with pPM0610 DNA and the same napE primer was used for the transcript mapping. The transcriptional start site is marked with an arrow. For RNA isolation, cells were cultured aerobically (Lanes 1 and 2) and anaerobically (Lanes 3 and 4) in YEM medium supplemented (Lanes 2 and 4) or not (Lanes 1 and 3) with 10 mM KNO_3.

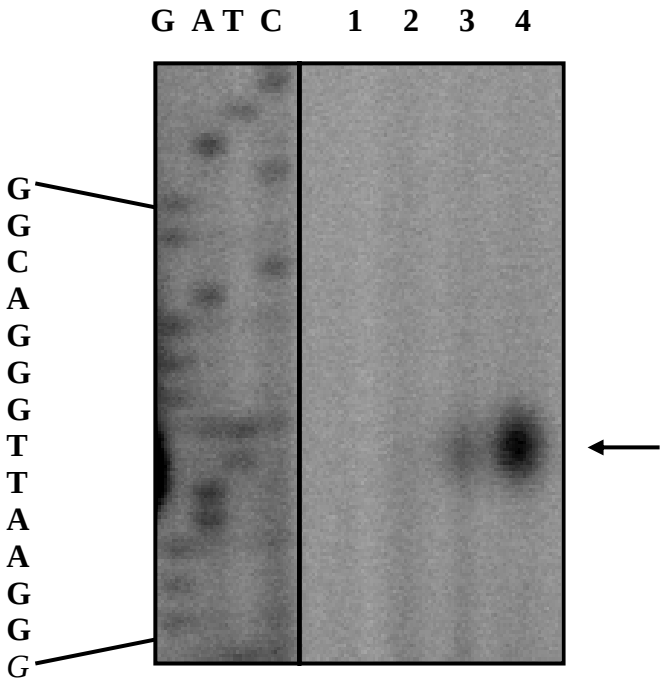
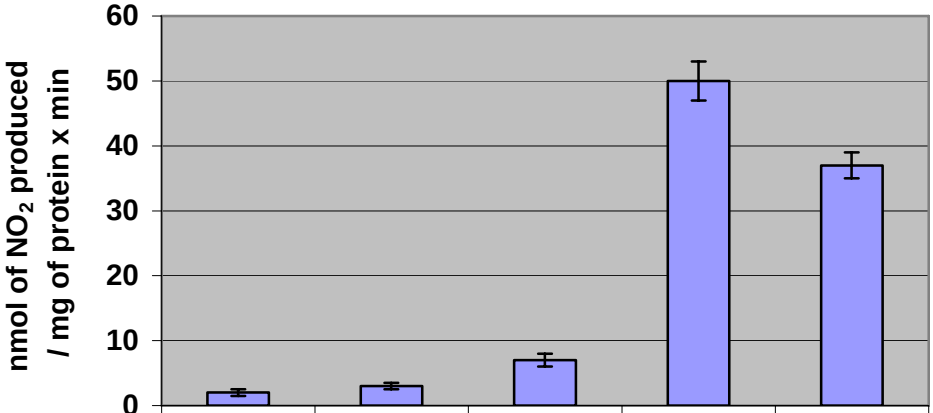


Figure 3.11. The *napE* promoter sequence. The putative FNR-binding sequence is boxed. The nucleotide at which transcription initiates is shown in bold and marked +1 above. A potential Shine-Dalgarno (RBS) sequence is underlined. The putative translational start codon is shown in bold. The N-terminal sequence of the predicted NapE protein is presented in one letter code.

4.2. Effect of oxygen, nitrate and nitrite on nitrate reductase activity

After growth of *B. japonicum* USDA110 cells aerobically or anaerobically in a medium supplemented or not with 10 mM nitrate or 1 mM nitrite, periplasmic nitrate reductase activity in whole cells was analyzed by using MV^+ as artificial electron donor. Only basal rates of periplasmic MV^+-dependent nitrate reductase activity were found in cells of *B. japonicum* USDA110 grown aerobically with or without nitrate (Figure 3.12). Values of activity increased when the cells were incubated anaerobically in the absence of nitrate, and maximal rates were obtained after growth under anaerobic conditions with nitrate (Figure 3.12).Similar levels of activity were observed when cells were grown anaerobically with nitrite and nitrate. These results agree with those observed in the transcript mapping experiments (Figure 3.10).

Figure 3.12. MV^+-dependent nitrate reductase activity in the parental strain USDA110 of *B. japonicum.* Cells were cultured aerobically and anaerobically in YEM medium supplemented or not with 10 mM KNO_3, or with 1 mM $NaNO_2$. Data, in nmol NO_2^- produced / mg of protein × min, are shown as means and standard error of the mean for at least three cultures which were assayed in duplicate.

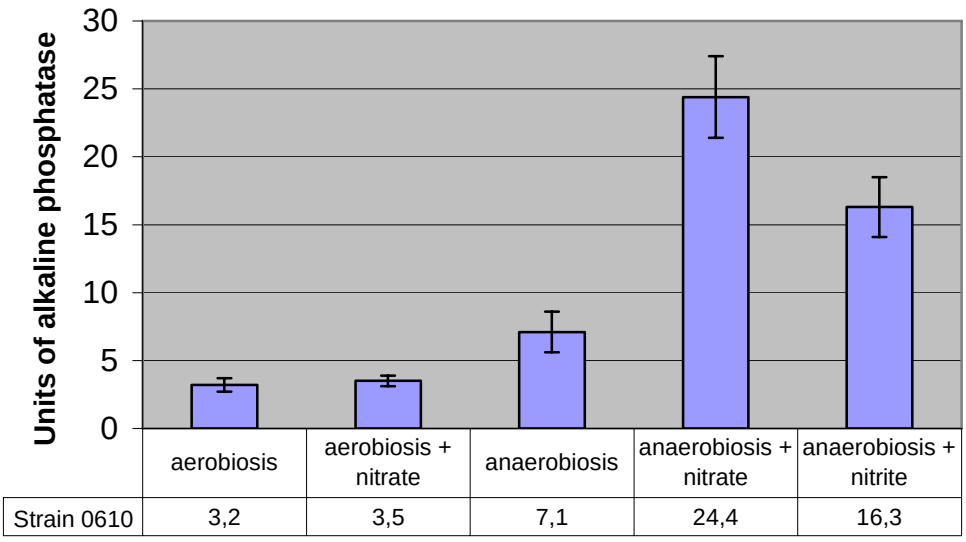


4.3. Effect of oxygen, nitrate and nitrite on expression of *nap* genes

Plasmid Tn*KPK2*-6 containing a Tn*KPK2* insertion in *napC* (Figure 3.4) was integrated by homologous recombination into the chromosome of *B. japonicum* strain USDA110, via conjugation using *E. coli* S17-1 as donor strain. Single recombination events were selected by resistance to kanamycin and gentamycin which resulted in strain 0610. The resulting recombinant strain was subsequently tested genetically by Southern blot analysis of genomic DNA preparations (data not shown). Strain 0610 containing a translational fusion of NapC with a '*phoA* truncated gene as described in Table 2 of Material and Methods was used to confirm the periplasmic or membrane bound location of NapC and to study expression rates of the NapC-*phoA* translational fusion. *B. japonicum* 0610 cells were grown aerobically or anaerobically in a medium supplemented or not with 10 mM nitrate or 1 mM nitrite. When cells of *B. japonicum* 0610 were grown aerobically in YEM medium supplemented or not with nitrate, only basal rates of alkaline phosphatase activity were detected after cell lysis and incubation with the substrate of the enzyme (Figure 3.13). Values of activity increased about 2 fold when the cells were incubated anaerobically in the absence of nitrate or nitrite. Induction of alkaline phosphatase activity of about 3.4 fold was observed after incubation under anaerobic conditions with nitrate (Figure 3.13). The presence of nitrite in the growth medium of 0610 cells under anaerobic conditions also induced levels of alkaline phosphatase activity of about 2.3 fold compared to those observed in anaerobic cultured cells without either nitrate or nitrite. These results agree with those observed in the transcript mapping

experiments (Figure 3.10) and in the MV^+-dependent nitrate reductase activity assays (Figure 3.6).

