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Versatility in rhizobial respiration under oxygen-limiting conditions: new insights in the denitrification regulatory network

Memoria de Tesis Doctoral presentada por la licenciada en Biología

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A mis padres que son mis cimientos y mi apoyo.

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1. SUMMARY

Las bacterias del suelo conocidas con el nombre genérico de rizobios son capaces de fijar el dinitrógeno atmosférico en asociación simbiótica con plantas leguminosas. Esta asociación es de gran interés agrícola y medioambiental, debido a la capacidad de la bacteria de proporcionar a la planta el nitrógeno necesario para su nutrición y desarrollo, reduciendo las necesidades de fertilización química. Por ello, el uso de inoculantes microbianos para mejorar la nutrición de los cultivos constituye una tecnología de gran interés por su impacto positivo sobre la sustentabilidad agrícola y su carácter respetuoso con el medio ambiente. Además de fijar nitrógeno, algunas especies de rizobios son capaces de desnitrificar tanto en vida libre como en simbiosis. La desnitrificación es un proceso clave en el ciclo biogeoquímico del N en la biosfera, ya que es el mecanismo mediante el cual se devuelve a la atmósfera el N_2 fijado. Este proceso es el mecanismo principal para eliminar el exceso de nitratos que, como consecuencia del abuso en la utilización de fertilizantes nitrogenados en la agricultura, contaminan los ecosistemas terrestres y acuáticos. La contaminación de acuíferos por nitratos y nitritos es un grave problema con repercusiones en la salud pública. Además, el óxido nítrico y el óxido nitroso, productos intermediarios de la desnitrificación, tienen también un enorme impacto sobre la contaminación atmosférica, ya que son gases que se liberan a la atmósfera e intervienen en la formación de la lluvia ácida, en el calentamiento global de la atmósfera, y en la destrucción de la capa de ozono. La desnitrificación tiene, por tanto, un gran impacto en la agricultura, medioambiente y salud humana.

La desnitrificación es una forma alternativa de respiración por la que, en condiciones limitantes de oxígeno, los microorganismos pueden utilizar el nitrato y sus óxidos de nitrógeno derivados (NOx) como aceptores de electrones en una cadena de

transporte hasta la formación de N_2. La reducción de los NOx está acoplada a la producción de ATP, lo que permite a la célula crecer en condiciones limitantes de oxígeno. La desnitrificación consiste en la completa reducción del nitrato (NO_3^-) o nitrito (NO_2^-) hasta nitrógeno molecular (N_2), a través de la producción de los intermediarios óxido nítrico (NO) y óxido nitroso (N_2O). Estas reacciones tienen lugar secuencialmente por la actuación de las enzimas nitrato reductasa (Nap/Nar), nitrito reductasa (CuNir/*cd*$_1$Nir), óxido nítrico reductasa (qNor/cNor) y óxido nitroso reductasa (Nos), codificadas por los genes *nap/nar*, *nirK/nirS*, c*nor/qnor* y *nos,* respectivamente (Zumft et al., 1997; van Spanning et al., 2005, 2007; Kraft et al., 2011; Richardson, 2011; Bueno et al., 2012).

Bradyrhizobium japonicum es el único rizobio en el que se han aislado y caracterizado los genes de la desnitrificación *napEDABC* (Delgado et al., 2003), nirK (Velasco et al., 2001), norCBQD (Mesa et al., 2002) y nosRZDFYLX (Velasco et al., 2004), implicados en la síntesis de las enzimas nitrato reductasa periplásmica (Nap), nitrito reductasa (NirK), óxido nítrico reductasa (cNor) y óxido nitroso reductasa (Nos), respectivamente (revisado por Potter et al., 2001; Richardson et al., 2001; Zumft, 2005; González et al., 2006; Richardson et al., 2007; de Vries et al., 2007; Richardson, 2011; van Spanning, 2011; Spiro, 2012).

Como en muchos otros desnitrificantes, la expresión de los genes de la desnitrificación, en *B. japonicum*, ocurre en condiciones limitantes de oxígeno y presencia de nitrato, o un NOx derivado de él (revisado por Bedmar et al., 2005; Delgado et al., 2007). En *B. japonicum* existe una sofisticada red de regulación formada por dos cascadas que coordinan la expresión genes implicados en la respiración

microóxica (cascada FixLJ/$FixK_2$) y en la fijación de nitrógeno (cascada RegSR/NifA) (Sciotti et al., 2003). El papel de la cascada FixLJ/$FixK_2$ está bien establecido, la cual en condiciones microóxicas (≤ 5% O_2 en la fase gaseosa), induce la expresión, entre otros, de los genes *fixNOQP*, responsables de la síntesis de la oxidasa terminal *cbb*$_3$ de alta afinidad por oxígeno (Nellen-Anthamatten, 1998). En dichas condiciones, $FixK_2$ controla también la expresión de los genes *nap* y *nirK*, pero no la de los genes *nor* (Mesa et al., 2008). Además, mediante el empleo de ensayos de transcripción *in vitro* (Mesa et al., 2005), hemos podido demostrar que $FixK_2$, en colaboración con la ARN polimerasa de *B. japonicum* activa la transcripción de los genes *nap* y *nirK*, pero no la de los genes *nor* (Bueno et al., enviado para su publicación). En *B. japonicum*, se ha identificado el gen *nnrR*, que codifica la proteína NnrR (nitrite and nitric oxide reductase regulator), homóloga a otros reguladores transcripcionales de la familia CR/FNR (Mesa *et al*., 2003). La activación microaeróbica de *nnrR* está controlada por $FixK_2$ (Mesa *et al*., 2003; Mesa et al., 2008). Recientemente, hemos sido capaces de demostrar, en ensayos de calorimetría en ausencia de oxígeno, que la proteína NnrR se une al promotor de los genes *nor* , pero no al de los genes *nirK* y *napE* (Robles *et al*., enviado para publicación). Por lo tanto, mientras que NnrR ejerce un control directo sobre los genes *nor*, el control sobre los genes *nirK* y *napE* es más bien indirecto (Robles *et al*., enviado para publicación).

La cascada RegSR/NifA principalmente induce la expresión de los genes necesarios para la fijación de nitrógeno en respuesta a concentraciones muy bajas de O_2 (≤ 0,5% en la fase gaseosa). Recientemente, se ha demostrado que la proteína NifA, además, es necesaria para la máxima expresión de los genes de la desnitrificación en *B. japonicum* (Bueno et al., 2010). En base a estos resultados, nos planteamos investigar

si la cascada RegSR también pudiera estar implicada en la regulación de los genes de la desnitrificación. RegSR pertenece a la familia de sistemas reguladores de dos componentes presentes en un amplio número de proteobacterias y que principalmente ejercen un control en respuesta a cambios en el potencial redox, donde RegS es la proteína que percibe la señal y RegR la proteína reguladora (revisado por Bueno et al., 2012). Para ello, llevamos a cabo un análisis comparativo del transcriptoma de la cepa parental y de una mutante *regR*, ambas crecidas en condiciones anóxicas con nitrato como aceptor final de electrones y con succinato como fuente de carbono. Los resultados obtenidos revelaron que más de 600 genes inducidos en condiciones desnitrificantes, están también regulados por RegR. Estos datos indican que RegR es un regulador global y de gran importancia en estas condiciones de cultivo. Entre los genes identificados se encuentran los genes *nor* y *nos*, genes que codifican la Nor y Nos, respectivamente. También se identificaron como dianas de RegR otros genes implicados indirectamente en la desnitrificación como son los genes *cycA* y *c2*, que codifican transportadores de electrones. Además de genes relacionados con desnitrificación, también se identificaron otros implicados en detoxificación de óxido nítrico (*blr2806-09),* así como genes reguladores (*bll3466*, *bll4130).*

Mediante diferentes aproximaciones experimentales que explicaremos en detalle más adelante, hemos podido demostrar en esta Tesis Doctoral, que tanto la anoxia como el nitrato están implicados en la inducción de los genes *nor* por la proteína RegR y que dicho control es independiente de su proteína sensora RegS.

Este nuevo nivel en el control de los genes de la desnitrificación en *B. japonicum* está en concordancia con las evidencias que nos indican la existencia de un

exhaustivo control de la expresión génica en respuesta a condiciones adversas como son la limitación de O_2. Otra proteína clave a este respecto, que permite a la bacteria tanto crecer en vida libre en condiciones limitantes de oxígeno, así como capacitarla para obtener energía en el ambiente microóxico que existe en el nódulo simbiótico, es la oxidasa terminal *cbb*$_3$ de alta afinidad por el oxígeno, codificada por el operón *fixNOQP*. Esta proteína ha sido ampliamente estudiada en *B. japonicum* (Preisig et al., 1993, 1996). En cambio, su caracterización es más limitada en *Ensifer meliloti*, que a diferencia de *B. japonicum*, tiene tres copias de los genes *fixNOQP*. De estas tres copias que están localizadas en el megaplásmido pSymA (Galibert et al., 2001), las copias 1 y 2 son muy parecidas entre sí. La copia 1 se localiza en un entorno genético donde se encuentran una serie de genes reguladores como *fixLJ, fixT1, fixK1, fixM*, convirtiendo a esta copia como la candidata potencial para ser la copia funcional de la oxidasa terminal *cbb*$_3$ en *E. meliloti*. La copia 3 se ha relacionado con el metabolismo del fósforo (Krol and Becker, 2004). En el transcurso de esta Tesis, hemos realizado un análisis fenotípico de la mutante *fixN1* de *E. meliloti* tanto en vida libre como en simbiosis. En vida libre, pudimos observar un defecto en crecimiento en una mutante *fixN1* con respecto a la cepa parental crecidas en medio mínimo tanto en condiciones aeróbicas como microaeróbicas, al igual que se observó una disminución de la actividad oxidasa dependiente de N,N,N´,N´,-tetrametil-p-fenilenediamina (TMPD). En condiciones simbióticas, las plantas inoculadas con la cepa mutante en *fixN1* mostraron una clara deficiencia en la fijación de nitrógeno tras 3 semanas de crecimiento con una solución nutritiva libre de nitrógeno. Este fenómeno no se observó cuando las plantas se crecieron hasta 8 semanas, indicando que la copia 1 de la *fixNOQP* no es necesaria para la fijación de nitrógeno en períodos de cultivo más

prolongados. Aunque, nuestros resultados no confirmaron que la copia 2 es la que se activa en dichas condiciones de crecimiento, sí nos permitieron concluir que ambas copias no tienen funciones redundantes, siendo la copia *fixNOQP1* de *E. meliloti* crucial para la fijación de nitrógeno en las etapas iniciales de la fijación de nitrógeno (Torres et al., 2013). Gracias a estos estudios, se ha podido identificar por primera vez que los citocromos *c* de membrana de 27 y 32 KDa se corresponden, respectivamente, con las subunidades FixO y FixP de la oxidasa terminal cbb_3 de *E. meliloti* (Torres et al., 2013).

E. meliloti también posee los genes *nap, nir, nor* y *nos* responsables de la desnitrificación localizados en el genoma del megaplásmido simbiótico pSymA. Además, estudios de transcriptómica habían demostrado previamente la inducción de estos genes en condiciones microóxicas (Becker et al., 2004) y que *E. meliloti* poseía actividad desnitrificante tanto en vida libre como en simbiosis (Garcia-Plazaola et al., 1993; 1996). Sin embargo, hasta el inicio de este trabajo, *E. meliloti* solo había sido considerado como un desnitrificante parcial debido a su incapacidad para crecer en condiciones limitantes de oxígeno a expensas del nitrato o del nitrito como aceptores finales de electrones. Durante el desarrollo de esta Tesis, hemos podido demostrar que *E. meliloti* es capaz de usar el nitrato o el nitrito como sustratos respiratorios en condiciones microóxicas, indicando que, a diferencia de *B. japonicum,* que es capaz de desnitrificar en condiciones anóxicas, la desnitrificación en *E. meliloti* requiere la presencia de bajas tensiones de oxígeno (Torres et al., 2011). Para completar estos estudios, hemos llevado a cabo una caracterización funcional de los genes *nap, nirK, nor* y *nos* de *E. meliloti*, demostrando la implicación de estos genes en la capacidad de usar nitrato como sustrato respiratorio en condiciones microóxicas por esta bacteria. Gracias a la técnica de tinción de grupos hemo, hemos podido identificar que el

citocromo tipo *c* de membrana de 16 kDa se corresponde con la proteína NorC, componente de la óxido nítrico reductasa. Además, las actividades NR y Nir y la actividad Nor, disminuyeron significativamente en las mutantes *napA, nirK* y *norC*, respectivamente. Así mismo, una mutante *nosZ*, acumuló N_2O en el medio de cultivo cuando se incubó en condiciones microóxicas con nitrato. En su conjunto, estos resultados muestran claramente la implicación de los genes de la desnitrificación de *E. meliloti* tanto en la respiración de nitrato y como en el proceso de desnitrificación en condiciones microóxicas. Por último cabe mencionar que los genes *napA, nirK, norC* y *nosZ* de *E. meliloti*, también se expresaron en condiciones de anoxia en presencia de nitrato (donde se alcanzan sus valores máximos de expresión). Además las enzimas Nap, Nir y Nor son activas en dichas condiciones. Por tanto, la incapacidad de *E. meliloti* de crecer anaeróbicamente con nitrato no se debe ni a un defecto en la expresión de los genes de la desnitrificación, ni a la ausencia de actividad de las enzimas desnitrificantes. Posibles hipótesis para explicar esta observación se estudiarán en profundidad en experimentos futuros (véase capítulo IV en la sección de resultados y discusión general).

2. GENERAL INTRODUCTION

2.1. The Nitrogen Cycle.

Nitrogen is the key component of all biological organisms since it is required to synthesize amino acids, proteins, nucleic acids and many additional cofactors. The total nitrogen combined in biology originates from the atmosphere to where it is ultimately returned as the gas, N_2. Figure 2.1 shows the best known, arguably, of all elemental cycles, the nitrogen cycle. N_2, present at 78.08 per cent (v/v) in the atmosphere, possesses one of the most stable chemical linkages known, namely, a chemical triple bond that requires almost 103 kJ M^{-1} of energy to break into its component N atoms. The triple bond of N_2 also has a very high-energy barrier towards breaking, necessitating the use of highly effective catalysts, or enzymes, to speed up the scission process being, then, assimilated by most life forms. Atmospheric N_2 is not available for plants, animals or human. Only diazotrophic microorganisms can convert bio-unavailable N_2 gas to bio-available ammonia (NH_4^+), thought the nitrogenase enzyme (Newton, 2007; Peters et al., 2011). This process initiates the N cycle in the biosphere (Fig. 2.1). Ammonia is subsequently incorporated into cellular biomass mainly via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway. Alternatively, glutamate dehydrogenase (GDH) may also be involved in aerobic ammonium assimilation. In addition to its incorporation into organic nitrogen compounds, ammonia can be oxidized to nitrate (NO_3^-) by nitrifying bacteria in a two-step process called nitrification (Ferguson et al., 2007). During nitrification, the enzymes ammonia monooxygenase and nitrite oxidoreductase (nitrite oxidase) oxidize ammonia to nitrite (NO_2^-) and nitrite to nitrate, respectively (Fig. 2.1). Nitrate can be reduced to ammonium for assimilatory purposes (Moreno-Vivian and Flores, 2007; Tischner and Kaiser, 2007). When oxygen becomes limiting, nitrate is used as electron acceptor instead oxygen through denitrification and is reduced to NO_2^-, nitric oxide (NO), nitrous oxide (N_2O) and N_2, which returns to the atmosphere, thus closing the N cycle in the biosphere (van Spanning et al., 2007). Some bacteria such as *Escherichia coli* or *Bacillus subtilis* are able to perform nitrate respiration, but they do not denitrify with N_2 as a product. Instead, they reduce nitrate to ammonium via nitrite with N_2O being produced, so-called dissimilatory nitrate reduction to ammonium (Mohan and Cole, 2007). In addition to denitrification, anaerobic ammonia oxidation (anammox)

converts nitrite and ammonium directly into N_2, thus largely contributing to production of N_2 (Jetten et al., 2009) (Fig. 2.1). To complete the global N cycle, many bacteria and fungi degrade organic matter, releasing fix nitrogen for reuse by other organisms through ammonification. Genome sequencing of several N-cycle organisms, the nitrite-dependent anaerobic methane oxidazing (Raghoebarsing et al., 2006) and hypertermophilic N_2-fixing methane producing archaea (Mehta and Baross, 2006) has unveiled the biodiversity and metabolic capacity of new nitrogen conversions within the N cycle (Jetten, 2008). Together, these processes form the global N cycle and microorganisms are essential for maintaining the balance between reduced and oxidized forms of nitrogen.