Figure 3.13. Rates of alkaline phosphatase activity in cells of *B. japonicum* 0610 containing a translational fusion of NapC to the reporter gene *phoA*. Cells were cultured aerobically and anaerobically in YEM medium supplemented or not with 10 mM KNO_3, or with 1 mM $NaNO_2$. Data, in units (U) of alkaline phosphatase are shown as means and standard error of the mean for at least three cultures which were assayed in duplicate.



5. Symbiotic phenotype of mutant strains GRPA1 and 0609 of *B. japonicum*

5.1. Effect of the *napA* mutation on nitrogen fixation in the symbiosis with Soybean plants

To test the symbiotic phenotype of the *B. japonicum napA* and *napC* mutant strains, cell suspension cultures of the wild type strain USDA110 and mutant strains GRPA1 and 0609 were used to inoculate surface sterilized, two days old soybean seeds (*Glycine max*). Plants were grown under controled conditions with a nitrogen-free nutrient solution during 35 days.

B. japonicum mutant strains GRPA1 and 0609 nodulated soybean plants similarly as the wild type strain since nodule fresh and dry weight (NFW and NDW) and nodules number (NN) observed among soybeans inoculated with either the wild type strain or any of the mutant strains mentioned above were very similar (Table 3.1). As schown in Table 3.1, no differences in acetylene reduction activity (ARA), plant dry weight (PDW) and nitrogen content were observed in plants inoculated with the mutant strains GRPA1 or 0609 compared with soybeans inocultated with the parental strain USDA110.

Table 3.1. Nodules number (NN), nodule fresh or dry weight (NFW or NDW), acetylene reduction activity (ARA, µmol ethylene / g NDW × h), plant dry weight (PDW, g / plant) and nitrogen content (mg of N / plant) of soybean plants inoculated with *B. japonicum* USDA110 and GRPA1 and 0609 mutant strains. These data are means of 3 independent experiments. Values in individual columns followed by the same letter are not significantly different according to the Fisher test ($P \leq 0.05$).

***B. japonicum* Strain**	**NN**	**NFW** (g/plant)	**NDW** (g/plant)	**ARA** (µmol/h/plant)	**PDW** (g/plant)	**[N]** (mg/plant)
USDA110	58 a	0.95 a	0.18 a	28.46 a	2.08 a	60.35 a
GRPA1	57 a	0.90 a	0.18 a	26.70 a	2.22 a	59.10 a
0609	56 a	0.85 a	0.17 a	27.50 a	2.15 a	58.60 a

5.2. Nitrate reductase activity in bacteroids of mutant strains GRPA1 and 0609

To test the nitrate reductase activity in bacteroids of the *B. japonicum napA* and *napC* mutant strains, cell suspension cultures of the wild type strain USDA110 and mutant strains GRPA1 and 0609 were used to inoculate surface sterilized, two days old soybean seeds (*Glycine max*). Plants were grown under controled conditions with a nitrogen-free nutrient solution during 35 days.

After bacteroids isolation from nodules, nitrate reductase activity was assayed by using MV^+ or BV^+ as electron donors. As shown in Table 3.2, a constitutive nitrate reductase activity was observed in bacteroids of the wild type strain. As observed in free-living conditions, no differences in activity were found in bacteroids of USDA110 when either MV^+ or BV^+ was used as electron donors (Table 3.2). Levels of MV^+- or BV^+-dependent nitrate reductase activity in bacteroids of mutant strains GRPA1 or 0609 were 6-fold lower than those detected in the bacteroids of the wild type strain (Table 3.2).

Table 3.2. Nitrate reductase activity in bacteroids infected with the parental strain USDA110 and the *napA* mutant derivative GRPA1 of *B. japonicum.* Data, in nmol NO_2^- produced / (mg of protein) × min, are shown as means and standard error of the mean for at least three cultures which were assayed in duplicate.

***B. japonicum* strain**	**Electron donor**	
	MV^+	**BV^+**
USDA110	115 ± 10	125 ± 34
GRPA1	18 ± 2	18 ± 1
0609	20 ± 3	19 ± 2

3. RESULTS

Chapter 3.2. Identification of new extracytoplasmic proteins of *B. japonicum* by using E-tag fusion clones obtained from a phage display library.

ABSTRACT

A *Bradyrhizobium japonicum* expression library based on the phagemid vector pG3DSS was used by Rosander *et al.* (2003) to identify and isolate a large number of clones encoding extracytoplasmic proteins. N-terminal parts of *B. japonicum* proteins fused to the artificial E-tag peptide were detected in a phage display system by a highly specific monoclonal antibody. Following PCR amplification, some selected gene fusions were used in this work to construct *B. japonicum* gene disruption mutants which were subsequently inoculated on soybean plants to test their symbiotic properties. While some of these mutants did not exhibit altered phenotypes, other mutants were severely affected in their symbiotic properties. These results allow us to propose this phage display strategy to identify unknown extracytoplasmic proteins with potential role on the symbiotic interaction between *Rhizobia* and legume plants.

1. E-tag fusion clones from the phage display library.

An expression library of the *B. japonicum* genome was constructed by Rosander *et al.* (2003), which was based on phagemid vector pG3DSS (see Introduction, Section 2.2.2.). The specificity of the phagemid developed is based on the fact that the protein III is truncated and lacks its original signal peptide sequence. After cloning of DNA fragments from *B. japonicum* genome in the phagemid, only extracytoplasmic proteins will be displayed on the surface of the phage as their sequence comprise an indigenous signal peptide sequence which replace the signal peptide of protein III. As an artificial peptide, the E-tag is fused to any translocated *B. japonicum* protein, a phage display system allows the detection and isolation of these constructs by a highly specific monoclonal antibody. In this work, we have performed a more detailed analysis and characterization of some of the E-tag fusion clones (Table 3.3) in order to identify novel extracytoplasmic proteins in *B. japonicum.*

2. Analysis of the DNA sequences of the selected E-tag fusion clones

Different clones from the phage display library created by Rosander *et al.* (2003) and listed in Table 3.3 have been selected according to the characteristics of their predicted amino acid sequences. Further studies were performed in order to analyze whether these genes are required for the symbiotic interaction of *B. japonicum* with soybeans. Two of these selected candidates and their characteristics are described in the following Sections 2.1 and 2.2.

Table 3.3. List of E-tag fusion clones that were selected for gene disruption mutagenesis. Clone designations and corresponding RhizoBase ORFs fused to the E-tag peptide are listed in the first and second columns. The predicted protein functions are described in the third column, and the references are compiled in the fourth column. No hit found in databases is indicated as nhf. blx4866b is not an original Rhizobase ORF designation, but it is located next to bll4867.

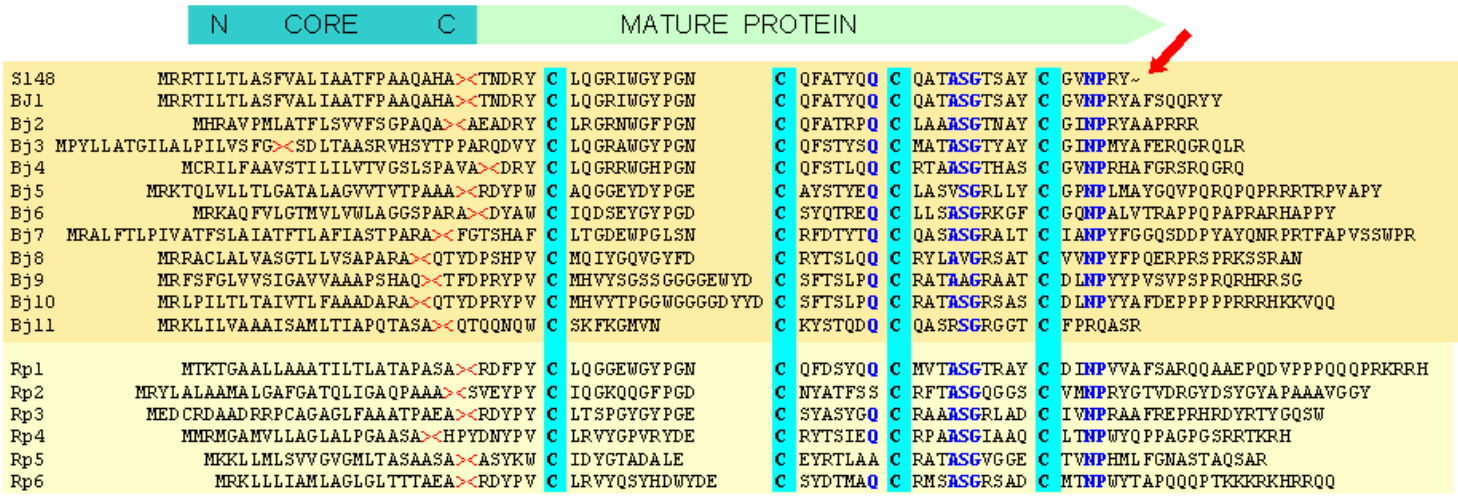
E-tag fusion clones from the phage display library inserted in pG3DSS (database reference)			
Clone designation	**Rhizobase designation**	**Predicted function**	**Reference**
S148	nhf	Predicted as a member of a small protein family with conserved Cys pattern	Rosander *et al*. (2003)

MR117	blx 4866b	Predicted as a member of a small protein family with conserved Cys pattern	“
FK248 and MR117	blx 4866b	Predicted as a member of a small protein family with conserved Cys pattern 4CBj7 (mr117)	This work
FK106	ORF not annotated in Rhizobase	Tyrosine-rich protein (99 aa), similar to chitin-binding domain of chitinase I	“
S105	bll7406	Predicted as outer membrane protein	Rosander *et al*. (2003)
MR048	blr0479	Predicted as outer membrane protein	“
MR055		Predicted as outer membrane protein	“
S112b	blr0276	Putative membrane protein	“
L108	nhf	Similar to Group 1 outer membrane protein precursor	“
FK225	bll1204	Similar to outer membrane immunogenic protein precursor	This work
FK333	blr7534	Similar to hypothetical heatstable antigenic protein of 120 kDa protein	“
MR021		Similar to P-binding protein	Rosander *et al*. (2003)
S101b and (MR021)	blr1091	Similar to phosphate-binding protein	“
S126b	blr4446	Similar to general L-amino acid-binding protein, AapJ precursor	“
S209	bll7600	Similar to glutamate/aspartate binding protein	“
S129b	bll0887	Similar to ANF-receptor, Receptor family ligand binding region	“
MR042	bll7946	Similar to phosphonates transport ATP-binding protein	“
FK209	blr1752	Similar to host inducible protein A homologue and ABC transporter	This work
MR039	bll2706	Similar to transglycosylase	Rosander *et al*. (2003)
MR037	blr 6654	Similar to soluble lytic murein transglycosylase	“
MR045	bll2692	Similar to UDP glycosyltransferase	“
(S106b) mr074	blr 7466	Similar to ribonuclease	“
FK243	blr5637	Similar to adenylate cyclase	This work
mr089	bll 4692	Similar to peptidyl-prolyl cis-trans isomerase B protein	Rosander *et al*. (2003)
S204	bll0332	Similar to heat resistance agglutinin I precursor	“
MR034	bll6484	hypothetical protein, similar to TolB precursor	“
FK240	blr2197	Putative toxin precursor	This work
FK242	bll3735	Putative outer membrane protein	“
FK305	blr2992	Similar to RlpA-like protein	“
FK309	bll6636	Similar to ATP synthase subunit	“
FK315	bll2431	Hypothetical protein, *sec*-independent protein	“
FK343	blr7958	Similar to multidrug resistance protein	“
FK409	blr5829	Similar to FlaD, distal basal body ring component of flagellum	“

2.1. The 4-cysteine protein family

E-tag clone S148 (Table 3.3) represents a small E-tag fusion protein which contains an N-terminal signal peptide and carries four cysteine residues. In this particular case the eight C-terminal amino acids are replaced by the E-tag peptide. As the native protein (77 amino acids) is shorter than the cut-off value of 100 amino acids, it has not been annotated in the Rhizobase database (http://www.kazusa.jp/rhizobase/) where the total sequence of the *B. japonicum* genome has been established (Kaneko *et al.* 2002). A careful examination of the genomic data has revealed that this genetic locus is a member of a family of proteins, which all have in common their small size, the presence of an N-terminal signal peptide and a highly conserved pattern of four cystein residues. These proteins therefore have been described as the 4-cysteine protein family (Figure 3.14).

Figure 3.14. 4-cysteine protein family (Rosander *et al.*, 2003). The signal peptidase cleavage site is indicated by > <. The red arrow indicates the location of the E-tag fusion in clone S148. The pattern of four highly conserved cystein residues is colored in blue. The last six sequences are from *Rhodopseudomonas palustris* (Rp).



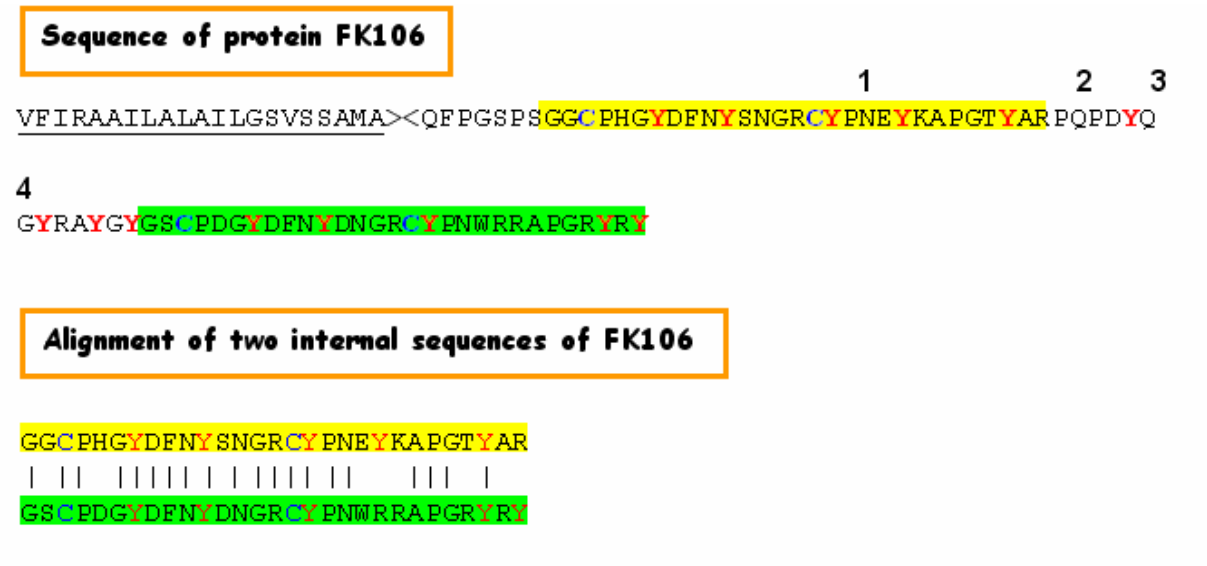
2.1.1. Detection of S148 protein

Preliminary experiments supported the idea that the insert of E-tag clone S148 is expressed in the *Escherichia coli* background and that the E-tag fusion protein can be regained from the growth medium suggesting that the fusion protein is secreted due to its small size (Rosander, personal communication). Using the *B. japonicum* mutant BJ-S148 (see Section 3), attempts were achieved to detect this protein in the *B. japonicum* genetic background and in which subcellular compartment the protein can be detected. The strain was cultivated in liquid medium up to the late logarithmic phase and the cells were separated from the medium by centrifugation. They were subjected to fractionation and separation of periplasmic space, membranes, and external growth medium. The external growth medium was first filtered by vacuum through a 0.2 µm pore membrane and then concentrated by lyophilization or by ammonium sulphate precipitation (60 to 80 %). Samples from each fraction were then purified by a column with immobilized E-tag antibody and further concentrated by ultrafiltration using Amicon Centriprep 3 and Centricon 3 filters (Millipore). After separation in SDS-PAGE, the proteins were detected by silver staining. However, the E-tag fusion protein could not be identified in any of the cell fractions analysed (data not shown). Although the detection is based on the fusion of the E-tag peptide to the extracytoplasmic protein, it is possible that in some cases this E-tag moiety prevents the putative fusion protein from translocation. Alternatively, it may be possible that the growth conditions tested are probably not suitable for the expression of this particular protein in the *B. japonicum* background.