Denitrification and nitrogen fixation appear to be antagonic processes revealing nitrogen fixation as an essential process that biologically compensates for nitrogen losses occurring via denitrification and anammox. But, in other hand, today it exists a problem of excess of nitrate coming from an imbalance of biological nitrogen fixation and denitrification of about 90-130 Tg N per year (Tg = Teragram = 1 billion grams). Additionally, the industrial manufacture of ammonium by the Haber-Bosch process contributes to nitrate formation with some additional 140 Tg per year. All these result in a considerable increase in soil nitrate concentration and this excess nitrate cannot be removed by denitrification, resulting in the accumulation of nitrate in soil, water, and sediments. This large increase in N load in the environment, in turn, leads to serious alterations in the cycling of N and will likely cause severe damage to environmental services at local, regional and global scales (Galloway et al., 2008).

2.1.1. Anthropogenic impacts on the nitrogen cycle

During the 20th Century, human activities, particularly the chemical reduction of nitrogen on an industrial scale to make synthetic fertilizers and the combustion of fossil fuels, have had an increasingly significant effect on the global nitrogen cycle (Galloway et al., 2008). Unsurprisingly, there is a disproportionate impact by human populations in developed countries, where vehicle emissions and industrial agriculture are most prevalent (Socolow, 1999). These influence climate change, human health

and the ecological functioning of natural ecosystems reducing biodiversity, especially aquatic systems and soils where nitrogen concentrations are increasing, causing eutrophication of lakes or rivers and oceanic dead zones through algal bloom-induced hypoxia (Howarth, 2004). The nitrogen compounds resulting from human activities that have the greatest impact on the environment are the following.

(i) Enhanced NO and N_2O emissions from fertilized soils due to denitrification. N_2O along with carbon dioxide (CO_2) and methane (CH_4) are the three most important greenhouse gases. Not only N_2O has a 300-fold greater global warming potential than CO_2, but also its atmospheric loading is increasing by 0.25% each year. As a consequence, it is essential that strategies to mitigate climate change include the reduction of N_2O emissions (Richardson et al., 2009). In addition, both N_2O and NO have deleterious effects on the stratosphere, where they act as catalysts in the destruction of atmospheric ozone (Lassey and Harvey, 2007; Ravishankara et al., 2009). Limiting future anthropogenic N_2O emissions would not only allow the recovery of the depleted ozone layer, but also reduce climate change.

(ii) Excess NO_3^- and NO_2^- derived from fertilizers are leached from soils and enter the groundwater. Elevated levels of nitrate in drinking water is a known risk factor for methaemoglobinaemia (a potential cause of blue baby syndrome) (Greer and Shannon, 2005) and colon cancer (Van Grinsven et al., 2010).

(iii) NH_3 in the atmosphere has tripled as the result of human activities. It acts as an aerosol, decreasing air quality and clinging on to water droplets (Harper et al., 2010).

Consequently, researchers in a number of disciplines, including microbiologists, biochemists, soil scientists, ecologists and atmospheric chemists, working on different aspects of the nitrogen cycle, have increasingly come together to explore some of the great challenges facing 21st Century humankind, including climate change (Duce et al., 2008; Richardson et al., 2009), food security (Socolow, 1999), waste-water treatment (Howarth, 2004) and human health (Greer and Shannon, 2005; Van Grinsven et al., 2010).

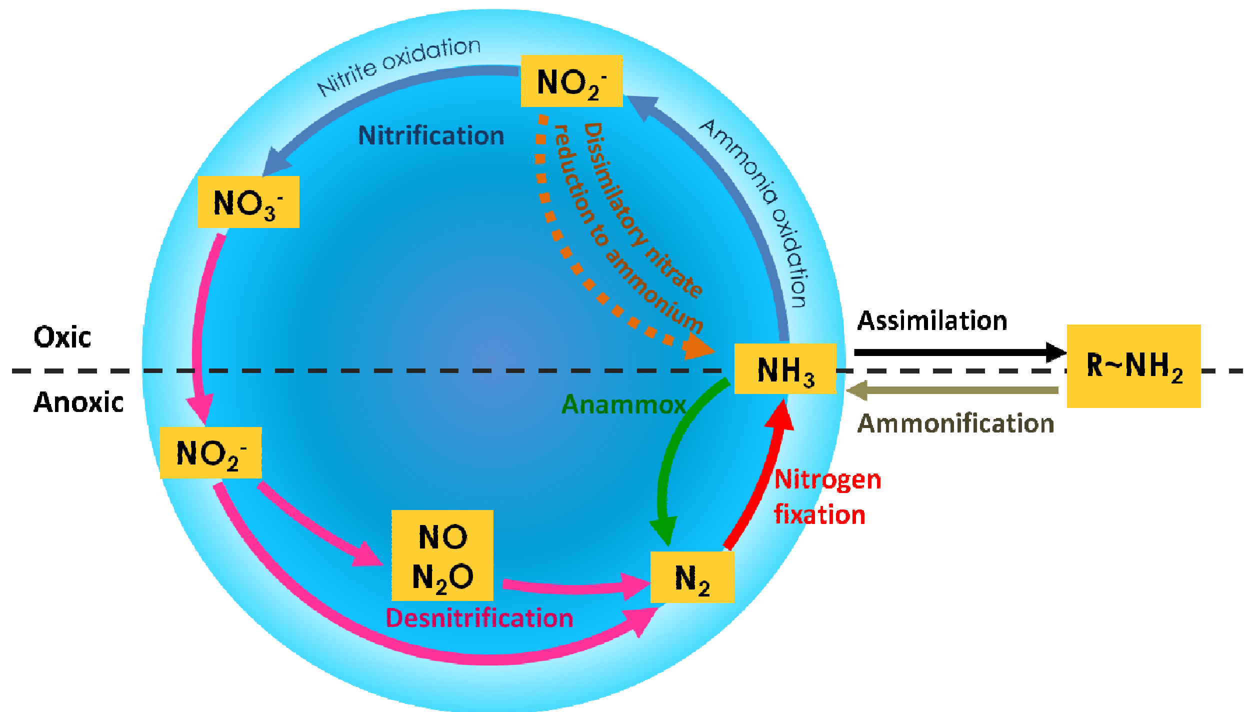


Figure 2.1. Schematic N_2 cycle (adapted from http://www.nature.com/scitable/knowledge/library/the-nitrogen-cycle-processes-players-and-human-15644632).

2.2. Symbiotic nitrogen fixation.

Biological nitrogen fixation process can be carry out by free-living, associative or symbiotic nitrogen fixers. The most effective fixation occurs in symbiotic relation with plants. Since atmospheric N_2 is an unlimited source of N, symbiotic nitrogen fixation (SNF) is of great potential for sustainable agriculture. Together with the actinorhizal plants, legumes are best characterized by their ability to establish N_2-fixing symbiotic associations with soil bacteria collectively referred as rhizobia. During this process, an exchange of molecular signals occurs between the two partners, leading to the formation of root nodules, where nitrogen fixation takes place. The plant provides sucrose to nodule host cells, where it is oxidized to dicarboxylic acids and used as energy source by the bacteroids to fix atmospheric nitrogen, which is converted into ammonium and assimilated as amides or ureides (for reviews see Graham and Vance, 2003; Stacey, 2007; Terpollini et al., 2012).

The Leguminosae (alternative name Fabaceae) is the third largest flowering plant family, containing 19,325 species, and accounts for over 8% of the world's

flowering plants. The legume family has 730 genera and currently is divided into three subfamilies: *Caesalpinioideae, Mimosoideae* and *Papilionoideae*. The legume (or bean) family, which includes lentils, peas, beans, peanuts and soya, is hugely important as a source of food due to its high protein content. It is second only to grasses (cereals) in agricultural importance, and many species are also used for forage, hay, silage and green manure, and it constitutes an important component for fodder animal feeding. Species of legume are found throughout the world in a wide variety of habitats that include arid environments and tropical rainforest, and range in size from small herbs to huge tropical forest trees (Carpena et al., 2006; Rodiño et al., 2001). Legumes have an important role for both human nutrition and animal feeding, however, soybeans are unique in legumes with contents of 40% protein and 21% oil as well as isoflavones. Thus, soybean is the most widely grown protein/oilseed crop in the world representing 77% of the N fixed by the crop legumes by fixing 16.4 Tg N annually. Fixation by soybean in the US, Brazil and Argentina is calculated at 5.7, 4.6 and 3.4 Tg, respectively (Herridge et al., 2008; *Soybean Physiology and Biochemistry*, In-Tech). Alfalfa, called the "Queen of the Forages" is the most widely legume crop in Spain which is one of the main producers of this forage legume in Europe with a surface of about 230,000 ha (20% of total forage crops surface, http://www.mapa.es/agricultura/pags/cultivos_herbaceos/forrajes/). In addition to the traditional uses of alfalfa as an animal feed, alfalfa has a great potential as a bioenergy crop because of its high biomass production, perennial nature, and ability to provide its own nitrogen fertilizer due its ability to establish symbiotic relations with nitrogen-fixing soil bacteria. Thus, different studies considered alfalfa (especially stems) as a good sustainable crop for second-generation bioethanol production (Samac and Lamb, 2006; González-Garcia et al., 2010; Dien et al., 2011).

Inoculation of legumes with rhizobia is an economical and environmental friendly recommended worldwide agricultural practice to increase crop yield and to improve soil fertility without adding N fertilizers. Until 2001, all known bacteria involved in root nodule symbioses with legume plants were classified as members of the order Rhizobiales of the Alphaproteobacteria, including the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (Graham 2008; Rivas et

al., 2009; Velázquez et al., 2010), and are collectively referred to as rhizobia. During the last 12 years, it has been reported non-rhizobial Alphaproteobacterial strains capable to carry on a nitrogen fixation symbiosis with legume plants such as *Mithylobacterum nodulans* (Jourand et al., 2004), *Devosia neptuniae* (Rivas et al., 2003), *Blastobacter denitrificans* (van Berkum et al., 2002), *Ochrobactrum lupini* (Trujillo et al 2005), *Phyllobacterium trifolii* (Valverde et al., 2005), and *Microvirga* (Ardley et al., 2011), among others. Futhermore, legume nodulation is not restricted to Alphaproteobacteria, since, Betaproteobacterial *Burkholderia* strains have been shown to nodulate and fix N_2 with species of *Mimosa* (Moulin et al., 2001; Elliott et al., 2009; Gyaneshwar et al., 2011;) or common bean (Talbi et al., 2010).

Soybeans perform symbiosis with rhizobia strains of the genera *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium*, being *B. japonicum* the specie most widely employed as commercial inoculants for soybean crops. *B. japonicum* occupies two distinct niches: free-living in the soil and establishing symbiotic associations with soybean (*Glycine max*), siratro (*Macroptilium atropurpureum*), mung bean (*Vigna radiata*) and other *Vigna* species. *B. japonicum* strain USDA110, which was originally isolated from soybean nodule in Florida, USA in 1957, has been widely used for the purpose of molecular genetics, physiology, and ecology. The genome of *B. japonicum* USDA110 is a single circular chromosome of about 9.1 Mb in length. No plasmid are detected in *B. japonicum*, but a 410-kb DNA segment containing clusters of genes for symbiotic nitrogen fixation has been assigned as a symbiotic region (Kaneko et al., 2002).

E. meliloti (formerly *Sinorhizobium meliloti)* is one of the best characterized endosymbionts of alfalfa. *E. meliloti* is an aerobic soil bacterium which establisches symbiotic N_2^- fixing associations with plants of the genera *Medicago, Melilotus* and *Trigonella*. The interaction between *E. meliloti* with the host legumes has been the subject of extensive biochemical, molecular, genetic (Jones et al., 2007), and evolutionary investigation (Bailly et al., 2006; 2007). The genome of *E. meliloti* consists of a single circular chromosome (3.65 Mb) plus two large symbiotic plasmids: pSymA (1.35 Mb) and pSymB (1.6 Mb) (Galibert at al., 2001). In *E. meliloti* the genes required

for forming nodules with legume hosts (including *nod*, *exo*, and *nif* genes) are distributed across both the chromosome and each of the two megaplasmids (MacLean et al., 2007). Also, pSymA contains a large fraction of genes known to be specifically involved in symbiosis such as genes involved in nodulation or in nitrogen fixation process, as well as genes involved in microoxic metabolism or in denitrication (Barnett et al., 2001; Torres et al., 2011).

2.2.1. Nodulation process.

Nodulation is a complex multi-step process that requires specific interactions between the symbionts, starting with the exchange of a variety of molecular signals between the host plant and the bacterium. Seed and root exudates comprise a variety of compounds that induce the expression of specific genes in compatible bacteria, generally preceded by a common conserved sequence denominated “nod-box”. Plant perceives a critical bacterial signal (the -Nod factor) and transduces the signal for the activation of downstream responses, leading to infection and nodule morphogenesis. Several reviews summarize the findings on the involvement of signals during nodule development (Jones et al., 2007; Stacey, 2007; Oldroyd and Downie, 2008). Nod factor is a lipochitooligosaccharide (LCO) signal molecule that elicits both nodule organogenesis and root hair deformation in the plant (Figure 2.2, recently reviewed in Oldroyd et al., 2011). Bacteria then infect plant roots either via root hairs or by crack entry, however the major crops legumes are infected by the former means (Sprent, 2009). Root hairs deform in such way as to entrap bacteria within a curl. Infection is initiated from these curled root hairs, with infection threads growing as tunnel-like invaginations of the host cell from the centre of the curl. The plants allow rhizobia access to an intracellular space. The infection thread is reminiscent of a growing cylinder of plant cell wall in which rhizobia replicate to remain at the growing tip. In parallel with this infection process, cell division is initiated in the cortical cells of the root, and this leads to the formation of a nodule. Release of the bacteria from the infection thread resembles endocytosis by resulting in the formation of a membrane-bound compartment in which bacteria exist as intracellular symbionts (Figure 2.2). This membrane-bound compartment has been termed the symbiosome and is the unit of biological N_2 fixation. Across the membrane of the symbiosome there is metabolite

exchange, including uptake of dicarboxylic acids, export of ammonia and cycling of amino acids with the host cell. Rhizobia within the symbiosomes differentiate and induce a variety of new enzyme systems (i.e. nitrogenase and the high-affinity cytochrome *cbb*$_3$-type oxidase) and often take on a larger, more extended shape. For these reasons, the special term bacteroid is used to define the intracellular symbiont (For a review see Terpolilli et al., 2012).

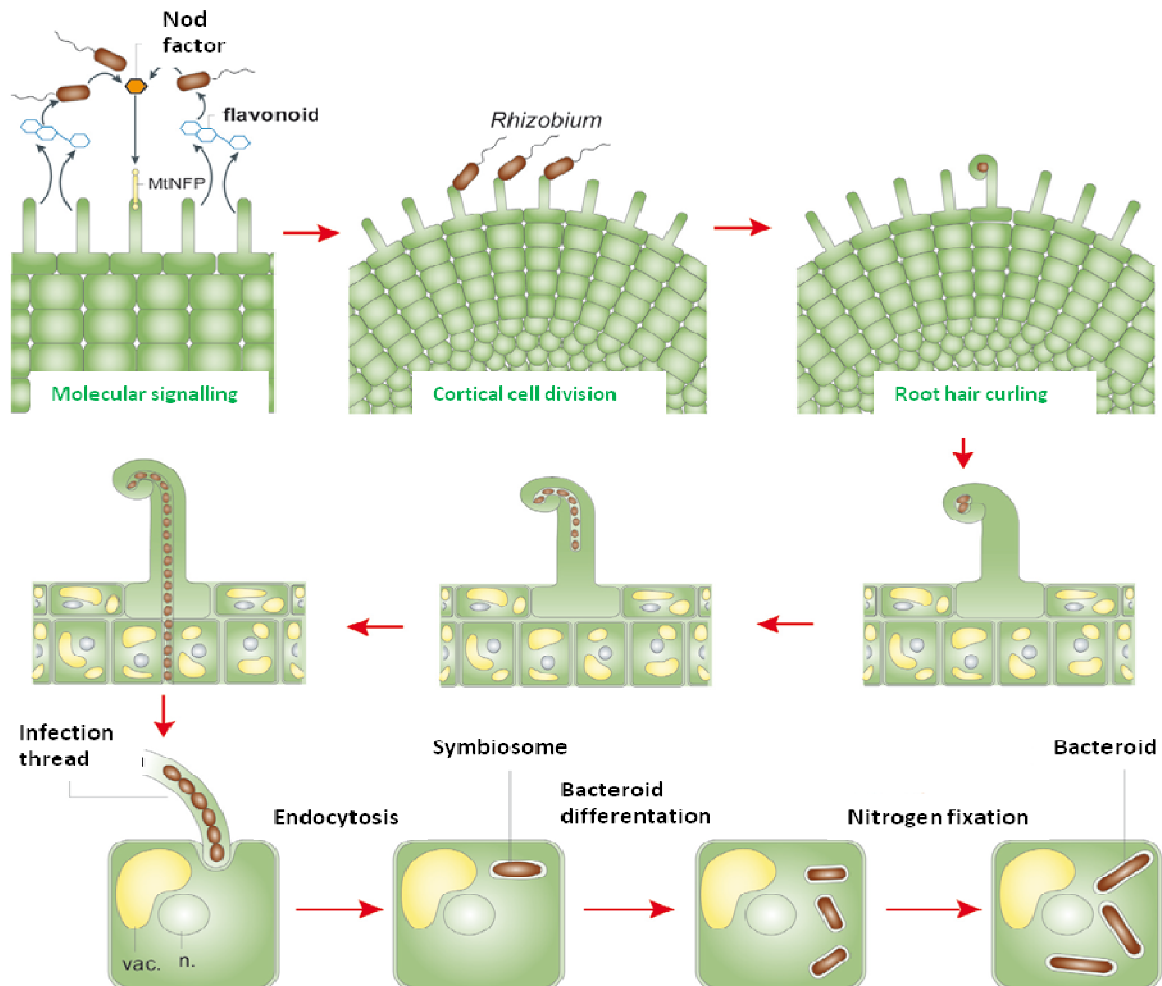


Figure 2.2. Schematic representation of nodulation process and bacteroids formation (adapted from Jones et al., 2007).

Two morphological types of nodules are known and they are determined by the plant host. The first type is called indeterminate such as those of pea (*Pisum sativum*), alfalfa (*Medicago sativa*), and broad bean (*Vicia faba*). They have a persistent meristem, and in longitudinal section, can be divided into four zones: (I) the meristem

at the nodule tip, (II) the invasion zone immediately behind the meristem with cells invaded by rhizobial-containing infection threads, (III) the N_2-fixing zone, where the cells contain fully differentiated bacteroids, and (IV) the senescent zone. The second type, called determinate nodules, which form on plants such as soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*) have a globular structure. Typical determinate legume nodules possess a central zone formed by bacteroids containing infected cells and uninfected cells, an inner cortex containing small cells with large intercellular spaces and a boundary layer of closely-packed cells, and an outer cortex containing large loosely packed cells with large intercellular spaces, sometimes surrounded by a periderm. In addition to their morphology, determinate and indeterminate nodules also differ in the developmental program by which rhizobia form N_2 fixing bacteroids (For a review see Terpolilli et al., 2012).

2.2.2. Nitrogenase complex

Along with the differentiation of bacteria into bacteroids comes the induction of a set of bacteroid genes, critical to the reduction of N_2 to NH_3. Generally, those genes which are common to both free-living diazotrophs and rhizobia, are referred to as *nif* genes whereas those that are important to symbiotic N_2 fixation but have no equivalents in free-living diazotrophs are referred to as *fix* genes. In rhizobia, the N_2 fixation onset necessitates the activation of a whole range of both *nif* and *fix* genes. Expression of the genes *nifH* and *nifDK* are central to the process of N_2 fixation as they code for the molybdenum-nitrogenase enzyme complex which all known diazotrophs possess (this complex catalyzes the reduction of N_2 to NH_3 with the overall stoichiometry under optimal conditions of:

$$N_2 + 8\,e^- + 8\,H^+ + 16\,MgATP \rightarrow 2\,NH_3 + H_2 + 16\,MgADP + 16P_i$$

Rhizobia possess a conventional molybdenum-based nitrogenase (for reviews about biochemistry and genetics of nitrogenase, see Dixon and Kahn, 2004; Seefeldt et al., 2009; Newton et al., 2007; Peters et al., 2011), which contain the larger

heterotetrameric NifDK component which receives the electrons from the smaller dimeric NifH component. NifDK contains the essential iron-molybdenum cofactor [FeMoCo ($MoFe_7S_9$·homocitrate)] and one P-cluster ([8Fe-7S] , whereas NifH is the homodimer containing a Fe-S cluster and sites for MgATP binding and hydrolysis (Rubio and Ludden, 2008). Other essential *nif* gene is NifA, a centrally regulator of N_2 fixation in rhizobia. There are another 16 *nif* genes described in free-living, however, not all of these genes are present in rhizobia, with *Azorhizobium caulinodans* and *B. japonicum* having 15 and 13, respectively, and *Rhizobium leguminosarum bv. viciae* 3841 and *E. meliloti* 1021 having only 8 and 9, respectively (Masson-Boivin et al., 2009). Among the *fix* genes, *fixABCX* are essential for N_2 fixation with mutation of any these genes abolishing N_2 fixation in *E. meliloti, B. japonicum* or *A. caulinodans* (Fischer, 1994). Their role, however, is still not clear, although it has been postulated that FixABCX might facilitate the transfer of electrons from the piruvate dehydrogenase complex to nitrogenase (Scott and Ludwig, 2004).

2.3. Microxia in root nodules.

Nitrogenase is rapidly inactivated by atmospheric concentrations of O_2 and even an oxygen concentration as low as 57 nM within a soybean nodule can reduce nitrogenase activity (Kuzma et al., 1993). Therefore, legume nodules keep the O_2 tension to the extremely low level of 5–30 nM (Appleby, 1984; Kaminski et al., 1998), compared with aerobic growth at 250 µM. The O_2 sensitivity nitrogenase is conferred by the surface-exposed [4Fe-4S] cluster that bridges the two subunits of the NifH dimer. So, unsurprisingly, low O_2 is a major signal for the activation of the *nif* and *fix* genes.

2.3.1 Control of oxygen diffusion.

In the nodule, maintenance of nitrogenase activity, which is irreversibly inhibited by O_2, is subject to a delicate equilibrium, since the necessity for low O_2 tension must be balanced with the O_2 requirement for ATP synthesis needed to energize nitrogenase. These competing needs are met primarily by the modulation of O_2 concentration via an oxygen diffusion barrier in the nodule (reviewed in Minchin et

al., 2008), by use of leghemoglobin to buffer O_2 concentration (Downie, 2005 and references therein) and by a terminal *cbb*$_3$ oxidase with a high affinity for O_2 encoded by *fixNOQP* genes (reviewed in Delgado et al., 1998). So, these systems maintain a delicate balance in O_2 supply and demand, solving this apparent conflict in order to keep the steady-state concentration of free-O_2 low (Figure 2.3).

The cortex of nodules acts as a diffusion barrier, which greatly limits permeability to O_2 (Hunt and Layzell, 1993). That barrier is a complex of structures involving intercellular space occlusions in the mid-cortex and osmocontractile responses in the inner cortex. There are also several metabolic or structural changes which could occur within the infected region (for a review, see Minchin et al., 2008).

Oxygen is delivered to the symbiosomes by the plant O_2-carrier leghemoglobin (Lb), which transports O_2 at a low but stable concentration allowing for the simultaneous operation of nitrogenase activity and bacteroid respiration (Downie, 2005 and references therein). The O_2-binding characteristics of Lbs are unusual in that they have an extremely fast O_2 association rate and a relatively slow O_2 dissociation rate, and so can buffer the free-O_2 concentration at around 7-11 nM. The presence of milimolar concentrations of Lb within the cytoplasm of nodule cells serves to buffer free O_2 in the nanomolar range while ensuring rapid transport of O_2 to the sites of respiration (Appleby 1984, 1992; Bergersen and Turner, 1993). It has been shown that removal of Lb from *Lotus* nodules via RNA interference (RNAi) resulted in an increase in nodule free O_2, destabilization of nitrogenase, and a failure of symbiotic N_2 fixation (Ott et al., 2005).

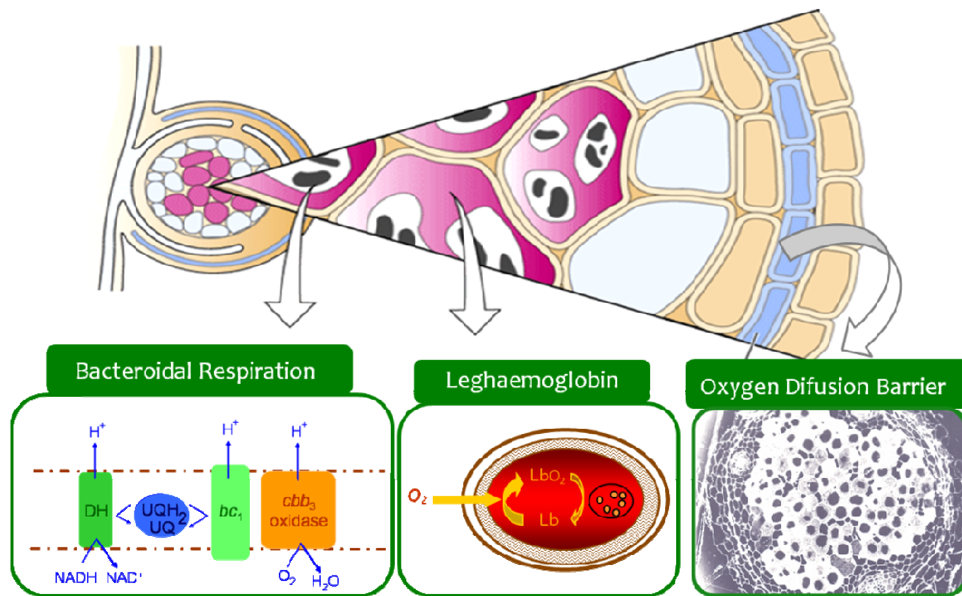


Figure 2.3. Schematic representation of the mechanisms involved in nitrogenase protection against oxygen in legume root nodules.

2.3.1.1. High affinity *cbb₃* oxidase

N_2-fixing bacteroids deal with the low levels of free O_2 by inducing a high-affinity cytochrome *cbb₃*-type oxidase. Genes encoding the *cbb₃* oxidase complex were isolated initially from rhizobial species and named *fixNOQP* for their role in symbiotic N_2 fixation (Preisig et al., 1993; Mandon et al., 1994; Delgado et al., 1998). Since then, orthologous genes have been identified in other Gram-negative bacteria and called *ccoNOQP* (for a review, see Pitcher and Watmough, 2004). The *cbb₃*-type oxidase is made up of three to four subunits (Figure 2.4): subunit I is encoded by *ccoN* and is a membrane-integral *b*-type cytochrome with a high-spin heme-Cu_B binuclear center and a low-spin heme. Subunits II and III are encoded by *ccoO* and *ccoP*, respectively, and are membrane-anchored *c*-type cytochromes. Cytochrome *cbb*$_3$ oxidases have been purified from several organisms including *Paracoccus denitrificans*, *R. sphaeroides*, *Rhodobacter capsulatus* and *B. japonicum* (reviewed by Pitcher and Watmough, 2004) The biogenesis of this multisubunit enzyme, encoded by the *ccoNOQP* operon, depends on the *ccoGHIS* gene products, which are proposed to be specifically required for cofactor insertion and maturation of *cbb*$_3$-type cytochrome *c* oxidases (Preisig et al., 1996). In the facultative photosynthetic model organism *R. capsulatus,* CcoN, CcoO and CcoQ assemble first into an inactive 210 kDa sub-complex, which is stabilized *via*

its interaction with CcoH and CcoS. Binding of CcoP, and probably subsequent dissociation of CcoH and CcoS, generates the active 230 kDa complex (Kulajta et al., 2006). Recent results have proposed that CcoH behaves more like a subunit of the cbb_3 oxidase rather than a transient assembly factor *per se* (Pawlik et al., 2010). The insertion of the heme cofactors into the *c*-type cytochromes CcoP and CcoO precedes sub-complex formation, while the cofactor insertion into CcoN could occur either before or after the 210 kDa sub-complex formation during the assembly of the cbb_3-type oxidase (Kulajta et al., 2006). CcoQ is required for optimal cbb_3-type oxidase activity because it stabilizes the interaction of CcoP with the CcoNO core complex, leading subsequently to the formation of the active 230-kDa cbb_3-type oxidase complex (Peters et al., 2008). Several additional proteins including SenC (Swem *et* al., 2005), PCu_AC (Banci *et* al., 2005; Abriata *et* al., 2008; Serventi *et* al., 2012), DsbA (Deshmukh *et* al., 2003) and CcoA (Ekici *et* al., 2012) might be also involved in cbb_3 biogenesis.

CcoNOQP has been extensively studied in *R. sphaeroides*, where it has multiple roles. It functions not only as a terminal oxidase (García-Horsman et al., 1994) but also as a redox sensor in a signal transduction pathway controlling photosynthesis gene expression (Oh and Kaplan, 2002; Kim et al., 2007).

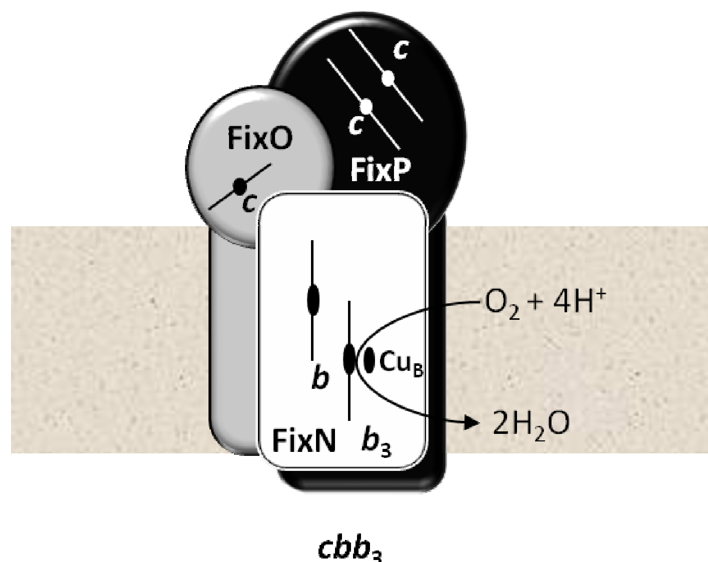


Figure 2.4. *B. japonicum* cbb_3 teminal oxidase model (Bueno et al., 2012).

Among rhizobia, the *cbb₃* terminal oxidase from *B. japonicum* is the only the enzyme in which substrate affinity has been measured showing a Km for dioxygen in the order of 7 nM (Preisig et al., 1996), a value which is consistent with its function in the bacteroid. *B. japonicum* free-living bacteria growing microoxically and N_2-fixing bacteroids use the cytochrome *cbb₃*-type oxidase (Preisig et al., 1993). Electron transfer to this high-affinity oxidase is via the cytochrome bc_1 complex (Thöny-Meyer et al., 1989). *Rhizobium etli* CFN42 has two copies of the *fixNOQP* operon: *fixNOQPd* (located on the symbiotic plasmid) and *fixNOQPf* (located on plasmid p42f) (Lopez et al., 2001). Moreover, these operons are differentially regulated and only the *fixNOPQd* copy is essential for symbiotic nitrogen fixation, that is, a mutation in *fixNd* (but not in *fixNf*) results in a N_2 fixation rate that is ~2% that of the wild-type rate on *P. vulgaris* (Girard et al., 2000; Lopez et al., 2001). However, *A. caulinodans* mutants in *fixNO* genes retain a significant ability to fix N_2 in both symbiotic and free-living conditions (Mandon et al., 1993, 1994). *E. meliloti* possess three different copies of the *fixNOQP* operon in a 290-kilobase (kb) region of pSymA (Barnett *et* al., 2001; Renalier *et* al., 1987). However, up today functional analyses of these *fixNOQP* copies are missing. The involvement of copy 1 of the *E. meliloti fixNOQP* operon in free-living respiration and symbiotic nitrogen fixation has been investigated in this work (see chapter II from results section).

2.3.2. Oxygen control of nitrogen fixation.

Microoxia is a prerequisite not only for nitrogenase activity but also for induction of N_2 fixation and symbiosis-related genes (*nif* and *fix*) (Fischer, 1994). Perception and transduction of the “low-O_2 signal” are mediated by conserved regulatory proteins that are integrated into species-specific networks in different rhizobia (Fischer, 1994; Dixon and Kahn, 2004; Terpolilli et al., 2012). In the α-Proteobacteria, *nif* and *fix* genes are invariably subject to transcriptional activation by the enhancer-binding protein NifA in conjunction with the indispensable alternative sigma factor σ^{54} (encoded by *rpoN*) (Fischer, 1994; Dixon and Kahn, 2004). Transcription of *nifA* and *fix* genes in rhizobia is predominantly controlled by the O_2-responsive two component FixL-FixJ system, a key component in the regulation of N_2

fixation (Green et al., 2009). The sensory domain of FixL contains a PAS (Per-Arnt-Sim) domain, which is found in a wide range of sensors including those that respond to oxygen, redox potential, voltage, or light (Taylor and Zhulin, 1999). FixL is in its “on” state in the absence of oxygen, in which it auto-phosphorylates prior to phosphor-transfer to FixJ. Phosphorylated FixJ is active for DNA binding and regulation of the transcription of target genes. When molecular oxygen binds to FixL it converts the ferrous iron of the haem from the PAS domain from high spin to low spin that brings a series of conformational changes that inhibit the kinase activity of FixL. There is a great deal of structural and biochemical information for FixL proteins, so the mechanism by which oxygen regulates the kinase activity is quite well understood (Rodgers et al., 2005; Green et al., 2009; Gilles-Gonzalez and Gonzalez, 2005). FixL can bind other haem ligands (such as CO and NO) but these do not inhibit the kinase activity, and so their interaction with FixL is probably not physiologically significant. Transcriptomic analyses have revealed that FixLJ influence a wide range of cellular processes (Bobik et al., 2006, Mesa et al., 2008), but for now, we are going to focus on components of the FixLJ regulon that are involved in activation of N_2 fixation and the differences in the FixLJ regulatory cascade between rhizobia. So, these key regulators are organized hierarchically either in a single cascade as in *E. meliloti* or in two parallel, largely independent cascades as in *B. japonicum* (Fischer et al., 1994; Sciotti et al., 2003; Dixon and Kahn, 2004; Terpolilli et al., 2012).