2.2. Tyrosine rich protein FK106

E-tag clone FK106 (Table 3.3) represents a small E-tag fusion protein which contains an N-terminal signal peptide and in its mature predicted protein carries four cysteine residues. In this particular case, different E-tag insertion clones were obtained in the phage display library (S146b, MR108, S104, FK106), as represented in Figure 3.15 (position 1, 2, 3, and 4). As the predicted full-length protein (99 amino acids) is shorter than the cut-off value of 100 amino acids, it has not been annotated in the Rhizobase database (http://www.kazusa.jp/rhizobase/) (Kaneko *et al.* 2002). Two unusual features characterize this protein, on one hand its richness in tyrosine residues raises 18 %, on the other hand two internal sequences of 27 amino acid residues present 74 % homology with each other (Figure 3.15).

Figure 3.15. Tyrosine rich protein FK106. The sequence of the signal peptide is underlined. The tyrosine and cysteine residues are represented in red and blue colors respectively. Positions 1, 2, 3, and 4 represent the locations of different E-tag fusion sites in clones S146b, MR108, S104, and FK106, respectively. In the alignment of the two fragments of the sequence, the homology is indicated by red vertical lines.



3. Construction of a *B. japonicum* mutants library by using the selected E-tag fusion clones

Construction of a mutants library affected in extracytoplasmic proteins was performed by site directed mutagenesis after cloning the gene fragments from the phage display library in an appropriate vector. First step of cloning was the amplification of the gene fragment cloned in the library using the G3delrev primer and another primer specifically designed according to the individual nucleotide sequence within the putative coding region of the signal peptides of the different clones (Material and Methods, Section 2.13.3., Table 2.5). After checking the PCR product size by separation in an agarose gel, they were purified from the gel and ligated into pTOPO-TA cloning vector. Unique restriction sites were used to excise the insert and religate it to the mobilizable vector pJQ200SK (Quandt and Hynes, 1993), before transformation of *E. coli* DH5α cells. After selection of the correctly transformed strain, its plasmid was isolated and transformed into *E. coli* S17.1 strain, and plasmids were subsequently checked by DNA sequencing. Finally, these hybrid plasmids were transfered into *B. japonicum* by conjugation using *E. coli* S17.1 as donor. Potential transconjugants were selected by growth on agar plates containing gentamycin, and checked

by Southern hybridization with an E-tag specific probe (data not shown). Alternatively, the recombination event was checked by cloning and sequencing of a gentamycin resistent *Xho*I fragment containing pJQ200SK plasmid, the inserted DNA amplification product and the adjacent DNA region. The obtained mutant strains that were used in plant test analysis are indicated in Table 3.4.

4. Symbiotic phenotype of the mutants

To test the symbiotic phenotype of the *B. japonicum* mutant strains obtained (Table 3.4), logarithmically growing cultures of each mutant strain were used to inoculate surface sterilized, two days old soybean seeds (*Glycine max*). Plants were grown under controled conditions with a nitrogen-free nutrient solution. *B. japonicum* mutant strains BJ-S148, BJ-FK106, BJ-MR021, BJ-MR042, BJ-MR045, BJ-MR048, BJ-MR055, BJ-FK243 and BJ-FK333 (Table 3.4) nodulated soybean plants similarly as the wild type strain. Plant dry weight, nitrogen content and nitrogenase activity observed among soybeans inoculated with either the wild type strain or any of the mutant strains mentioned above were very similar (data not shown). The structure and color of the nodules obtained after inoculation with the mutant strains were similar to those obtained by the wild type strain (data not shown).

Table 3.4. Symbiotic phenotypes of the *B. japonicum* mutants carrying the E-tag fusions. Symbiotic phenotype similar to the wild type strain is indicated by WT.

Mutant strain	Description	Symbiotic phenotype
BJ-S148	Member of the 4 C family	WT
BJ-FK106	Tyrosine rich protein	WT
BJ-MR021	Similar to phosphonate transport binding protein	WT
BJ-MR042	Similar to phosphate binding protein	WT
BJ-MR045	UDP-glycosyltransferase	WT
BJ-MR048	Outer membrane protein	WT
BJ-MR055	Outer membrane protein	WT
BJ-FK243	Similar to adenylate cyclase	WT
BJ-FK333	Hypothetical heatstable antigenic protein	WT
BJ-FK242	Outer membrane protein	altered
BJ-FK305-12	Unknown protein, RlpA-like protein	altered
BJ-FK309-13	Similar to ATP synthase subunit	altered
BJ-FK343-22	Similar to multidrug resistance efflux pump	altered
BJ-FK409-25	Similar to FlaD, distal basal body ring component of flagellum	altered

By contrast, mutants BJ-FK242, BJ-FK305-12, BJ-FK309-13, BJ-FK343-22, and BJ-FK409-25 were severely affected in their symbiotic properties (Tables 3.4 and 3.5).

Nodules formed by mutant BJ-FK242 showed a markedly reduced nitrogenase activity in the plant test as indicated by the acetylene reduction assay (Table 3.5). This is consistent with the reduced plant dry weight and nitrogen content observed in plants inoculated with BJ-FK242 mutant (Table 3.5). These plants showed higher nodule number than those inoculated with the wild type strain, but their fresh or dry weight was similar to wild type strain (Table 3.5). Indeed, the nodules formed were small and most of them were white suggesting that leghemoglobin was strongly reduced or missing entirely. The analysis of the nodule ultrastructure showed that the nodule occupancy by mutant strain BJ-FK242 was significantly lower compared with the wild-type strain. Furthermore, large starch granules were found within infected cells (Figure 3.16.A and .B), bacteroids contained many polybetahydroxybutyric acid (pHBA) droplets and showed symptoms of deformation, and several vacuoles were present around the nuclei of infected cells (Figure 3.16.B).

Nodules formed by mutant BJ-FK305-12 were less active in nitrogen fixation activity compared with wild type strain nodules (Table 3.5). Plant dry weight and nitrogen content measured in plant inoculated with BJ-FK305-12 were also reduced (Table 3.5). However, the number of nodules counted and their fresh or dry weight measured in plants inoculated with the BJ-FK305-12 mutant were similar to the wild type strain. It seems then that the nodulation was not affected but the nodules formed were less efficient. Sections of the nodules exhibited a high number of starch granules which were very large in noninfected cells and still considerable in size and number within infected cells (Figure 3.16.C). The number of symbiosomes was clearly reduced, and in many cases the bacteroids were degradaded. As a consequence, the infected cells contained amyloplasts in the peripherical parts, whereas the centre was filled with several vacuolar structures, which were located next to symbiosomes in different degradation steps. Occasionally infection threads were observed (Figure 3.16.C), indicating that the infection process had occurred normally, suggesting that this mutant was disturbed in bacteroid maintenance.

Mutant BJ-FK309-13 exhibited a less pronounced symbiotic phenotype. The nitrogenase activity of nodules produced by strain BJ-FK309-13 was similar to that of nodules infected by the wild type strain (Table 3.5). No significant differences were observed

regarding the plant dry weight, nitrogen content, number of nodules and nodule fresh or dry weight (Table 3.5). The infected cells were filled with numerous bacteroids surrounded by symbiosome membranes (Figure 3.16.D). However, at the same time, several amyloplasts were observed within the infected cells. Furthermore, the bacteroids showed symptoms of deformation and degradation, occasionally lytic degradations of the plant cytosol were found (Figure 3.16.D). These data indicate that the infection process and the nodule efficiency were not affected, but nodules maintenance was disturbed.