2.3.2.1. *Ensifer meliloti*.

In *E. meliloti*, FixL is anchored to the membrane (Lois et al., 1993) and in the absence of oxygen, FixL autophosphorylates and then phosphorylates FixJ (Figure 2.5). DNA binding sites for FixJ are mostly found in the pSymA replicon (Ferrieres et al., 2004), being confirmed by transcriptional analyses that the 97% of FixJ-activated genes are located on the symbiotic plasmid (Bobik et al., 2006). The direct regulon of FixJ was found to include *nifA* and also *fixK*, which encodes a FNR (fumarate/nitrate reductase regulator)/CRP (cAMP receptor protein)-type family of transcriptional regulator, FixK, required for the expression of the *fixNOQP* operon among other genes involved in microoxic energy metabolism (Figure 2.5) (Foussard et al., 1997). FixK also functions as

the transcriptional regulator in the FixLJ control of *fixT*. FixT was found to prevent expression of *fixK* and *nifA* under microoxic free-living conditions (Figure 2.5) (Foussard et al., 1997; Garnerone et al., 1999). This effect occurs at the top of the signaling pathway where FixT might act as an antikinase, preventing autophosphorylation or accumulation of autophosphorylated FixL (Garnerone et al., 1999). Thus, FixT appears to be integral to a FixLJ-feedback mechanism but *fixT* is not essential for N_2 fixing nodules on alfalfa and the physiological meaning of this kind of control remains to be elucidated (Bergés et al., 2001)..

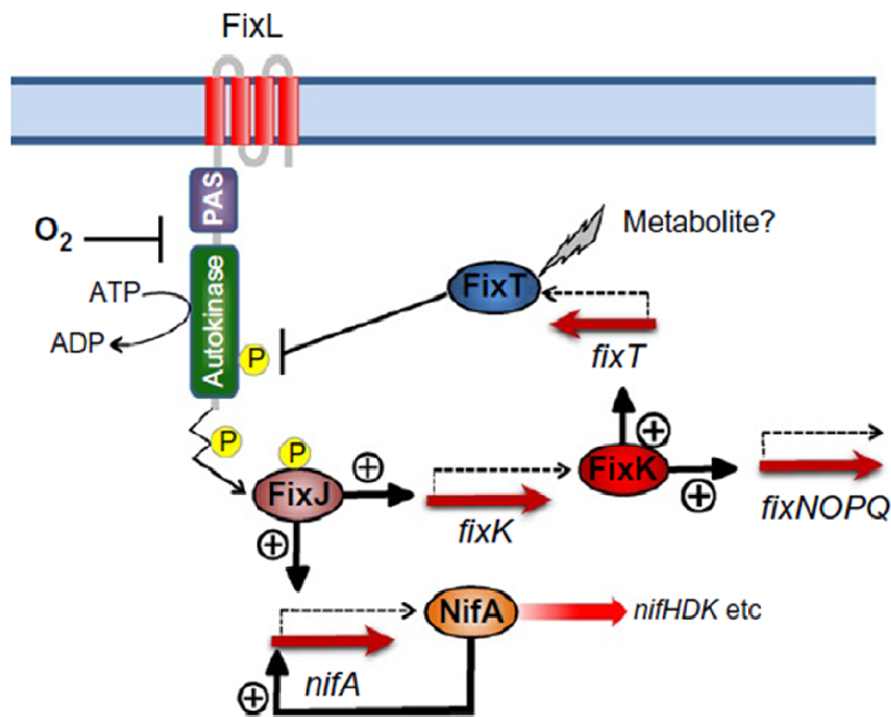


Figure 2.5. Governing pathway of the expression and activity of NifA in *E. meliloti*. Red arrows symbolize genes and dotted arrows represent their transcription. Black arrows illustrate regulation with circles containing (+) indicate positive control and perpendicular lines indicate protein inactivation (Terpolilli et al., 2012).

2.3.2.2. *Bradyrhizobium japonicum.*

In *B. japonicum*, the FixL protein does not possess the transmembrane segments of *E. meliloti* FixL and is soluble as a consequence (Gilles-Gonzalez et al., 1994; Rodgers, 1999). In *B. japonicum*, only a moderate decrease to 5% O_2 concentration is required to bring about autophosphorylation of FixL and the subsequent phosphorylation of FixJ (Figure 2.6). The FixLJ cascade in *B. japonicum* utilizes the CRP/FNR-type regulatory intermediates, $FixK_2$ and $FixK_1$ (Figure 2.6). The gene *fixK₂* is activated by FixJ and is directly or indirectly negatively autoregulated (Nellen-Anthamatten et al., 1998). A *fixT*-like gene (*bll2758*) has also been identified in *B. japonicum* located between the *fixLJ* and *fixK*$_2$ genes (Nellen-Anthamatten et al., 1998; Kaneko et al., 2002). However, the product of *bll2758* does not appear to interfere with the expression of *fixK*$_2$ (Reutimann et al., 2010). Thus, an alternative scenario for the negative regulation of *fixK*$_2$ has been proposed (Reutimann et al., 2010). $FixK_2$ activity is regulated at a posttranslational level by reactive oxygen species, basing in the observation of that the levels of the $FixK_2$ protein *in vivo* do not vary greatly between cells grown in oxic, microoxic, or anoxic conditions. Then, the oxidation of a critical single cysteine residue near the DNA-binding domain causes its inactivation. This posttranslational control might prevent $FixK_2$-activating genes too early-on during symbiosis (Mesa et al., 2009). In microoxic conditions, $FixK_2$ controls a large regulon including *fixNOQP*, *fixGHIS,* genes involved in heme biosynthesis, denitrification genes (*nap*, *nirK*, *nnrR*), *rpoN* (encoding the alternative σ^{54}) and *fixK*$_1$ (Nellen-Anthamatten et al., 1998; Mesa et al., 2008) among others. $FixK_1$ is not essential for N_2 fixation (Anthamatten et al., 1992; Nellen-Anthamatten et al., 1998; Mesa et al., 2008) controlling an small regulon relative to the $FixK_2$ regulon. It is noteworthy that a substantial proportion of the genes in the $FixK_1$ regulon is negatively controlled and belonged also to the NifA regulon. This shows that there is cross-talk between the FixLJ/$FixK_2$–$FixK_1$ cascade and NifA regulation. Moreover, induced gene expression dependent on *nifA* requires σ^{54} factor which is part of the $FixK_2$ regulon (Bauer et al., 1998). This cross-talk might allow a adequate activation of genes essential for N_2 fixation, that is, when the O_2 concentration drops to intermediate levels (5%), the FixLJ/$FixK_2$ cascade is activated and, as part of its response, represses

the activation of NifA-dependent genes via $FixK_1$. But as the O_2 concentration drops further to microoxic concentrations (0.5%), the build-up of active NifA protein overcomes this $FixK_1$ repression; thus, a fine-tuning of the N_2 fixation genes is achieved (Mesa et al., 2008). Microarray based experiments have led to a substantial expansion of the NifA regulon, revealing a total of 65 genes for N_2 fixation and other diverse processes (Hauser et al., 2007). Expression of *nifA* in *B. japonicum* is autoregulated but is also dependent on RegR which forms a two-component regulatory system with RegS. RegSR regulatory system will be widely described in section 2.5.2.1 from the Introduction. *nifA* forms part of the *fixR–nifA* operon, which is preceded by two overlapping promoters: one of these was found to be activated by RegR under oxic and microoxic conditions and the other one is dependent on RpoN and activated by NifA (Figure 2.6). However, under oxic conditions, NifA is not active and is degraded (Morett et al., 1991) and once under microoxic conditions, NifA is active and maximal expression of the *fixR–nifA* operon can occur via activation from the RpoN-NifA-dependent promoter. It is proposed that expression of FixL-FixJ targets allows the bacteria to adapt their respiratory metabolism to the microoxic environment of the nodule, whereas the O_2 sensitivity of NifA is compatible with the very low-O_2 conditions that are required for nitrogenase activity in particular zones of the nodule (Sciotti et al., 2003). Sensing of O_2 by NifA is likely to be through the coordination of a metal cofactor (Fischer et al., 1988), though this needs to be explored further as does the degradation of NifA. Metal deprivation has also been shown to cause degradation of NifA (Morett et al., 1991).

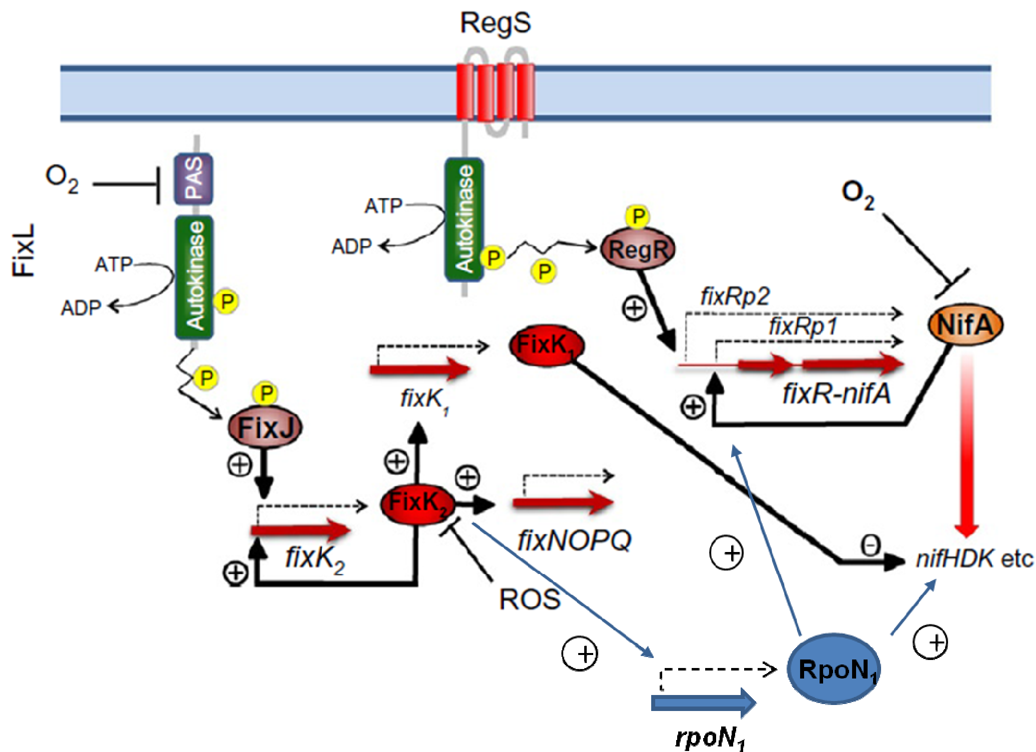


Figure 2.6. Regulatory circuits that control the expression and activity of NifA in *B. japonicum*. Red arrows symbolize genes and dotted arrows represent their transcription. Black arrows illustrate regulation with circles containing (+) or (-) indicating positive or negative regulation, respectively. Perpendicular lines indicate protein inactivation (adapted from Terpolilli et al., 2012).

2.4. Dentrification.

Denitrification is an alternative form of respiration in which bacteria sequentially reduce nitrate (NO_3^-) or nitrite (NO_2^-) to N_2 by the intermediates nitric oxide (NO) and nitrous oxide (N_2O) when oxygen concentrations are limiting, according to the following reaction:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

The switch from oxygen to nitrate respiration leads to a reduction in the ATP yield rates, but allows bacteria to survive and multiply (Zumft 1997, Simon et al., 2008). Although denitrification was believed to be performed exclusively by bacteria, there are evidences that some fungi (Takaya et al., 2002, Prendergast-Miller et al 2011)

and archaea (Treush et al., 2005) are also able to denitrify. Moreover, nitrifiers also have genes involved in denitrification (Cebron et al., 2005, Shaw et al., 2006). A list of archaeal, bacterial and fungal genera for which at least one denitrifying gene has been characterized has been reported by Philippot et al., (2007). Some bacteria like *E. coli* or *B. subtilis* are able to perform nitrate respiration and release gaseous nitrogen oxides, but they do not denitrify with dinitrogen as a product. Instead, they perform the so-caller nitrate-ammonification, i.e. the reduction of nitrate to ammonium (Cole and Richardson, 2008).

A very important aspect of denitrification in the N-cycle is its intermediate product, nitrous oxide (N_2O), since N_2O is a powerful atmospheric greenhouse gas and cause of ozone layer depletion. Global N_2O emissions continue to rise and more than two-thirds of these emissions arise from bacterial and fungal denitrification processes (Richardson et al., 2009; Thomson et al., 2012). There are diverse sources of N_2O, being produced as a by-product during ammonia oxidation, the first step of nitrification, being the final product of fungal denitrification because the enzyme N_2O reductase is absent in this group, being produced from nitrate-ammonifying (DNRA: dissimilatory nitrate reduction to ammonia) bacteria. In contrast to the multiplicity of mechanisms by which N_2O can be generated, only a single dominant sink for N_2O is known, the respiratory N_2O reductase (N_2OR) typically found in denitrifying bacteria that reduce N_2O to N_2. From the point of view of mitigating N_2O release from denitrification, there is an absence of regulation by N_2O because it is not a toxic gas, so the denitrifying populations do not apparently respond to N_2O accumulation by making more of the N_2O reductase. So, it is necessary to determinate N_2O production mechanism and conditions in order to avoid or reduce, as far as possible, its emission to atmosphere (Thomson et al., 2012; Spiro et al., 2012).

2.4.1. Denitrification enzymes.

Denitrification reactions are catalysed by periplasmic (Nap) or membrane-bound (Nar) nitrate reductase, nitrite reductases (CuNir/*cd*$_1$Nir), nitric oxide reductases (cNor, qNor, or qCuANor) and nitrous oxide reductases (Nos) encoded by *nap*/*nar*,

nirK/*nirS*, *nor* and *nos* genes, respectively. Reviews covering the physiology, biochemistry and molecular genetics of denitrification have been published elsewhere (Zumft et al., 1997; van Spanning et al., 2005, 2007; Kraft et al., 2011; Richardson, 2011; Bueno et al., 2012).

2.4.1.1. Respiratory Nitrate Reductases.

The first reaction of denitrification, this is the conversion of nitrate to nitrite, is catalyzed by two biochemically different enzymes, a membrane-bound nitrate reductase (Nar), or a periplasmic nitrate reductase (Nap) (Figure 2.7). Nar enzymes have been most studied in *E. coli* and *Paracoccus* (reviewed in Potter et al., 2001, Richardson et al., 2001; González et al., 2006; Richardson et al., 2007; Richardson, 2011). It is a 3-subunit enzyme composed of NarGHI (Bertero et al., 2003, Jormakka et al., 2004), where NarG is the catalytic subunit of about 140 kDa that contains a bismolybdopterin guanine dinucleotide cofactor and a [4Fe-4S] cluster. NarH, of about 60 kDa, contains one [3Fe-4S] and three [4Fe-4S]. NarG and NarH are located in the cytoplasm and associate with NarI, an integral membrane protein of about 25 kDa with five transmembrane helices and the N-terminus facing the periplasm. Nar proteins are encoded by genes of a *narGHJI* operon. Whereas *narGHI* encode the structural subunits, *narJ* codes for a cognate chaperone required for the proper maturation and membrane insertion of Nar (Figure 2.7) (Blasco et al., 1992). The organization of this operon is conserved in most species that express Nar. *E. coli* has a functional duplicate of the *narGHJI* operon named *narZYWV*, which physiologically has a function during stress response rather than anaerobic respiration (Blasco et al., 1990, Spector et al., 1999). In some archaea and bacteria the NarGH subunits are on the outside rather than the inside of the cytoplasmic membrane. This enzyme is supposed to be the evolutionary precursor of the Nar system (Martínez-Espinosa et al., 2007). In the hyperthermophile *Thermus thermophilus*, the Nar system is very similar to that described in *E. coli*. However, Nar gene cluster in *T. thermophilus* codes for additional redox proteins that include the subunits of an NADH dehydrogenase and a diheme cytochrome *c* (NarC) (Cava et al., 2008; Richardson, 2011). The Nar enzyme couples quinol oxidation with proton translocation and energy conservation. This respiratory

function permits cell growth under oxygen-limiting conditions (Potter et al., 2001, Simon et al., 2008).