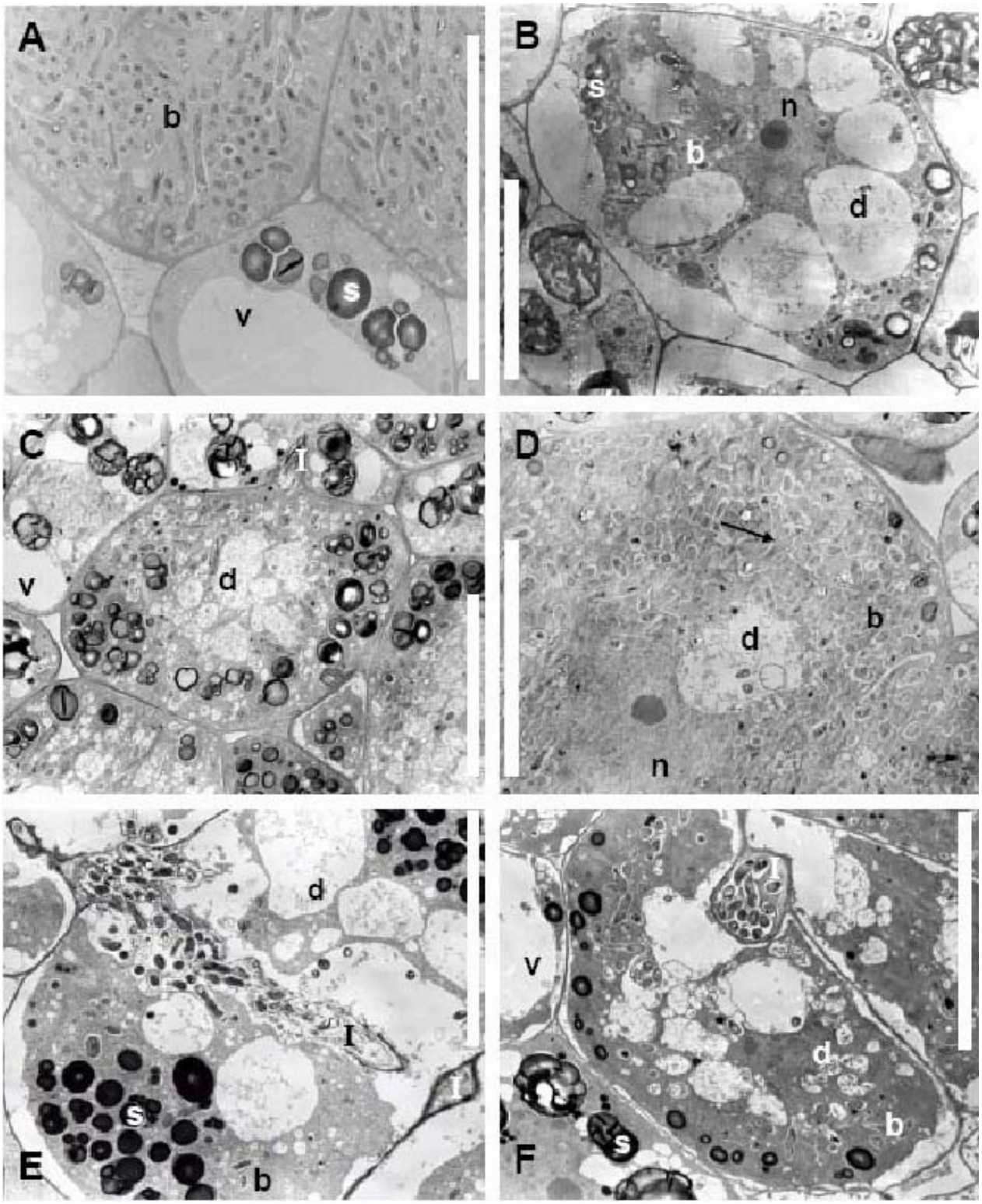
The symbiotic phenotype of mutant BJ-FK343-22 exhibited similar but even more severe characteristics than that of mutant BJ-FK305-12 as indicated by the values of acetylene reduction activity, plant dry weight, nitrogen content (Table 3.5). In particular, the number of intact symbiosomes within infected cells was extremely low. Instead, numerous vacuolar structures filled with electron-dense material, were found (Figure 3.16.F). The close vicinity to structurally intact symbiosomes and to transient stages of bacteroid degradation at various degrees suggests that this material is cell debris, originating from degraded bacteroids. In contrast to this observation, bacteroids within an infection thread were densely packed and looked intact (Figure 3.16.F). The *B. japonicum* blr7958 gene fused to the E-tag, is predicted to encode a multidrug resistance efflux pump, related to Gram-negative RTX secretion protein D and to secretion protein HlyD. The transmembrane prediction server indicates a transmembrane segment (TMS) which is made by amino acid residues 72-94 of the blr7958 encoded protein, where the short N-terminus sticks to the inner leaflet of the inner membrane and the longer C-terminus resides in the periplasmic space.

B. japonicum mutant BJ-FK409-25 induced the formation of ineffective nodules where the cells of the central nodule tissue were extremely poorly infected with bacteroids but rich in starch granules (Figure 3.16.E). This is in line with the highly reduced acetylene reduction activity values, the plant dry weight and nitrogen content (Table 3.5). The number of nodules was higher but they were affected in their weight and size. As seen in the micrograph of ultrastructural analyses, infection threads with a wide lumen can be found in the central nodule tissue (Figure 3.16.E).

Table 3.5. Nodules number (NN), nodule fresh or dry weight (NFW or NDW), acetylene reduction activity (ARA), plant dry weight (PDW) and nitrogen content ([N]) in soybean plants (*Glycine max* L. *Merr*., cv. Williams) inoculated with *B. japonicum* USDA110*spc*4 and BJ-FK242, BJ-FK305-12, BJ-FK309-13, BJ-FK343-22 and BJ-FK409-25 mutant strains. Values of ARA are expressed in µmol ethylene/h × (g NDW); PDW is expressed in g/plant and nitrogen content is expressed in mg N/plant. These data are means of 3 independent experiments. Values in individual columns followed by the same letter are not significantly different according to the Fischer test ($p \leq 0.05$).

B. japonicum **Strain**	**NN**	**NFW** (g/plant)	**NDW** (g/plant)	**ARA** (µmol/h x g NDW)	**PDW** (g/plant)	**[N]** (mg/plant)
USDA110	59 a	0.95 a	0.18 a	303.80 a	2.10 a	56.80 a
BJ-FK242	80 b	0.87 a	0.19 a	203.33 b	1.7 b	45.81 b
BJ-FK305-12	65 a	0.95 a	0.18 a	235.85 c	1.9 c	51.90 c
BJ-FK309-13	58 a	0.87 a	0.17 a	291.00 a	2.0 a	54.13 a
BJ-FK343-22	56 a	0.85 a	0.16 a	204.40 b	1.7 b	46.30 b
BJ-FK409-25	85 b	0.95 a	0.18 a	152.01 d	1.5 d	40.95 d

Figure 3.16. Micrographs of ultrathin soybean nodule sections infected with *B. japonicum* strain USDA110*spc*4 and mutants. (A) *B. japonicum* 110*spc*4, (B) BJ-FK242, (C) BJ-FK305-12, (D) BJ-FK309-13 (E) BJ-FK409-25 and (F) BJ-FK343-22. White bars indicate 10 µm. Abbreviations: **b,** bacteroid, **d,** defective symbiosome, **I,** infection thread containing bacteria, **n,** nucleus of plant cell, **s,** starch granule, **v,** vacuole in noninfected cell. The arrow points to a structurally deformed bacteroid.


A
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4. DISCUSSION

Chapter 4.1. Identification and characterization of the periplasmic nitrate reductase from *B. japonicum*

1. Isolation and sequencing of the *napEDABC* genes

B. japonicum is a facultatively anaerobic soil bacterium with the capability to reduce nitrate simultaneously to ammonia and nitrogen when cultured anaerobically with nitrate as terminal electron acceptor and sole source of nitrogen (Vairinhos *et al.*, 1989). In *B. japonicum*, the denitrification process depends on the *nirK* (Velasco *et al.*, 2001), *norCBQD* (Mesa *et al.*, 2002) and *nosRZDYFLX* (Velasco *et al.*, 2004) gene clusters encoding nitrite reductase, nitric oxide reductase and nitrous oxide reductase, respectively. The first step of denitrification, the two-electron reduction of nitrate to nitrite, is catalysed by the nitrate reductase enzyme. Two types of enzymes have been found in denitrifying bacteria, a membrane-bound respiratory nitrate reductase (Nar) that generates a proton motive force for energy, and a periplasmic dissimilatory nitrate reductase (Nap) encoded by the *nar* and *nap* genes, respectively (Richardson *et al.*, 2001).