Nap is widespread in all classes of denitrifying and non-denitrifying proteobacteria (reviewed in Potter et al., 2001, Richardson et al., 2001, González et al., 2006, Richardson et al., 2007; Richardson, 2011). The best studied Nap enzymes were isolated from *P. pantotrophus*, *E. coli*, *R. sphaeroides* and *Desulfovibrio desfuromonas* (Dias et al., 1999, Arnoux et al., 2003, Jepson et al., 2007). Nap is a 2-subunit enzyme composed of the NapAB complex located in the periplasm for which a crystal structure has been solved from *Rhodobacter sphaeroides* (Arnoux et al., 2003) and a transmembrane NapC component. The catalytic subunit NapA contains the bis-MGD cofactor at its active site and a FeS center. NapB is diheme cytochrome c_{552}, and NapC is a *c*-type tetra-heme membrane-anchored protein that is involved in the electron transfer from the quinol pool to NapAB (Figure 2.7) (Roldán et al., 1998, Cartron et al., 2002, Figure 2.7). Eight different genes have been identified as components for operons that encode Naps in different organisms (Richardson et al., 2001). Most bacteria studied thus far have the *napABC* genes in common. The remaining *napDEFKL* genes encode for different proteins that are not directly involved in the nitrate reduction. NapD is a redox maturation cytoplasmic chaperone which interacts with NapA. NapD structure has recently been solved (Maillard et al., 2007). NapF is a cytoplasmic iron–sulfur containing protein with four loosely bound [4Fe–4S] clusters, and is thought to participate in the assembling of the iron–sulphur cluster of NapA (Olmo-Mira et al., 2004, Nilavongse et al., 2006). The *napEKL* genes encode for proteins with so far unknown functions. In *E. coli*, the *nap* operon includes *napGH* genes encoding a periplasmic and an integral membrane protein with [4Fe–4S] clusters. NapH and NapG interact, making an electron transfer supercomplex that can channel electrons from both menaquinol and ubiquinol to NapA (Brondijk et al., 2002, 2004). Although Nap is also linked to quinol oxidation, it does not synthesize ATP (Simon et al., 2008). Physiological functions for Nap systems include the disposal of reducing equivalents during aerobic growth on reduced carbon substrates (e.g. *Rhodobacter* species or *P. denitrificans*), or anaerobic nitrate respiration as a part of bacterial ammonification (e.g. *E. coli*) or denitrification (e.g. *B. japonicum*) pathways

(Potter et al., 2001; Richardson, 2011). Quinol oxidation by the periplasmic Nap is not directly coupled to the generation of a proton motive force (PMF) and is independent of the cytochrome *bc*$_1$ complex. Thus, nitrate reduction via Nap can only be coupled through the quinone reductase NADH dehydrogenase which generates an H^+-electrochemical gradient (Figure 2.7, Richardson, 2000; Simon et al., 2008).

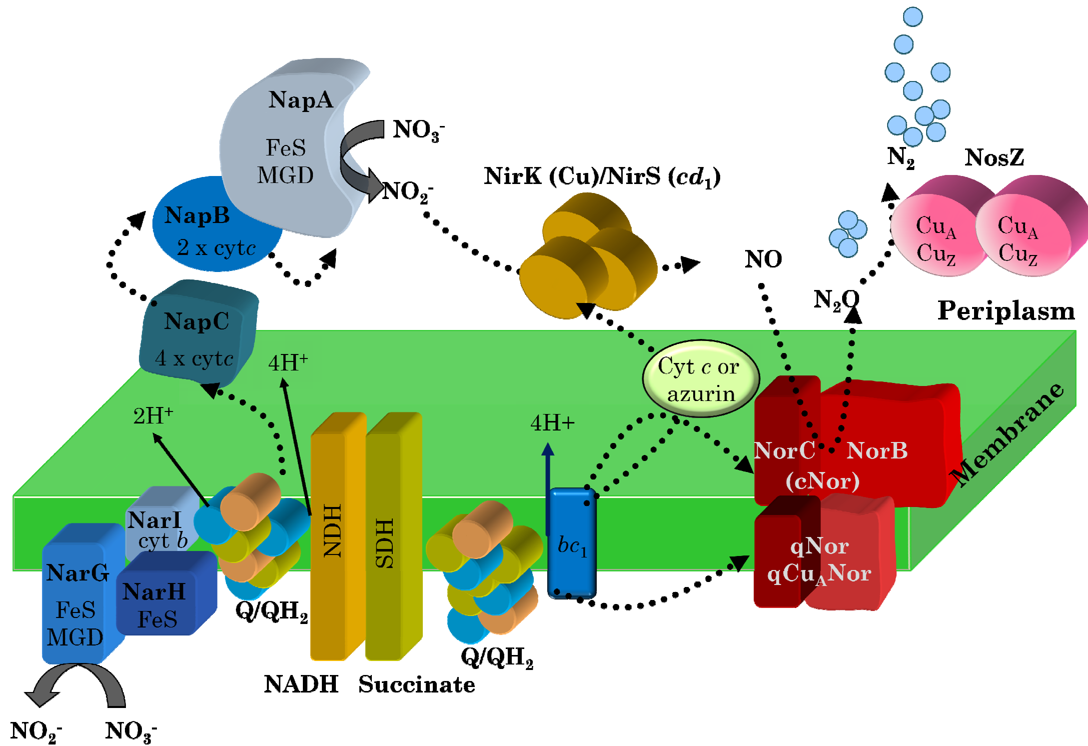


Figure 2.7. Denitrification pathway in bacteria as a topological organization of denitrification enzymes. The membrane-bound (NarGHI), and periplasmic, (NapABC) nitrate reductases as well as the nitrite reductases (Cu-type or *cd1*-type), nitric oxide reductases (cNor, qNor, and qCuANor), and nitrous oxide reductase (NosZ) are shown (adapted from Bueno et al., 2012).

2.4.1.2. Respiratory Nitrite Reductases.

Two types of respiratory nitrite reductases (Nir) have been described in denitrifying bacteria, NirS and NirK (Rinaldo and Crutuzzolá, 2007; Rinaldo et al., 2008, van Spanning, 2011). They catalyze the one-electron reduction of nitrite to nitric oxide, however, neither of the enzymes is electrogenic. Both are located in the periplasmic space, and receive electrons from cytochrome *c* and/or a blue copper protein, pseudoazurin, via the cytochrome *bc*$_1$ complex (Figure 2.7, Pearson et al., 2003). The *cd*$_1$ NirS nitrite reductase is a homodimeric enzyme with hemes *c* and *d*$_1$. Electrons are

transferred via the heme *c* of NirS to heme d_1, where nitrite binds and is reduced to nitric oxide (Rinaldo et al., 2008). The best-characterized *nirS* genes clusters are those from *P. aeruginosa* (*nirSMCFDLGHJEN*) and *P. denitrificans* (*nirXISECFDLGHJN*). In the model denitrifier *P. stutzeri,* there are two *nir* clusters (*nirSTBMCFDLGH* and *nirJEN*) which are separated by one part of *nor* gene cluster encoding nitric oxide reductase. The *nirS* gene encodes the functional subunits of the dimeric NirS. All other genes are required for proper synthesis and assemblage of the d_1 heme and related functions (reviewed by van Spanning, 2011).

The Cu-containing NirK enzymes are homotrimeric complexes harboring three type I copper centers, and three type II copper centers, which form the active site. Nitrite binds to the type II site where it is reduced to nitric oxide by electrons transferred from the type I copper site. In contrast to the complex organization of the genes encoding the NirS proteins, the Cu-NirK enzyme is encoded by the *nirK* gene (Rinaldo and Crutuzzolá, 2007; van Spanning et al., 2011). Here it must be noted that expression of NirK requires only a single gene, sometimes accompanied with a second one expressing a protein called NirV. The latter enzyme is related to desulfurates and may well be required for proper insertionof the copper reaction centre. As yet, there has been no organism found to have both types of reductases, so apparently the presence of either type of reductase excludes the option of gaining the other type.

2.4.1.3. Respiratory Nitric Oxide Reductases.

Three types of nitric oxide reductases (Nor) have been characterized, cNor, qNor, and qCuANor (Figure 2.7, reviewed in Zumft 2005, de Vries et al., 2007, Richardson, 2011; Spiro, 2012). The cNor is an integral membrane enzyme composed of two subunits, the heme *c* containing-NorC, and NorB, which contains hemes *b* and a non-heme iron. Electron transfer to cNor is mediated by the cytochrome bc_1 complex and a soluble cytochrome *c* or pseudoazurin. Electrons are transferred to the heme *c* and then via the heme *b* to the active site. There, two molecules of nitric oxide are reduced to form nitrous oxide (de Vries et al., 2007). The best-characterized cNORs are those from *P.* denitrificans, *Pseudomonas stutzer*i and *Pseudomonas aeruginosa*. The

structure of the NorBC complex from *P. aeruginosa* (Hino et al., 2010) confirmed the predicted presence of 12 membrane-spanning α-helices in NorB. Biochemical experiments indicated that the protons required for NO reduction are taken from the periplasmic side of the membrane, and that NorB does not function as a proton pump (Bell et al., 1992). The latter is confirmed in the structure by the absence of trans-membrane proton channels in NorB analogous to those found in the proton-translocating haem-copper oxidases, which are otherwise structurally related to NorB (Hino et al., 2010). The qNor uses quinol or menaquinol as electron donors. The enzyme has been found not only in denitrifying archaea and soil bacteria, but also in pathogenic microorganisms that do not denitrify (de Vries et al., 2003). The qCuANor has been described in the Gram-positive bacterium *Bacillus azotoformans* (Suharti et al., 2004). This enzyme is bifunctional using both menahydroquinone (MKH_2) and a specific *c*-type cytochrome c_{551} as electron donor. It was suggested that the MKH_2-linked activity of qCuANor serves detoxification and the c_{551} pathway has a bioenergetics function.

The subunit II and I of cNor is encoded by the *norCB* genes, respectively, which are usually co-transcribed with accessory genes designed *norD*, *norE*, *norF* and *norQ.* The gene order *norEFCBQD* is typical thought not universal. The *norQ* and *norD* genes are always linked to *norCB*; the other accessory genes may be distantly located or absent in some genomes (Zumft, 2005). The functions of the accessory genes and their protein products are not well understood. NorE is a predicted membrane protein with some sequence similarity to part of subunit III of the cytochrome *c* oxidase. This led to speculation that NorE might be a component of the Nor complex (de Boer et al., 1996). Mutation of the accessory genes tends to lead to variable phenotypes in different organisms (Zumft, 2005), but the biochemical function of the accessory proteins is not known.

2.4.1.4. Respiratory Nitrous Oxide Reductase

The final step in denitrification consists of the two-electron reduction of nitrous oxide to N_2, a reaction catalysed by the nitrous oxide reductase (Nos) (reviewed in Zumft and Kroneck 2007; van Spanning, 2011; Spiro, 2012). The enzyme is located in the periplasmic space and has been purified from some denitrifying strains, including *P. denitrificans*, *P. pantotrophus*, and *P. stutzeri*. Nos is a homo-dimer of a 65 kDa copper-containing subunit. Each monomer is made up of the Cu_A and Cu_Z domains (Figure 2.7). Electron input into Cu_A is usually via *c*-type cytochromes or cupredoxins (Berks et al., 1993). Three-dimensional structures are available for the enzymes from *Marinobacter hydrocarbonclasticus* (*Pseudomonas nautical*) (Brown et al., 2000). The recently reported structure of purple N_2OR from *P. stutzeri* has revealed that N_20 binds at Cu_Z in close proximity to Cu_A (Pomowski et al., 2011). The Z-type Nos is encoded by *nosZ* that is usually linked to other *nos* genes, whose products have roles in the maturation of the active enzyme. The functions of the accessory Nos proteins are not well understood, but they include an ABC transporter (NosFYD) that may export a sulphur compound to the periplasm, an outer membrane copper porin (NosA), an outer membrane anchored copper protein (NosL), a periplasmic flavoprotein (NosX) and NosR (Zumft and Kroneck, 2007). NosR is a membrane-bound iron-sulphur flavoprotein, which is required for the transcription of *nosZ* by an unknown mechanism, but this control is likely indirect. NosR might be also involved in electron transfer between the quinone pool and Nos (Wunsch and Zumft, 2005).

2.4.2. Control of denitrification.

The general requirements for biological denitrification are: a) the presence of bacteria possessing the metabolic capacity; b) suitable electron donors such as organic carbon compounds; 3) restricted O_2 availability; and 4) the presence of a nitrogen oxide (NO_3^-, NO_2^-, NO, or N_2O) as terminal electron acceptor. Thus, the key molecules that act as signals for the regulation of denitrification genes are oxygen, nitrate, nitrite, and NO (for reviews see van Spanning et al., 2011; Shapleigh, 2011; Spiro, 2012; Bueno et al., 2012).

2.4.2.1. Oxygen control.

Oxygen strongly influences the growth and physiology of bacteria catalyzing reactions in the nitrogen cycle. Generally, denitrification is regarded as an anoxic or microoxic process. Interestingly, no strictly anaerobic denitrifier has ever been isolated. Since denitrifiers are facultative aerobes, this means that they must choose between oxygen and nitrate if both are available. Due to the organization and structural features of the denitrification enzymes, the maximum efficiency of free energy transduction during denitrification is only 60% of that during aerobic respiration (Richardson, 2000; Simon et al., 2008). Thus, oxygen is preferred as terminal electron acceptor than nitrate, and hence the regulation of expression of either type of respiration occurs according to an energetic hierarchy. In all species, the onset of denitrification is triggered by oxygen depletion and nitrate availability. However, with the exception of Nos, none of the terminal nitrogen oxide reductases are oxygen-sensitive, making it possible that the denitrification enzymes could be used under oxic conditions (Morley et al., 2008).

The expression of the periplasmic nitrate reductase is quite variable, with this enzyme being maximally expressed under oxic conditions in some bacteria, but under microoxic conditions in others, adjusting to fit the physiological role it plays. In general, in organisms where its primary function is redox homeostasis it is expressed under oxic conditions. However, when Nap is being used for respiration it is maximally expressed under microoxic conditions (Shapleigh, 2011). By contrast to *nap* expression, expression of *nir, nor* and *nos* genes in most denitrifiers is more tightly controlled, only occurring under microoxic conditions (Shapleigh, 2011, Bergaust et al., 2012).

Regarding the O_2-sensing protein regulators, the two most important types of O_2 sensors involved in regulation of denitrification are FixL and FNR. FixL is a membrane-bound O_2 sensor found in rhizobial species which together with its cognate response regulator FixJ, belong to the group of two-component regulatory systems (see section 2.3.2.; 2.5.2 and 2.5.3 from introduction). In *B. japonicum*, phosphorylated FixJ activates transcription of *fixK*$_2$. In turn, FixK$_2$ activates expression of genes involved

in denitrification, among others (Mesa et al., 2003, 2008; Bedmar et al., 2005; Robles et al., 2006) (see section 2.3.2.; 2.5.2 and 2.5.3 from introduction).

FNR is an oxygen responsive regulator that belongs to the CRP/FNR superfamily of transcription factors. Four conserved cysteines of FNR coordinate a $[4Fe\text{-}4S]2^+$ cluster, which is converted to a $[2Fe\text{-}2S]2^+$ cluster on exposure to oxygen. This transition is accompanied by a reduced tendency of FNR to dimerize, and so a reduced affinity for its DNA target. Details of the mechanism of the reaction of the cluster with oxygen are beginning to emerge (Green et al., 2009; Fleischhacker and Kiley, 2011). Orthologous of FNR from other organisms (such as FnrP, ANR, and FnrN) are presumed to work in a similar way. For example, the *na*r and *nap* operons in *E. coli* and *B. subtilis* are activated by Fnr under anoxic conditions (Reents et al., 2006; Stewart and Bledsoe, 2005; Tolla and Savageau, 2011). *P. denitrificans* FnrP controls expression of the *nar* gene cluster and the *cco*-gene cluster encoding the cbb_3-type oxidase (Veldman et al., 2006; Bouchal et al., 2010). Oxygen tension is sensed in *P. aeruginosa* by the Anr regulator, which activates transcription of the *narK1K2GHJI* operon encoding nitrate reductase and two transporters in response to oxygen limitation (Schreiber *et* al., 2007).