In this work, the cloning and sequence analysis of *B. japonicum napEDABC* genes have been achieved. The deduced primary sequences of NapA, NapB and NapC have between 46% and 76% identity with the translated sequences from other denitrifiers. *napA* encodes the catalytic subunit containing the molybdopterin guanine-dinucleotide cofactor (MGD) and a [4Fe-4S] cluster. The Mo-dependent activity of NapA has been recently demonstrated in our Group (Bonnard *et al.*, 2005). *napB* encodes an electron-transfer subunit, dihaem cytochrome *c*, and *napC* a membrane-bound *c*-type tetrahaem cytochrome, respectively. Overlapping coding regions between *napA* and *napB*, as well as between *napB* and *napC* stop and start codons suggest translational couplings between *napA*, *napB*, and *napC*, which has also been found in many other bacterial genomes (Richardson *et al.*, 2001). However, unlike a translational coupling between *napD* and *napA* which was found in all cases analysed so far (Richardson *et al.*, 2001), in *B. japonicum* there is a short intergenic region of 16 nucleotides between these two genes. *napE* encodes a transmembrane protein of unknown function, and the *napD* gene product is a soluble protein which is assumed to play a role in the maturation of NapA prior to export to the periplasm (Berks *et al.*, 1995; Potter and Cole, 1999).

Additional *nap* genes such as *napF*, *napG*, *napH* or *napK* have not been detected in the *B. japonicum nap* genetic region described in this work. The complete genome sequence of *B. japonicum* USDA110 (Kaneko *et al.*, 2002) confirms the existence of the *napEDABC* genes in *B. japonicum* and the absence of other related *nap* genes. However, DNA sequences

showing homology with those published coding for the Nar system (*narGHI* genes) have not been found in the genome sequence of *B. japonicum* USDA110 [(Kaneko *et al.*, 2002), see also http://www.kazusa.or.jp/rhizobase]. Similarly as described in *B. japonicum*, the rhizobia *Pseudomonas* sp. G-179 (actually *Rhizobium galegae*) (Bedzyk *et al.*, 1999), *Sinorhizobium meliloti* [http://www.kazusa.or.jp/rhizobase] and *Azospirillum brasilense* (Steenhoudt *et al.*, 2001), contain *nap* genes, and lack *nar* genes. Neither the genes encoding Nar or Nap enzymes have been detected in other symbiotic rhizobia such as *Mesorhizobium loti, Rhizobium etli* or *Rhizobium leguminosarum* (http://www.kazusa.or.jp/rhizobase).

2. Involvement of the *B. japonicum* periplasmic nitrate reductase in nitrate metabolism

To establish the function of Nap in *B. japonicum* a *napA* and *napC* mutant strains were constructed and their capacity to grow aerobically or anaerobically with nitrate and to express nitrate reductase activity was studied. Both mutants were unable to grow under nitrate respiring conditions. The fact that a similar nitrate reductase activity was detected in *B. japonicum* USDA110 using methyl viologen (MV^+) or benzyl viologen (BV^+) as artificial electron acceptor, demonstrated the periplasmic location of the nitrate reductase activity in *B. japonicum* under anaerobic conditions. Considering that cell membranes are permeable to BV^+, the presence of a membrane-bound nitrate reductase would have resulted in an higher nitrate reductase activity when BV^+ was used as artificial electron donor. The use of MV^+ and BV^+ has been therefore a useful tool for distinguishing between nitrate reductases with active sites in the periplasm and cytoplasm and the results obtained confirm the presence in *B. japonicum* of a periplasmic nitrate reductase type rather than a membrane-bound type. The *napC* mutant strain 0609 could not reduce nitrate when succinate was used as physiological electron donor, whereas similar levels of nitrate reductase activity were detected when BV^+ or MV^+ were used as artificial electron donors. These results suggest that *napC* gene product is responsible for the electron transfert to the NapAB catalytic components of periplasmic nitrate reductase.

Periplasmic location of the *B. japonicum* nitrate reductase was supported by inmuno-stained and heme-stained detection of NapA and NapB, respectively, in the periplasm of *B. japonicum*. This is consistent with the presence of a signal peptide and a consensus Sec-independent twin arginine translocon (TAT) motif in *napA* gene product, and a type I signal peptide at N-terminal end in NapB corresponding to a translocation by the general secretory

pathway. The TAT-dependent transport of NapA is not valid for all bacteria since although NapA from *Pseudomonas* sp. G-179 is found in the periplasm, it lacks this signal peptide. As indicated above, sequence analysis in NapB and NapC also identified a di-haem and tetra-haem cytochrome *c* binding site respectively. *napB* and *napC* gene products could therefore be detected by haem *c* detection in the periplasm and the membrane of *B. japonicum* and they were identified as a 15 kDa and a 25 kDa *c*-type cytochrome, respectively. The fact that in the *napA* mutant, NapB and NapC proteins were not detected indicates the existence of a polar effect of the *napA* mutation on *napBC*, suggesting that *napEDABC* are transcribed in one transcription unit as deduced from their sequence.

Taken together, the results of this work clearly indicate that the periplasmic nitrate reductase encoded by the *napEDABC* genes is the primary enzyme carrying out nitrate reduction in *B. japonicum* under oxygen-limiting conditions. This work clearly gives another example among the *Rhizobiales* order where, as it has been shown in *Pseudomonas* sp. G-179 (Bedzyk *et al*., 1999), the Nap enzyme can support anaerobic growth by reducing nitrate to nitrite which can be further reduced in the reactions of denitrification.

3. Regulation of the *nap* genes

By performing primer extension experiments it has been demonstrated that expression of *B. japonicum nap* genes is maximally induced in cells grown anaerobically with nitrate. This maximal expression of *nap* genes under low oxygen conditions in the presence of nitrate has also been demonstrated by using a NapC-*phoA* translational fusion. Moreover, levels of gene expression correlate with maximal rates of nitrate reductase activity detected after growth under anaerobic conditions with nitrate. Anaerobic induction of the *nap* genes is in line with the presence of a putative FNR-box upstream of the transcriptional start of *napE*. The region homologous to the FNR-binding domain is centred 66.5 bases upstream of the transcriptional start site, which suggests that *B. japonicum napE* promoter could be considered as a class I FNR-dependent promoter (Wing *et al.*, 1995). The *Escherichia coli* FNR-dependent *napF* promoter is also a type I promoter centred 64.5 bases upstream of the transcriptional start site (Darwin *et al*., 1998). Recentlty, it has been demonstrated in our group that the Fnr homologue responsible for anaerobic control of *B. japonicum nap* genes expression is $FixK_2$ (Robles *et al.*, 2006). Similarly, anaerobic expression of the *B. japonicum nirK* (Velasco *et al.*, 2001), *norCBQD* (Mesa *et al.*, 2002) and *nosSRZDYFLX* (Velasco *et al.*,

2004) denitrification genes is regulated by $FixK_2$, a member of the FNR- (fumarate and nitrate reductase regulator) and CRP- (cyclic AMP receptor) like transcriptional regulators from bacteria. $FixK_2$ has also been shown to activate genes involved in anaerobic and microaerobic metabolism (Fischer, 1994; Nellen-Anthamtten *et al.*, 1998). As indicated above, besides the absence of oxygen, expression of the *B. japonicum nap* genes was further induced by the presence of nitrate. Induction of *nap* transcription by nitrate under anaerobic conditions has also been demonstrated in *E. coli* (Darwin *et al*., 1998), but in this bacteria *nap* genes are induced only at low nitrate concentrations. By contrast, expression of the *nap* operon of *Paraccocus pantotrophus* is negatively regulated during anaerobiosis such that the expression is restricted to aerobic growth, but only when the organism is grown on a highly reduced carbon source such as butyrate (Ellington *et al.*, 2002). In phototrophic bacteria such as *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* DSM158 (Richardson 2000; Gavira *et al.*, 2002), expression of nap genes occurs in the presence of nitrate under either oxic or anoxic conditions. Those findings highlight differences in the regulation of functionally different Nap systems.