2.4.2.2. Nitrogen oxides control.

Species that can denitrify or those that reduce anaerobically nitrate to ammonium respond to nitrate/nitrite through three types of regulatory systems: NarXL, NarQP, and NarR. NarXL and NarQP are members of two-component regulatory systems being the NarX and NarQ proteins the signal sensors, and NarL and NarP proteins their cognate response regulators, respectively (Stewart, 2003). The sensing mechanism of the kinase NarX has been recently established (Cheung and Hendrickson, 2009; Stewart and Chen, 2010). In *E. coli* NarL and NarP bind DNA to control induction of the *nar* and *nap* operons (Stewart, 2003; Darwin et al., 1998: Stewart and Bledsoe, 2005) (Figure 2.8). The effects of nitrate and nitrite on the *E. coli* transcriptome during anaerobic growth have been investigated, revealing in a novel group of operons that are regulated by all Fnr, NarL and NarP (Constantinidou et al., 2006). To date, *narXL* and *narQP* genes are confined to species classified in the γ and β

subdivisions of the proteobacteria such as *Escherichia, Salmonella, Klebsiella, Yersinia, Burkholderia*, *Ralstonia*, *Neisseria* and *Pseudomonas* species among others. In *P. aeruginosa,* NarL in concert with the regulators Anr and Dnr and an integration host factor (IHF) activate transcription of the *narK1K2GHJI* operon encoding nitrate reductase and two transporters in response to oxygen limitation, nitrate and N-oxides (Schreiber *et* al., 2007). Recently, it has been shown that during anaerobic growth of *P. aeruginosa* PAO1, NarL directly represses expression of periplasmic nitrate reductase, while induces maximal expression of membrane nitrate reductase (van Alst et al., 2009).

NarR is a member of the CRP/FNR family of transcription activators, but it lacks a [4Fe-4S] cluster. Genes encoding NarR are found in the α-proteobacteria *Brucella suis*, *B. melitensis*, *P. denitrificans* and *P. pantotrophus*. There are no indications that they have counterparts of *narXL*. It therefore seems that NarR substitutes the NarXL system in the α-proteobacteria (for reviews see van Spanning et al., 2007; Bueno et al., 2012). NarR of *Pa. pantotrophus* and *P. denitrificans* is specifically required for transcription of the *narKGHJI* genes and a nitrate transport system in response to nitrate and/or nitrite (Wood et al., 2001).

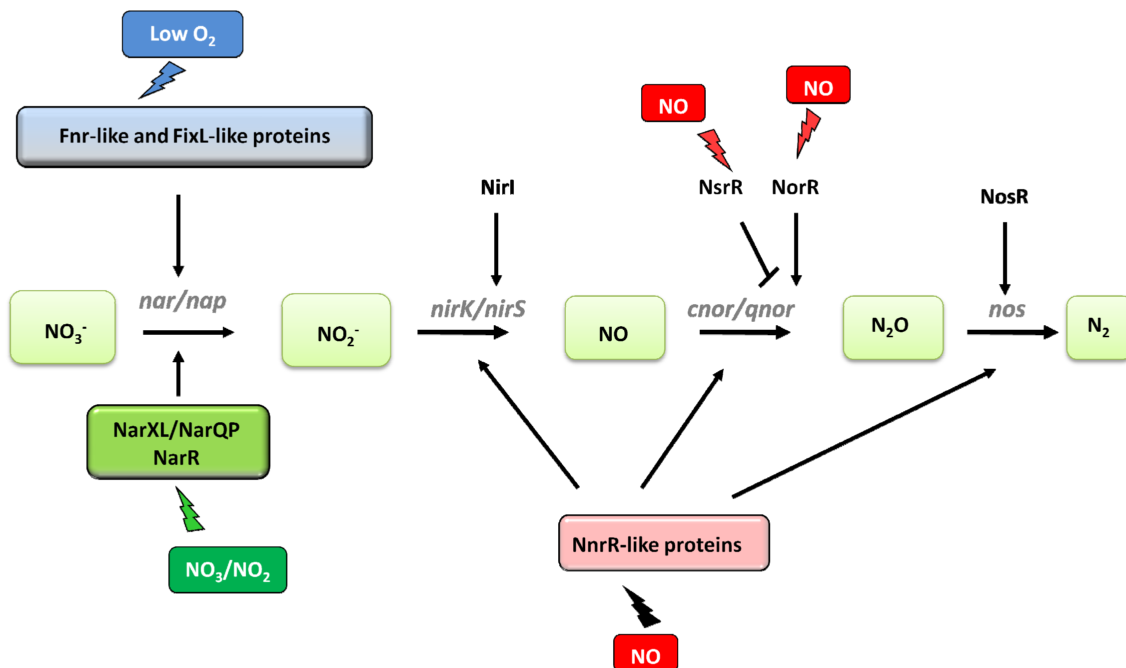


Figure 2.8. Regulatory network of denitrification genes in response to O_2 concentration, nitrate/nitrite, and nitric oxide (NO). Positive regulation is denoted by arrows, and negative regulation is indicated by perpendicular lines (Bueno et al., 2012).

Oxygen and nitrate/nitrite are not the only signals that control denitrification. An additional fine-tuned regulation of denitrification genes is also required in order to keep the free concentrations of nitrite and nitric oxide (NO) below cytotoxic levels. In this context, NO has been proposed as an additional key molecule that is involved in denitrification genes regulation (reviewed by van spanning, 2011). As yet, three different types of NO-responsive transcriptional regulators have been characterized in denitrifying species. These are: NnrR, NorR and NsrR (Figure 2.8).

NnrR (Nitrite and Nitric oxide Reductase Regulator) also belongs to the CRP/FNR family of transcription activators, but, just like NarR, it lacks the cysteines to incorporate a [4Fe-4S] cluster. NnrR orthologs, sometimes named as Nnr, Dnr or DnrR have been described in denitrifying bacteria including *P. denitrificans*, *P. stutzeri*, *P. aeruginose*, *B. japonicum* and *E. meliloti* and they orchestrate the expression of the *nir* and *nor* gene clusters (reviewed by van spanning, 2011). The promoters of these operons contain NnrR binding sites that resemble the consensus Fnr-box to a large extent. In *P. aeruginosa* and *P. denitrificans* Nnr homologs, also control the expression of *nos* genes in response to NO (Arai et al., 2003; Bergaust et al., 2012. The mechanism of NO sensing by these proteins is difficult to be established. Nnr from *P. denitrificans* and Dnr from *P. aeruginosa* require haem for their NO-dependent activity in heterologous reporter systems in *E. coli* (Lee et al., 2006; Castiglione et al., 2009). The structure of the sensory domain of Dnr reveals a hydrophobic pocket that might be a haem-binding site, and purified apo-Dnr can bind haem (Giardina et al., 2008). The current model proposes that DNA binding activity of Dnr *in vitro* requires haem and NO, though the complete details of this mechanism remains to be established (Giargina et al., 2011).

NorR is another NO-responsive protein which was first identified in *Ralstonia eutropha*. In this bacterium, NorR activates transcription of *norB,* which encodes a single-subunit nitric oxide reductase of the qNor type (Pohlmann et al., 2000). The

NorR protein of *E. coli* activates transcription of the *norVW* genes, which encodes a flavorubredoxin involved in NO reduction to N_2O (Gardner et al., 2003). NorR-dependent transcription requires RNA polymerase containing the alternative sigma factor, σ^{54}, so NorR belongs to the σ^{54}-dependent enhancer-binding protein (EBP) family of transcriptional activators. NorR has a three-domain structure that is typical of EBPs, with a C-terminal DNA-binding domain, a central domain from the AAA^+ family that has ATPase activity and interacts with RNA polymerase, and an N-terminal signalling domain. The N-terminal GAF domain of NorR contains a mono-nuclear nonhaem iron, which is the binding site for NO. Formation of a mono-nitrosyl complex at this centre disrupts an intra-molecular interaction, by which the GAF domain inhibits the activity of the AAA^+ domain in the absence of NO (Tucker et al., 2008).

NsrR is an iron–sulfur-containing negative regulator that senses NO directly via a [2Fe–2S] cluster. Nitrosylation of this cluster leads to a loss of DNA binding activity and, hence, derepression of NsrR target genes. In *E. coli,* this transcription repressor was shown to sense reactive nitrogen species (RNS) and to switch on a regulon of at least 60 genes, including genes involved in nitrate respiration (Filenko et al., 2007; Tucker et al., 2010). In denitrifying bacteria, NsrR appears to have a specific role in coordinating production of the nitrite and NO reductase enzymes to prevent the build-up of NO. Intriguingly, the same role is performed by Nnr homologues in denitrifying bacteria that do not contain NsrR. In the denitrifying pathogenic organisms *Moraxella catarrhalis*, *Neisseria meningitidis* and *Neisseria gonorrhoeae*, NsrR is a repressor of the *norB* gene encoding the respiratory NO reductase (Overton et al., 2006; Rock et al., 2007; Wang et al., 2008).

In addition to the regulatory proteins that can monitor oxygen, nitrate, nitrite, and NO, regulation of the on-set and fine-tuning of denitrification in some bacteria involves copper responsive regulators, redox sensing mechanisms and the NosR and NirI proteins (reviewed by van Spanning, 2011; Bueno et al., 2012; Spiro, 2012).

2.4.2.3. Redox control.

In addition to external oxygen concentration, other signals such as redox changes can regulate the expression of genes involved in denitrification (for reviews

see van Spanning, 2011; Bueno et al., 2012; Spiro, 2012). Redox-responsive two-component regulatory systems are present in a large number of Proteobacteria. These proteins are named RegBA in *R. capsulatus, Rhodovulum sulfidophilum*, and *Roseobacter denitrificans* (Elsen et al., 2004; Wu and Bauer, 2008), PrrBA in *R. sphaeroides* (Oh et al., 2001; Happ et al., 2005), ActSR in *E. meliloti* (Emmerich et al., 2000; Fenner et al., 2004) and *Agrobacterium tumefaciens* (Baek et al., 2008), and RoxSR in *P. aeruginosa* (Comolli and Donohue, 2002). In *Rhodobacter* species, the RegBA/PrrBA regulon encodes proteins involved in numerous energy-generating and energy-utilizing processes such as photosynthesis, carbon fixation, nitrogen fixation, hydrogen utilization, aerobic respiration and denitrification, among others (reviewed by Swem et al., 2001; Elsen et al., 2004; Wu and Bauer, 2008; Bueno et al., 2012). The RegBA/PrrBA two-component systems comprise the membrane-associated RegB/PrrB histidine protein kinase, that senses changes in redox state, and its cognate PrrA/RegA response regulator. Under conditions where the redox state of the cell is altered due to generation of an excess of reducing potential, produced by either an increase in the input of reductants into the system (e.g. presence of reduced carbon source) or a shortage of the terminal respiratory electron acceptor (e.g. oxygen deprivation), the kinase activity of RegB/PrrB is stimulated relative to its phosphatase activity. This increases phosphorylation of the partner response regulators RegA/PrrA, which are transcription factors that bind DNA and activate or repress gene expression. The membrane-bound sensor kinase proteins RegB/PrrB contain an H-box site of autophosphorylation (His^{225}), a highly conserved quinone binding site (the heptapeptide consensus sequence GGXXNPF, which is totally conserved among all known RegB homologues), and a conserved redox-active cysteine (Cys^{265}, located in a “redox box”). The mechanism by which RegB controls kinase activity in response to redox changes has been an active area of investigation. A previous study demonstrated that RegB Cys^{265} is partially responsible for redox control of kinase activity. Under oxidizing growth conditions, Cys^{265} can form an intermolecular disulfide bond to convert active RegB dimers into inactive tetramers (Swem et al., 2003). The highly conserved sequence, GGXXNPF, located in a short periplasmic loop of the RegB transmembrane domain has also being implicated in redox sensing by interacting with the ubiquinone pool (Swem et al., 2006). Recently, kinase activity assays together with

isothermal titration calorimetry (ITC) measurements indicated that RegB with a substitution in the cytosolic cysteine by serine in position 265 (RegB $C^{265}S$), binds both oxidized and reduced ubiquinone with almost equal affinity. However, only the oxidized ubiquinone inhibits RegB kinase activity (Wu and Bauer, 2010). The observation that the RegB $C^{265}S$ mutant is still redox responsive suggests that ubiquinone binding is a signal input able of functioning independently from Cys^{265}. However, the contribution of each redox sensing inputs is unknown.

In *R. sphaeroides*, the PrrB histidine kinase is a bifunctional enzyme that possesses both kinase and phosphatase activities (Oh et al., 2004). Several reports proposed that the *cbb*$_3$ oxidase transduced an inhibitory signal to the PrrBA under oxic conditions to prevent gene expression. The dual function of the *cbb*$_3$ oxidase as both terminal oxidase and O_2/redox sensor and modulator of PrrB kinase/phosphatase activity represents a new model of redox sensing. In this model, the ubiquinone binding site within the PrrB transmembrane domain is not required for monitoring the PrrB kinase activity. Instead, a control based in direct interaction between components of the terminal oxidase *cbb*$_3$ and PrrB is strengthened (Kim et al., 2007).

The photosynthetic regulatory response protein (PrrC) is a Sco homolog present in *R. sphaeroides* (Eraso and Kaplan, 2000). Sco is thought to be involved in donating copper to the Cu_A centre and thus it has a central role in cytochrome oxidase synthesis (Balatri et al., 2003). *R. sphaeroides* PrrC, which reduces Cu^{2+} to Cu^{+}, and possesses disulfide reductase activity, is required for the correct functioning of the sensor kinase/phosphatase PrrB (Badrick et al., 2007). Similarly, the *R. capsulatus* SenC protein, homologous to PrrC, which is required for synthesis of a functional cytochrome *c* oxidase (Swem et al., 2005) might act as a signal mediator between the Q-pool and the sensor kinase RegB. However, at present there is no direct evidence that SenC or *cbb*$_3$ oxidase directly modulate the activity of the RegBA regulatory system.

RegA/PrrA contain conserved domains that are typical in two-component response regulators such as a phosphate accepting aspartate, an “acid box” containing two highly conserved aspartate residues and a H-T-H DNA-binding motif. The

phosphorylated form of RegA/PrrA has increased DNA binding capacity (Laguri et al., 2006; Ranson-Olson et al., 2006). Under oxidizing conditions, RegB/PrrB shifts the relative equilibrium from the kinase to the phosphatase mode resulting in a dephosphorylated inactive RegA/PrrA form. Despite this evidence, it has been reported that inactivation of the *regA* gene affects expression of many different genes under oxidizing (aerobic) conditions suggesting that both, phosphorylated and unphosphorylated RegA/PrrA, may be active transcriptional regulators (Swem et al., 2001). In this context, it has been shown that both phosphorylated and unphosphorylated forms of RegA/PrrA are capable of binding DNA *in vitro* and activating transcription (Ranson-Olson et al., 2006).

The PrrBA and ActSR proteins control denitrification processes in *R. Sphaeroides* and *A. tumefaciens*, respectively. In *R. sphaeroides* 2.4.3, inactivation of *prrA* impaired ability to grow both photosynthetically and anaerobically in the dark on nitrite-amended medium (Laratta et al., 2002). The PrrA-deficient strain exhibited a severe decrease in both nitrite reductase activity and expression of a *nirK-lacZ* fusion when environmental oxygen tension was limited. This regulation is not mediated by NnrR, since *nnrR* is fully expressed in a PrrA mutant background. Instead, Laratta and colleagues (2002) proposed a model where, under low oxygen tension, the kinase activity of PrrB is increased relative to its phosphatase activity, resulting in an increased concentration of PrrA-P. Thus, under microoxic conditions in the presence of NO, PrrA-P activates transcription of *nirK* in collaboration with NnrR.

Insertional inactivation of the response regulator ActR in *R. sphaeroides* significantly reduced *nirK* expression and Nir activity but not *nnrR* expression. In *A. tumefaciens*, a putative ActR binding site was identified in the *nirK* promoter region using mutational analysis and an *in vitro* binding assay (Baek et al., 2008). These studies also shown that purified ActR bound to the *nirK* promoter but not to the *nor* or *nnrR* promoter. Finally, it has been recently reported that the NtrY/X two-component system of *Brucella* spp. acts as a redox sensor and regulates the expression of *nar*, *nir*, *nor* and *nos* operons in response to microoxic conditions (Carrica et al., 2012; Roop and Caswell, 2012).

2.5. Denitrification in rhizobia.

Denitrification among rhizobia is rare, and most species do not contain the whole set of denitrification genes. *Pseudomonas* sp. G-179 (actually *Rhizobium galegae*) (Bedzyk et al., 1999) has been shown to contain Nap, Nor and CuNir. *R*. *sullae* (formerly *R. hedysari*) only expresses CuNir (Toffanin et al., 1996). The genetic determinants for expression of CuNir and cNor are present in *R. etli* (Bueno et al., 2005, Gomez-Hernandez et al., 2011). *E. meliloti* (Galibert et al., 2001; Holloway et al., 1996; Torres et al., 2011a), and *B. japonicum* (Kaneko et al., 2002; Bedmar et al., 2005) contain *nap*, *nirK*, *nor*, and *nos* genes (see http://www.kazusa.or.jp/rhizobase). Denitrification genes are not present neither in the complete genome sequence of *M. loti* strain MAFF303099 nor in the symbiotic island of *M. loti* strain R7A (see http://www.kazusa.or.jp/rhizobase). However, *M. loti* fast-growing strains isolated from *Lotus* sp. showed a hybridization band with the *B. japonicum nirK* (Monza et al., 2006). Although the ability to denitrify may enhance bacterial survival and growth capability in soils subjected to anoxic conditions, only *B. japonicum (*Bedmar et al., 2005)*, Pseudomonas* sp. G-179 (Bedzyk et al., 1999), *A. caulinodans* (Raju et al., 1997) and *E. meliloti* (Torres et al., 2011a) have been shown to grow under O_2-limiting conditions with nitrate through denitrification pathway.

2.5.1. Denitrification in root nodules.

Besides of the latent paradox of nitrogen fixation and denitrification are dramatically opposed processes usually regarded as independent and separated by space, if not also by time, it has been proposed that both reactions could take place at the same time under an adequate ambient such as low-oxygen and the presence of nitrate. These conditions could be found inside the nodule, being recently suggested, that the anaerobic respiration by denitrification might have an important role in symbiotic compatibility between different *E. meliloti* strains with *M. truncatula* (Sugawara et al., 2013). The significance of denitrification in rhizobia-legume symbiosis can be appreciated when O_2 concentration in soils decreases during environmental stress such as flooding of the roots, which causes hypoxia. Under these conditions,

denitrifying activity could work as a mechanism to generate ATP for survival of rhizobia in the rhizosphere and also to maintain nodule functioning.

Expression of *B. japonicum* 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* promoter regions to the reporter gene *lacZ* (Mesa et al., 2004). The symbiotic phenotype of *B. japonicum* strains carrying a mutation in any of the *nirK*, *norC*, or *nosZ* structural genes has also been reported (Mesa et al., 2004). In soybean plants not amended with nitrate, *nirK*, *norC* or *nosZ* genes are not essential for symbiotic N_2 fixation. Similarly, nodulation, plant growth, and rates of N_2 fixation in *H. coronarium* were similar after inoculation of the wild-type and a *nirK*-deficient strain (Casella et al., 1986). None of the Tn5 insertions in the *E. meliloti* strain JJ1c10 *nos* region affected N_2-fixing ability in symbiosis with alfalfa, which demonstrated that denitrification is not essential for N_2 fixation (Holloway et al., 1996). However, in soybean plants grown with nitrate, mutation of either the *nirK* or *norC* genes confers on *B. japonicum* a reduced ability for nodulation (Mesa et al., 2004).

An associated role of denitrification in nodules could also be detoxification of the cytotoxic compounds nitrite and NO produced as intermediates during denitrification reactions or emerging from the host plant. In fact, Meakin and associates (2007) and Sanchez and associates (2010) have demonstrated that nitrate reduction by Nap in *B. japonicum* USDA110 bacteroids contributes to the formation of nitrite, and NO in soybean nodules in response to hypoxia. Similarly, in *M. truncatula* nodules, recent findings have demonstrated that *E. meliloti napA* and *nirK* denitrification genes contribute to nitric oxide production (Horchani et al., 2011). Nitrite and NO have been reported as inhibitors of nitrogenase activity (Trinchant and Rigaud, 1980, 1982; Sasakura et al., 2006; Kato et al., 2009; Shimoda et al., 2009). Recent results from our group have demonstrated that, NO formed by *B. japonicum* NirK enzyme in soybean nodules in response to flooding and nitrate has a negative effect on both nitrogenase activity and expression of the *nifH* and *nifD* genes (Sánchez et al., 2010). In fact, inoculation of soybeans with a *B. japonicum nirK* mutant, which

does not produce NO from nitrate, increases the tolerance of symbiotic nitrogen fixation to flooding (Sanchez et al., 2011).

Similarly, in *Lotus japonicus* has been observed that a decrease in NO production in root nodules results in an increase in N_2 fixation activity (Shimoda et al., 2009; Tominaga et al., 2010), which suggests that adequate concentrations of NO might be necessary for nitrogenase activity. As such, NO could also interfere with N_2 fixation by binding to Lb which would therefore impair Lb functionality by competing with O_2 for binding sites, thus diminishing the O_2 supply available to bacteroids and thereby reducing N_2 fixation (Kanayama et al., 1990). Meakin and coworkers (2007) have demonstrated that nitrate reduction by Nap in *B. japonicum* USDA110 bacteroids contributes to the formation of nitrosylleghaemoglobin (LbNO) complexes in soybean nodules in response to hypoxia. However, it was proposed that since only a small proportion if Lb is bound to O_2, and given that affinity of Lb for NO is higher than that for O_2, then Lb could act as NO scavenger modulating NO bioactivity (Herold and Puppo, 2005). Supporting this hypothesis, Sánchez et al. (2010) have recently suggested that Lb has a major role in detoxifying NO and nitrite produced by bacteroidal denitrification in response to flooding conditions.

2.5.2. *B. japonicum* as a model: genes, enzymes and regulators

B. japonicum is the only rhizobial species able to denitrify under both free-living and symbiotic conditions where denitrification has been characterized. In *B. japonicum*, denitrification is dependent on the *napEDABC*, *nirK*, *norCBQD* and *nosRZDYFLX* genes that encode a periplasmic nitrate reductase, a Cu-containing nitrite reductase, a *c*-type nitric oxide-reductase and a nitrous oxide-reductase enzymes, respectively (Bedmar *et* al., 2005; Delgado et al., 2007). Figure 2.9 shows the genetic organization of *B. japonicum* denitrification genes

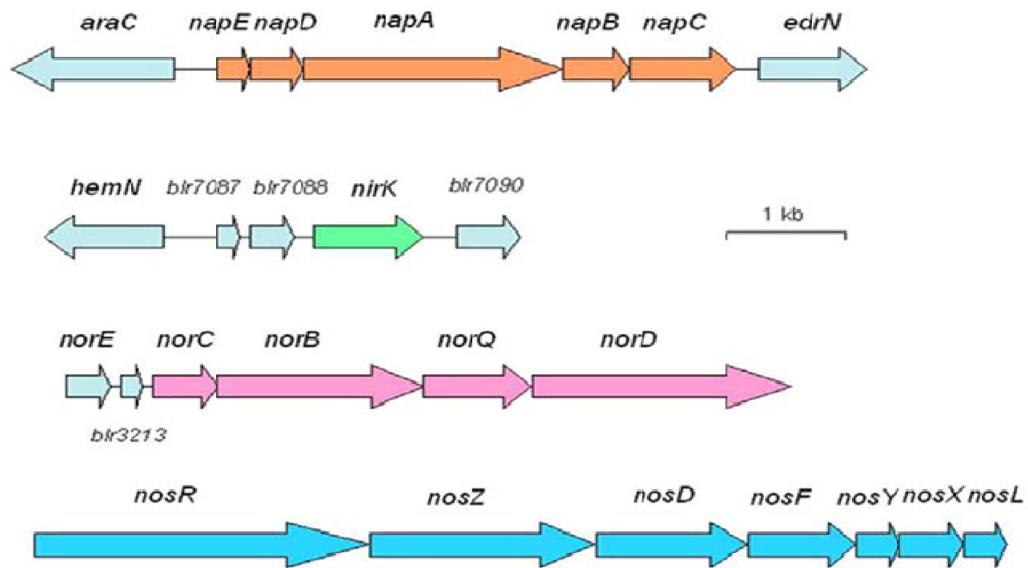


Figure 2.9. *B. japonicum nap*, *nir*, *nor* and *nos* genes organization. Identification codes from the *Rhizobase* (http://www.kazusa.or.jp/rhizobase) are between the identified genes (adapted from Bedmar et al., 2005).

In a shotgun cloning experiment, a DNA fragment of *B. japonicum* USDA110 was sequenced and found to contain the *napEDABC* genes (Delgado et al., 2003). The *napA* gene encodes the catalytic subunit (90 kDa) containing the molybdopterin guanine-dinucleotide cofactor (MGD) and a [4Fe-4S] cluster, *napB* an electron-transfer subunit, dihaem cytochrome *c* of about 15 kDa, and *napC* a membrane-bound *c*-type tetrahaem cytochrome of about 25 kDa, respectively (see Figures 2.10 and 2.11) (Delgado et al., 2003). Because a *napA* mutant was incapable of growing under nitrate-respiring conditions, lacked nitrate reductase activity, and did not show the NapA, NapB and NapC protein components, the *B. japonicum* Nap system is the primary enzyme responsible for nitrate respiration under anoxic conditions.

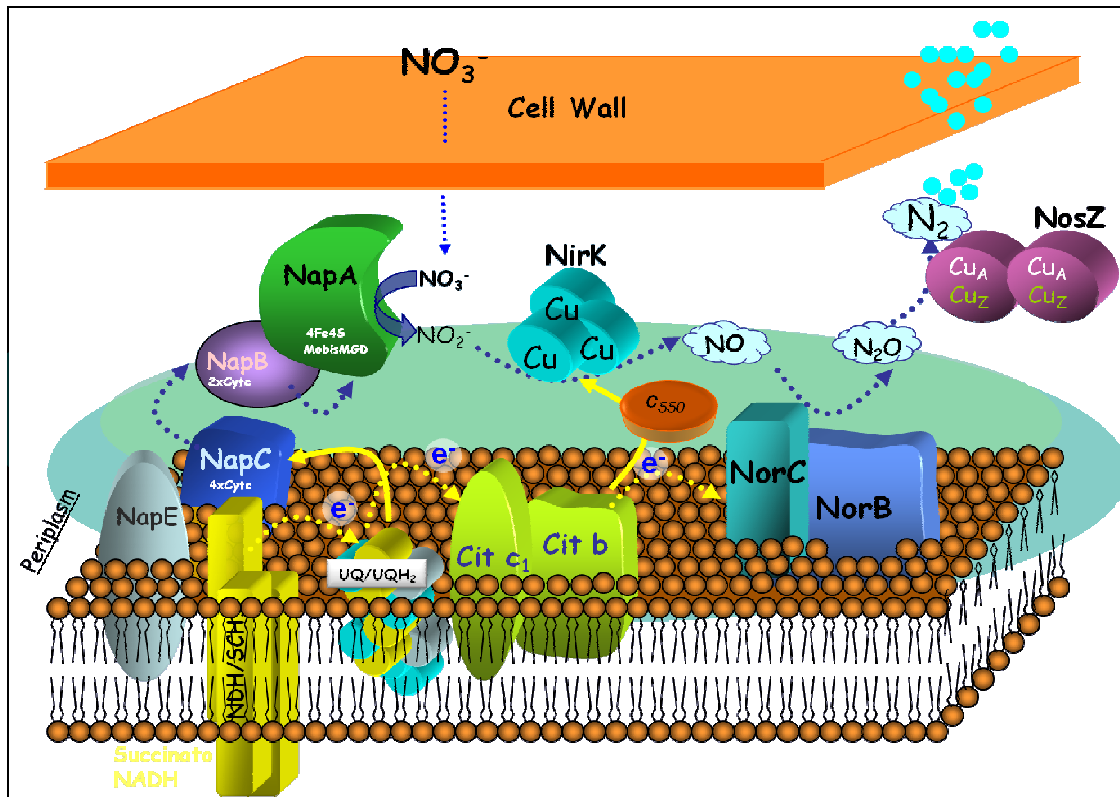


Figura 2.10. Schematic representation of *B. japonicum* denitrifying proteins location. UQ,ubiquinone; UQH_2, ubihydroquinone; NDH, NADH dehydrogenase; SDH, succinate dehydrogenase (adapted from Bueno et al., 2008).

The *B. japonicum nirK* gene, responsible for the synthesis of CuNir, was identified by Velasco et al., (2001). Implication of *B. japonicum nirK* in denitrification was shown in *nirK* mutants that were incapable of growing when cultured under anoxicc conditions in the presence of either nitrate or nitrite. Recently, it has been shown that cytochrome c_{550}, encoded by the *cycA* gene, is involved in electron transfer from the cytochrome bc_1 complex to the CuNir of *B. japonicum* USDA110. A *cycA* mutant strain is unable to consume nitrite and, consequently, to grow under denitrifying conditions with nitrite as the electron acceptor (see Figure 2.10, Bueno et al., 2008). Although mutation of *cycA* had no apparent effect on methylviologen-dependent nitrite reductase activity, succinate-dependent nitrite reduction was largely inhibited, which suggest that c_{550} is the *in vivo* electron donor to CuNir (Bueno et al., 2008).

The *B. japonicum nor* genes are organized in the *norCBQD* gene cluster. Mutational analysis indicated that the two structural *norC* and *norB* genes are required for growth under nitrate-respiring conditions, and that NorC corresponds to a 16 kDa *c*-type cytochrome found in membranes from wild-type cells (Mesa et al., 2002). Inspection of the complete genome sequence of *B. japonicum* USDA110 shows the existence of an open reading frame, *blr3212*, whose sequence has more than 60% identity with *norE* genes from various denitrifiers (see chapter 2.4.1.3 from the introduction).

The *B. japonicum* USDA110 *nos* genes were identified using a major internal portion of the *P. stutzeri nosZ* gene as a probe (Viebrock and Zumft, 1987), and found to be organized in the *nosRZDFYLX* gene cluster (Velasco et al., 2004). *B. japonicum* strains carrying either a *nosZ* or a *nosR* mutation grew well when cultured anoxically with nitrate as the final electron acceptor. Nevertheless, Nos enzyme was not active enzyme in a *B. japonicum nosZ* mutant, since nitrous oxide was accumulated when cells were grown anoxically in the presence of nitrate (Velasco et al., 2004).

2.5.2.1. Regulatory network.

Similar to many other denitrifiers, expression of denitrification genes in *B. japonicum* requires both oxygen limitation and the presence of nitrate or a derived nitrogen oxide (Bedmar et al., 2005). In *B. japonicum*, a sophisticated regulatory network, consisting of two linked regulatory cascades, co-ordinates the expression of genes required for microaerobic respiration (the FixLJ/FixK$_2$ cascade) and for nitrogen fixation (the RegSR/NifA cascade). In these two cascades, different oxygen-sensing mechanisms are responsible for a stepwise activation of downstream events (Sciotti et al., 2003). A moderate decrease in the oxygen concentration in the gas phase to 5% is sufficient to activate expression of FixLJ/FixK$_2$-dependent targets (Sciotti et al., 2003). However, in the RegSR/NifA cascade, the low oxygen-responsive NifA protein activates the transcription of essential symbiotic nitrogen-fixation genes at an oxygen concentration at, or below, 0.5% in the gas phase.

In the FixLJ/FixK_2 regulatory cascade, the FixLJ regulatory system senses the low-oxygen signal and induces the expression of *fix*K_2 whose product encodes the FNR/CRP-type transcriptional regulator FixK_2 (see chapter 2.3.2 from Introduction, Figure 2.11).

In addition to microoxically induced genes as *fixNOQP*, or *fixGHIS* among others, FixK_2 also activates denitrification genes such as *nap*, *nirK, nor and nos* (Bedmar et al., 2005; Mesa et al., 2008), as well as *nnrR* which encodes the CRP/FNR-type regulator NnrR (Mesa et al., 2003). Induction of the *norCBQD* promoter is completely abolished in the absence of a functional *nnrR* gene (Mesa et al., 2003). By contrast, microoxic induction of the *nap* or *nirK* promoters is retained in a *nnrR* mutant background, implying that the *napEDABC* or *nirK* and the *norCBQD* promoters exhibit slight differences with regard to their dependence on FixK_2 (Robles et al., 2006; Mesa et al., 2003). In this context, recent results from our group have demonstrated that purified FixK_2 activates transcription from *nap* or *nirK* promoters but not from the *nor* promoter (Bueno et al., unpublished results). By contrast, isothermal titration calorimetry allowed us to demonstrate that NnrR bound to a specific DNA fragment from the promoter region of the *norCBQD* genes, but not to those from the *napEDABC* and *nirK* genes and that this interaction requires anaerobic conditions but not the presence of an N oxide (Robles et al., unpublished results). Supporting these observations, a genome-wide transcription profiling of *B. japonicum fixJ* and *fix*K_2 mutant strains grown in free-living microoxic conditions have shown that *napEDABC*, *nirK* and *nnrR* but not *norCBQD* are targets of FixK_2 (Mesa et al., 2008).