Inmuno-staining experiments and nitrate reductase activity assays showed that NapA is expressed in the *napC* mutant strain 0609 at similar levels to that from USDA110 cells grown anaerobically with nitrate. Since cells of 0609 strain are unable to reduce nitrate under physiological conditions, these results indicate that nitrate reduction is not essential for the expression of *nap* genes. When cells of strains USDA110 or 0609 were incubated anaerobically with nitrite similar levels of nitrate reductase activity and NapA expression were found compared to those from cells incubated anaerobically with nitrate. Morover, alkaline phosphatase levels of USDA110 cells containing the NapC-*phoA* fusion were similar in cells grown with either nitrate or nitrite. Taken together, these results indicate that nitrate and nitrite can induce synthesis and activity of NapA. Since endogenous NO is not produced in the *napC* mutant, the results also sugests that NO may not be required for induction of the *nap* genes. In *E. coli* nitrate and nitrite control of *nap* genes is mediated by the DNA-binding response regulator NarP, which communicate with homologous sensor protein NarQ (Darwin *et al.*, 1998). The NarP protein recognizes heptamer binding sites that resemble the consensus TACYYMT (where Y=C or T and M= A or C). Specific DNA-sites for binding phosphor-NarP are not present in the promoter region of *B. japonicum nap* genes. Morover, a NarP-like protein has not been annotated in the genome sequence of *B. japonicum* (http://www.kazusa.jp/rhizobase/). However, besides $FixK_2$, other protein, NnrR has been

shown recently to be required for N-oxide regulation of the *B. japonicum napEDABC* (Robles *et al.*, 2006), *nirK* and *norCBQD* genes (Mesa *et al.*, 2003). However, the regulation mechanism of *B. japonicum nap* genes by nitrate and nitrite through the NnrR regulator is still unknown.

4. Symbiotic phenotype of the *napA* and *napC* mutant strains

When *napA* and *napC* mutant strains GRPA1 and 0609 were inoculated in soybean plants, no significant differences were observed compared to the plants inoculated with the strain USDA110 regarding the values of nodules fresh or dry weight and number of nodules indicating that those mutations did not affect nodulation of soybean plants. Similarly, nitrogen fixation capacity of those mutants measured as acetylene reduction activity, plant dry weight and nitrogen content were similar to that of the wild type strain. From these results we can conclude that the *B. japonicum* periplasmic nitrate reductase is not essential for symbiotic nitrogen fixation. Similarly, it has been demonstrated that although the *nirK*, *nor* and *nos* denitrification genes are expressed in soybean nodules, however they are not required for nodulation and nitrogen fixation of nitrogen-dependent grown soybean plants (Mesa *et al.*, 2004). In other rhizobia such *S. meliloti* and *Rhizobium sullae* (formerly *R. hedysari*) nodulation, plant growth and rates of nitrogen-fixation are similar between wild type and a *nosZ*- or *nirK*-deficient strains, respectively (Toffanin *et al.*, 1996; Holloway *et al.*, 1996).

The presence of nitrate reductase activity in bacteroids of nodules from soybean plants has been previously demonstrated (Delgado *et al.*, 1998), however the involvement of the periplasmic nitrate reductase in this activity is unknown. In this work, we have demonstrated that the *B. japonicum* periplasmic nitrate reductase is responsible for the 84 % of the nitrate reductase activity detected in bacteroids since a low level of this activity was detected in bacteroids of the *napA* or *napC* mutant strains. Moreover, similar levels of MV^+- or BV^+-dependent nitrate reductase activity of the wild type bacteroids confirm the periplasmic nature of the nitrate reductase activity detected in bacteroids. Taken together, these results suggest that the *B. japonicum* periplasmic nitrate reductase is active in bacteroids, however it is not important for symbiotic nitrogen fixation in nitrogen-dependent grown plants. Another growth conditions such as the presence of nitrate in the root medium need to be included in future experiments to fully understand the involvement of the periplasmic nitrate reductase in the symbiotic nitrogen fixation.

4. DISCUSSION

Chapter 4.2. Identification of new extracytoplasmic proteins of *B. japonicum* by using E-tag fusion clones obtained from a phage display library.

It has been recently shown that phage display can be used to identify genes encoding signal peptides (Rosander *et al.*, 2002). The phage display method applied by Rosander (2002) on the genome of *B. japonicum* has led to the isolation of a large number of E-tag fusions within *B. japonicum* genes that encode putative extracytoplasmic proteins. Statistical analyses revealed that the E-tag fusion site occurred most frequently within the range of 70 N-terminal amino acids, E-tag fusions towards C-terminal positions of larger proteins were found less frequently (Rosander *et al.*, 2003). Based on the results of this analysis a number of plasmids was selected for further investigations, which was based on the following criteria:

Firstly, the putative protein functions which could be predicted from the full length sequence of the *B. japonicum* genome (Kaneko *et al.*, 2002). Secondly, the ORFs should be organized preferably in small operons to avoid polar effects on downstream located genes. Finally, the E-tag fusion should truncate a considerable part of the C-terminus and at the same time the fusion site should leave a DNA fragment in the upstream region which was long enough to allow site-specific recombination.

1. Construction and characterization of *B. japonicum* mutants lacking extracytoplasmic proteins

The different PCR amplification products which were obtained when a combination of two primers was applied (one general primer binding to the nucleotide sequence of the E-tag moiety, and specific primers recognizing the nucleotide sequences of the various signal peptides) in a range between 150 and 600 nucleotides. Finally, these PCR products were ligated to the mobilizable vector pJQ200SK and introduced into *B. japonicum* by conjugation. Due to the small sizes of the homologous DNA inserts, the frequency of exconjugants was rather low. Southern hybridisation experiments revealed that very often (up to 80%) the E-tag sequence could not be detected by a specific probe. This observation can be explained either by the appearance of *B. japonicum* derivatives which had spontaneous resistance to Gentamycin, or by secondary deletions which removed the E-tag moiety after the recombination events. The choose of restriction enzymes was also critical to avoid the generation of troncated or too small fragments which could also be responsible for the lack of signal in Southern analysis. In some cases, from the Southern analysis, the hybridising fragments had not the correct sizes, suggesting that the recombination did not occurred precisely as expected according to the sequence data. Additional experiments, repeating the mutagenesis or using other probes for Southern analysis are required to check the correct

genetic manipulation in these strains or to elucidate which genetic mutation is responsible for their phenotype. By contrast, in strains BJ-S148, BJ-MR021, BJ-MR042, BJ-MR045, BJ-MR048, BJ-MR055, BJ-FK106, BJ-FK243 and BJ-FK333 the Southern hybridisation experiments confirmed the correct genetic manipulation exactly as predicted by the sequence information.

2. Symbiotic phenotype analysis of the *B. japonicum* mutant strains obtained

Although the genetic manipulation was confirmed by the Southern hybridisation experiments in mutant strains BJ-S148, BJ-MR021, BJ-MR042, BJ-MR045, BJ-MR048, BJ-MR055, BJ-FK106, BJ-FK243 and BJ-FK333, none of these mutant strains was disturbed in their symbiotic interaction with soybean under standart conditions. The absence of phenotype could be due in some cases to the fact that the function of the affected gene could be covered by genes of the same family. This could be the case for the E-tag fusion FK333 inserted within blr7534 which is one of several heatstable antigenic proteins in *B. japonicum*. Probably a clear phenotype can be observed only if double or multiple mutants are constructed. The same argument could be true for strain BJ-S148, where one of the eleven 4C proteins has been fused to the E-tag. This does not necessarily mean that these genetic loci do not play a role in the symbiotic interaction. Furthermore, the E-tag fusion of BJ-S148 replaces only a few C-terminal amino acid residues but does not affect the four cysteine residues which are assumed to define the most important characteristics of these proteins. As this is a rather small protein and the four cysteine pattern remains intact, probably the mutation does not affect its function.

As a consequence of the E-tag fusion of FK106 in the *B. japonicum* genome, the second half of the putative mature protein is truncated. Nevertheless, the symbiotic phenotype of the BJ-FK106 mutant strain is not affected and does not show any difference with the wild type strain. By sequence analysis, no similarities have been detected and, due to its small size (99 amino acids) this coding region has not been annotated in the RhizoBase. No information is available regarding the putative conditions for the expression or for the function of this protein.

Regarding strains BJ-MR021, BJ-MR042, BJ-MR045, BJ-MR048, BJ-MR055, BJ-FK243 and BJ-FK333, no symbiotic phenotype has been observed, which indicates that the mutation in the corresponding genes does not have effect on the symbiotic nitrogen fixing phenotype. All together these mutants show that the introduction of the E-tag peptide per se

does not have a negative influence on the symbiotic properties of *B. japonicum*. Despite no nitrogen fixing phenotype was observed, these extracytoplasmic proteins can be involved in important cell processes like metabolic pathways, biogeochemical pathways, stress tolerance. This was the case of the periplasmic nitrate reductase which was identified and characterized in Chapter one. This suggests that the characterization of these proteins identified by the phage display system requires some individually designed experiments.

In order to get molecular information about these proteins and their expression, attempts have been achieved to perform a preliminar protein detection of the E-tag fusion product of the *B. japonicum* mutant BJ-S148. Although no information is available regarding the conditions of expression of this protein, a putative constitutive expression under free-living conditions could not be discarded. Cells were grown aerobically under standard free-living conditions. After treatment of the cells, protein samples were submitted to separation through a column with specifique anti-E-tag antibodies. The pure protein sample obtained were concentrated and separated in PAGE gel with different SDS concentration, detection was performed by silver staining. Results did not allow us to detect or discard any expression, as similar signals were detected in the wild type strain (data not shown). Possible explanation for this result could be that the E-tag moiety can not be processed correctly in the *B. japonicum* background or that some specific conditions for the expression are not applied to the cells.

Although all attempts failed so far to verify that the genetic manipulations were as expected in the mutant strains BJ-FK240, BJ-FK305-12, BJ-FK-309-13, BJ-FK343-22 and BJ-FK409-25, their symbiotic phenotypes have been strongly affected. Bacteroid isolation experiments from surface sterilised nodules confirmed that the nodules were infected, although at different degrees. This was confirmed by electron microscope analysis of ultrathin sections of these nodules.

The gene blr2992 fused to E-tag in clone FK305 exhibits sequence similarity with the rare lipoprotein A (RlpA). In *Escherichia coli* this cell envelope component of the outer membrane contains a conserved region that has the double-psi-beta-barrel (DPBB) fold. The function of RlpA is not well understood, but it has been shown to act as a *prc* mutant suppressor in *E. coli* (Tadokoro *et al.*, 2004). The DPBB fold is often an enzymatic domain. The members of this family are quite diverse and may have several different functions.

Sequence similarities indicate that the E-tag fusion of FK309 has occurred within gene blr6636, which has been designated as an ATP synthase subunit. In *B. japonicum* this

sequence shares considerable similarity with ten other proteins that have a transmembrane segment. This family of proteins is found in a range of bacteria. The conserved region contains a histidine and cysteine, suggesting that these proteins have an enzymatic activity. Several members of this family contain peptidoglycan binding domains. So these proteins may use peptidoglycan or a precursor as a substrate.

Gram-negative bacteria produce a number of proteins which are secreted into the growth medium by a mechanism that does not require a cleaved N-terminal signal sequence. These proteins, while having different functions, require the help of two or more proteins for their secretion across the cell envelope. These secretion proteins include members belonging to the ABC transporter family and a protein belonging to a family which includes e.g. *hlyD* (Hemolysin) of *E. coli* and *rtxD* (Toxin-III) of *Actinobacillus pleuropneumoniae* and *emrA* (drugs and toxins) of *E. coli* (Borges-Walmsley *et al.*, 2003). The secretion proteins are evolutionary related and consist of 390 to 480 amino acid residues. They seem to be anchored in the inner membrane by a N-terminal TMS. Their exact role in the secretion process is not yet known. Based on sequence similarity and secondary structure predictions the protein encoded by blr7958, defective in BJ-FK343-22, belongs to this category of membrane proteins.

The E-tag fusion FK409 affects a gene, blr5829, which encodes the distal basal body ring component of the flagellum. It is part of an operon of genes with related functions. A mutation in this locus therefore can be predicted to affect the formation of intact flagella. If the poor nodule occupancy rate is related to this particular defect the question arises whether the bacteria within the infection thread require flagella for penetrating into the plant cells.

The symbiotic phenotypes of these mutants are related to those of the signal peptidase mutants (Muller *et al.*, 1995a; Bairl *et al.*, 1998) which indicates that, although the correct E-tag fusion insertion could not been checked, the mutation affect extracytoplasmic proteins as expected, and that they are involved in the symbiotic interaction between *B. japonicum* and soybean plants.

5. CONCLUSIONS

1. The *napEDABC* gene cluster that encodes the periplasmic nitrate reductase from *Bradyrhizobium japonicum* USDA110 has been isolated and characterized. *napA* encodes a periplasmic protein with a molecular size of about 90 kDa which corresponds to the catalytic subunit. *napB* and *napC* gene products are a periplasmic *c*-type cytochrome and a membrane-bound *c*-type cytochrome, with molecular masses of 25 kDa and 15 kDa, respectively. In *B. japonicum*, NapC is the physiological electron donor of the NapAB catalytic components of periplasmic nitrate reductase.

2. The periplasmic nitrate reductase is the enzyme responsible for anaerobic growth of *B. japonicum* under nitrate-respiring conditions.

3. The promotor region of *napEDABC* genes has been characterized by primer extension. A major transcript initiates 66.5 bp downstream of the centre of a putative FNR-like binding site.

4. Primer extension experiments and expression analysis of a translational fusion of the NapC protein to the reporter gene *phoA* showed that maximal expression of *nap* genes requires low oxygen conditions and the presence of nitrate.

5. Although *B. japonicum* periplasmic nitrate reductase is responsible for the 84 % of the nitrate reductase activity detected in bacteroids, *napA or napC* genes are not essential for nodulation and nitrogen fixation of soybean plants.

6. In addition to the periplasmic nitrate reductase, new extracytoplasmic proteins have been identified and *B. japonicum* gene disruption mutant strains have been constructed by using E-tag fusion clones obtained from a phage display library.

7. Proteins encoded by the *B. japonicum* ORFs designated as FK106, MR021, bll7946, bll2692, blr0479, MR055 and blr5637 are not essential for the establishment and functioning of the symbiosis with soybean plants. However, *B. japonicum* mutant strains affected in the ORFs FK242, FK305-12, FK309-13, FK343-22, and FK409-25 showed a altered symbiotic phenotype compared to that showed by the parental strain *B. japonicum* USDA110.

8. We propose the phage display strategy to identify unknown extracytoplasmic proteins with potential role on the symbiotic interaction between *Rhizobia* and legume plants.

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7. ABBREVIATIONS

A	adenine
ABC	ATP binding cassette
Abs.	Absorbance
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
ARA	acetylene reduction activity
bp	base pairs
BV	bencyl viologen
C	cytosine
Ca	calcium
cm	centimeters
Cu	copper
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
dUTP	deoxy-uridine triphosphate
EDTA	ethylenediaminetetraacetic acid
EPS	exopolysaccharide
FeS	iron sulfur
[4Fe-4S]	iron-sulfur cluster
g	gram
G	guanine
GTP	guanosine triphosphate
h	hour
Kb	kilo base pairs
kDa	kilo Daltons
l	litre
LPS	lipopolysaccharide
mA	milli Ampere
Mb	mega base pairs
mbar	milli bar
mg	milligram

MGD	molybdopterin guanine-dinucleotide cofactor
min	minute
ml	millilitre
mm	millimeters
mM	millimolar
mmol	millimole
Mo	molybdenum
mPa	milli Pascal
MV	methyl viologen
N	normal
Nap	periplasmic nitrate reductase
Nar	membrane nitrate reductase
ng	nanogram
Nir	nitrite reductase
nM	nanomolar
nmol	nanomole
Nor	nitric oxide reductase
Nos	nitrous oxide reductase
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMF	proton motive force
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RNase	ribonuclease
r.p.m.	rounds per minute
SDS	sodium dodecyl sulfate
T	thymine
TEMED	tetramethylethylenediamine
U	Units
UV	ultra violet
µg	microgram
µl	microlitre

µm	micrometer
µM	micromolar
µmol	micromole
v	volume
V	volts
w	weight