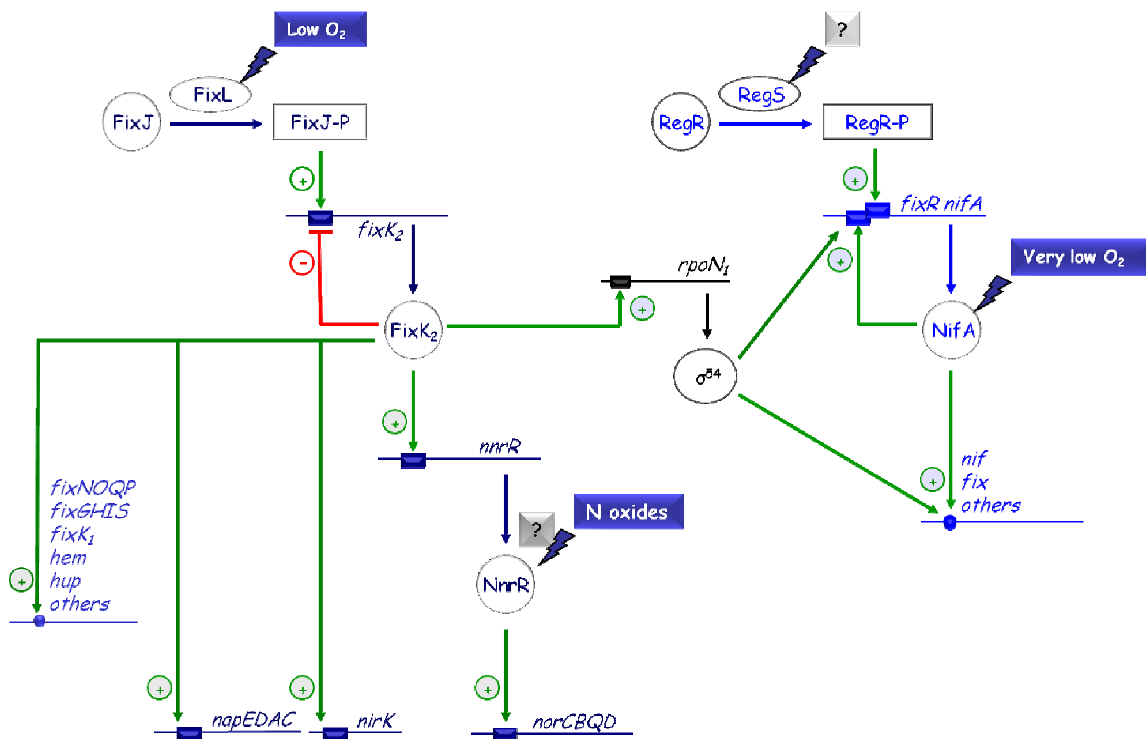


Figure 2.11. Regulatory network of *B. japonicum* denitrification (Torres et al., 2011b).

Activation of the second oxygen sensing cascade, RegSR/NifA**,** is initiated by the RegSR two-component regulatory system which induces expression of the *fixR-nifA* operon in aerobic and microoxic conditions (see chapters 2.3.2 and 2.3.2.2 from the introduction). However, the precise nature of the signal that is transduced by the *B. japonicum* RegSR is unknown. Recent results from our group showed that NifA regulatory protein is required for maximal expression of *napEDABC, nirK* and *norCBQD* genes (Bueno et al., 2010). In that study, it was shown that disruption of *nifA* caused a growth defect in *B. japonicum* cells when grown under denitrifying conditions, as well as decreased activity of Nap and Nir enzymes and on the expression of NapC and NorC. Furthermore, expressions of *napE–lacZ*, *nirK–lacZ* or *norC–lacZ* transcriptional fusions, as well as levels of *nirK* transcripts, were significantly reduced in the *nifA* mutant after incubation under nitrate-respiring conditions. These results suggest a role for RegSR/NifA regulatory cascade in the control of the denitrification process in *B. japonicum* (Bueno et al., 2010). Concerning the involvement of the two-component regulatory system in denitrification, it has been previously shown that free-living growth of *regR* mutants under anoxic conditions with nitrate as the terminal electron acceptor was severely impaired (Bauer et al., 1998). Moreover, recent results from our

group have demonstrated that expression of NorC was significantly lower in membranes from cells of the *regR* mutant incubated under denitrifying conditions in minimum medium with succinate as carbon source (Torres et al., 2011b). Supporting our findings, disruption of *R. sphaeroides prrA* or *prrB* causes a significant decrease in both *nirK* expression and Nir activity. Similarly, purified *A. tumefaciens* ActR binds to the promoter region of the *nirK* gene, but not to the *nor* or *nnrR* promoters. In *B. japonicum* the involvement of RegR in *nirK* expression is at the moment unknown, and further investigations are needed to demonstrate the involvement of RegR on *norCBQD* gene expression and to establish whether these genes are direct, or indirect, targets of RegR.

2.5.3. Denitrification in *E. meliloti*.

The ability to denitrify is widely distributed among the slow-growing rhizobia *(Bradyrhizobium)* and more rarely within fast-growing rhizobia. Nevertheless, except for *R. leguminosarum* bv. *phaseoli* (Bourguignon, 1987; Daniel et al., 1982), fast-growing *Rhizobium* strains able to denitrify have been identified. In fact, *E. meliloti* had the greatest proportion of denitrifying strains among the fast-growing group (Chan, 1989; Myshkina and Bonartseva, 1990; García-Plazaola 1993). This denitrifying capability may enhance their survival and growth in microoxic soils, and it could also reduce the effect of nitrate inhibition of nodulation and nitrogen fixation (O'Hara and Daniel, 1985), since denitrification is carried out both in the symbiotic and free-living states of *E. meliloti* strains (García-Plazaola et al., 1993).

However, denitrification has not been studied in *E. meliloti* as well as in *B. japonicum*, mainly because up to date, it has been considered a partial denitrifier due to its inability to grow under low-oxygen conditions with nitrate or nitrite as terminal electron acceptors. *E. melitoti* possess the complete set of denitrification genes (see figure 3.1 from chapter III) which are located in a 53 kb segment of pSymA (Barnett et al., 2001). Among them, the *napEFDABC*-type genes (*sma1232*, *sma1233*, *sma1236* and *sma1239–41*) which encode the periplasmic nitrate reductase, the gene *sma1250* encoding a copper-containing nitrite reductase, NirK, and is associated with a NirV-type protein which is encoded by the *nirV* gene (*sma1247*). Genes *norECBQD* (*sma1269*, *sma1272*, *sma1273*, *sma1276* and *sma1279*) and *nosRZDFYLX* (*sma1179*,

sma1182–v*86* and *sma1188*) encoding a nitric oxide reductase and a nitrous oxide reductase, respectively, are also located in the pSymA (Torres et al., 2011a). Furthermore, transcriptomic analyses have shown that *E. meliloti nap, nir, nor* and *nos* genes are induced in response to O_2 limitation (Becker et al., 2004). Under these conditions, denitrification genes expression is coordinated via a two-component regulatory system FixLJ and via a transcriptional regulator, FixK (Bobik et al., 2006). Recent transcriptomic studies demonstrated that denitrification genes (*nirK* and *norC*), as well as other genes related to denitrification (*azu1*, *hemN*, *nnrU* and *nnrS*) are also induced in response to nitric oxide (NO), and that the regulatory protein NnrR is involved in such control (Meilhoc et al., 2010). In contrast to all that has been carried out about regulation and symbiotic characterization (see chapter IV) of *E. meliloti* denitrification genes, the role of these genes under free-living conditions is not known.

3. OBJECTIVES

La fijación biológica de nitrógeno y la desnitrificación son dos procesos clave en el ciclo del nitrógeno. En concreto, la fijación simbiótica de nitrógeno, que llevan a cabo las bacterias del suelo conocidas con el nombre genérico de rizobios en asociación con plantas leguminosas es de gran relevancia agrícola, dado que mejora la calidad nutricional de los suelos reduciendo las necesidades de fertilización nitrogenada de los cultivos. Por otro lado, la desnitrificación es el proceso que permite la eliminación de nitratos del suelo mediante su reducción a óxidos de nitrógeno tales como el óxido nitroso (N_2O) y el nitrógeno molecular (N_2). Por ello, este proceso contribuye considerablemente a paliar los problemas de contaminación ambiental, que además pueden ser perjudiciales para la salud, provocados por el uso intensivo de fertilizantes químicos nitrogenados en la agricultura. Pero, por otro lado, la desnitrificación es una fuente de emisión de N_2O, un potente gas de efectoinvernadero, a la atmósfera. Además de fijar nitrógeno, los rizobios son también capaces de desnitrificar tanto en vida libre como en simbiosis. Sin embargo, aunque se ha avanzado considerablemente en el conocimiento del proceso de fijación simbiótica de nitrógeno, la información disponible sobre la desnitrificación en los rizobios es más escasa. Así, debido a su interés agrícola e impacto sobre la salud y el medio ambiente, es necesario profundizar en el conocimiento de ambos procesos en rizobios, y fundamentalmente, en el proceso de desnitrificación. Por ello, un mejor conocimiento de los factores medioambientales y los procesos de regulación que influyen en la emisión de N_2O por rhizobios desnitrificantes presentes en los suelos, cobra un enorme interés para la predicción y el desarrollo de adecuadas prácticas en la agricultura que contribuyan a mitigar las emisiones de N_2O.

En este sentido, los estudios llevados a cabo en el Departamento de Microbiología del Suelo y Sistemas Simbióticos de la Estación Experimental del Zaidín, han permitido caracterizar los genes *napEDABC*, *nirK*, *norCBQD* y *nosRZDFYLX* de *B. japonicum*, cuyos productos se han identificado como las enzimas desnitrificantes nitrato reductasa periplásmica (Nap), nitrito reductasa (NirK), óxido nítrico reductasa (Nor) y óxido nitroso reductasa (Nos), respectivamente. En cuanto a los estudios de regulación de estos genes, se ha demostrado la implicación de la cascada de regulación FixLJ/$FixK_2$/NnrR en la inducción de la expresión de los mismos en respuesta a

limitación de oxígeno y presencia de nitrato o un óxido de nitrógeno derivado de él. Estudios posteriores han demostrado que la máxima expresión de los genes de la desnitrificación depende también de la proteína NifA, otorgándole por primera vez un papel a la cascada RegSR/NifA en la regulación de la desnitrificación en respuesta a microoxia.,. Puesto que la expresión del operón *fixR-nif*A está controlada por el sistema regulador RegSR, éste podría ser un sistema regulador candidato para intervenir en un nuevo nivel de control de los genes de la desnitrificación. No obstante, al inicio de este trabajo de investigación, las posible implicación del sistema RegSR en el control de la desnitrificación en *B. japonicum* no había sido objeto de estudio.

*Ensifer meliloti*es el simbionte de alfalfa, leguminosa forrajera de gran interés en la producción agrícola de nuestro país. Esta bacteria posee los genes de la desnitrificación, sin embargo, no se consideraba como una auténtica especie desnitrificante, ya que, hasta la realización de este trabajo, se desconocía su capacidad para utilizar el nitrato o nitrito como sustratos respiratorios para obtener energía, lo cual permite a las células crecer en condiciones limitantes de oxígeno. Otro aspecto interesante de la respiración microóxica de los rizobios es la implicación de la oxidasa terminal de alta afinidad por el oxígeno cbb_3, codificada por el operón *fixNOQP*. Esta oxidasa, además de mantener la respiración aeróbica en células en vida libre en condiciones de microoxia, es clave para que se lleve a cabo la fijación simbiótica del nitrógeno. En *E. meliloti*, existen tres copias del operon *fixNOQP*, que codifica la cbb_3. Cabe mencionar que en la naturaleza existen otros casos similares al de *E. meliloti*, en el que las diferentes copias del operón *fixNOQP* tienen funciones redundantes ó bien específicas para cada una de ellas.Al inicio de este trabajo se desconocía cuál era la copia funcional de la oxidasa cbb_3 en *E. meliloti*, tanto en vida libre como en simbiosis.

De acuerdo con lo expuesto, en esta Tesis Doctoral se plantearon los siguientes objetivos:

1. Estudiar la implicación del sistema RegSR en la regulación de la desnitrificación de *B. japonicum*.
2. Investigar la implicación de la copia 1 de los genes *fixNOQP de E. meliloti,* que codifican la oxidasa terminal cbb_3, en su capacidad de respirar en vida libre, así como en su capacidad de fijar nitrógeno en simbiosis con *Medicago sativa*.

3. Demostrar la capacidad de *E. meliloti* para utilizar nitrato en condiciones limitantes de oxígeno y para desnitrificar, así como establecer el papel de los genes *napA, nirK, norC* y *nosZ* en dicho proceso.

4. RESULTS

CHAPTER I

4.1. RegSR-dependent expression of *Bradyrhizobium japonicum* *norCBQD* genes.

4.1.1. Abstract.

Bradyrhizobium japonicum can grow anoxically via denitrification. *B. japonicum* RegSR proteins belong to the family of two-component regulatory systems present in a large number of proteobacteria that they globally control gene expression mostly in a redox-responsive manner. In this work, we have performed a transcriptional profiling of wild type and *regR* mutant cells grown under anoxic conditions with nitrate as electron acceptor. The comparative analyses of wild type and *regR* revealed that almost 620 genes induced in the wild type under denitrifying conditions are regulated by RegR pointing out the important contribution of this protein as a global regulator of denitrification. Among the genes controlled by RegR are *nor* and *nos* structural denitrification genes encoding the nitric oxide and nitrous oxide reductase, genes encoding electron transporters (*cycA*, c_2), genes involved in nitric oxide detoxification (*blr2806-09*), as well as regulatory genes (*bll3466, bll4130*). Purified RegR interacted with the promoters of *norC*, *nosR*, the *fixK*-coding (*bll3466*), and the LysR-coding (*bll4130*) genes. By using fluorescently labeled oligonucleotide extension, we were able to identify two transcriptional start sites located at about 35 (P1) and 22 (P2) bp from the putative translational start codon of NorC. Whereas P2 is the principal start site and is modulated by RegR, P1 matched with the previously mapped 5' mRNA end previously proposed to be under $FixK_2$ control. Moreover, qRT-PCR experiments, expression assays of a *norC-lacZ* fusion and haem *c* staining analyses revealed that anoxia and nitrate are the signals involved in the RegR-dependent induction of *nor* genes. Further, this control seems to be independent of the sensor protein RegS

4.1.2. Introduction.

The *Rhizobiales* order of α-Proteobacteria cover Gram-negative soil nitrogen-fixing bacteria collectively named as rhizobia with the unique ability to establish N_2-fixing symbiosis on legume roots and on the stems of some aquatic legumes, leading to

the formation of a new structure called nodules. Expression of nitrogen fixation and symbiosis-related genes requires low-oxygen conditions (Fischer, 1994; Dixon and Kahn, 2004). To face with a shortage of oxygen, such as microoxic free-living conditions and bacteroid state (inside the nodules), rhizobial species express the high affinity *cbb*$_3$ oxidase encoded by the *fixNOQP* operon (Delgado et al., 1998). Moreover, some rhizobial species are able to use nitrate as final electron acceptor to support respiration under microoxic or anoxic conditions (Bedmar et al., 2005, Delgado et al., 2007; Sanchez et al., 2011, Torres et al., 2011a). This switch from oxygen to nitrate respiration leads to a reduction in the ATP yield rates, but allows bacteria to survive and multiply under oxygen-limiting conditions (Simon et al., 2008). Denitrification has been defined as the dissimilatory reduction of nitrate (NO_3^-) or nitrite (NO_2^-) to a gaseous N-oxide (N_2), via the gaseous intermediates nitric oxide (NO) and nitrous oxide (N_2O) concomitant with free energy transduction (Zumft 1997). This process requires four separate enzymatic reactions catalyzed by nitrate-, nitrite-, nitric oxide-, and nitrous oxide reductases, encoded by *nar*/*nap*, *nir*, *nor* and *nos* genes, respectively (van Spanning et al., 2007; Kraft et al., 2011; Richardson, 2011). In recent years, it has emerged that many rhizobial species have denifrication genes (Bedmar et al., 2005, Delgado et al., 2007; Sanchez et al., 2011). Among them, the soybean symbiont *B. japonicum* is considered the model organism for studying rhizobial denitrification. In this bacterium, denitrification is dependent on the *napEDABC*, *nirK*, *norCBQD* and *nosRZDYFLX* genes that encode Nap (periplasmic nitrate reductase), NirK (copper-containing nitrite reductase), cNor (*c*-type nitric oxide reductase) and Nos (nitrous oxide reductase) enzymes, respectively (Bedmar et al., 2005). Similar to many other denitrifiers, expression of denitrification genes in *B. japonicum* requires both oxygen limitation and the presence of nitrate or a derived nitrogen oxide (Bedmar et al., 2005). Perception and transduction of the ‘low-oxygen signal’ are mediated by conserved regulatory proteins that are integrated into species-specific networks in different rhizobia (Fischer, 1994; Dixon and Kahn, 2004). Two interlinked oxygen responsive regulatory cascades are present in *B. japonicum*, the FixLJ-FixK$_2$ and the RegSR-NifA cascades (Sciotti *et al.*, 2003). A moderate decrease in the oxygen concentration in the gas phase (≤ 5%) is sufficient to activate expression of FixLJ-FixK$_2$ dependent targets (Sciotti et al., 2003). This ‘low-oxygen’ signal is sensed by the haem-

based sensory kinase FixL, which auto-phosphorylates and then transfers the phosphoryl group to the FixJ response regulator, which activates transcription of *fixK*$_2$. In turn, FixK$_2$ activates the expression of *rpoN*$_1$ and the regulatory genes *fixK*$_1$ and *nnrR* (Nellen-Anthamattem, 1998; Mesa et al., 2003; Mesa et al., 2008), genes associated with microoxic metabolism such as *fixNOQP* (Nellen-Anthamattem, 1998; Mesa et al., 2005; Mesa et al., 2008), as well as structural genes involved in denitrification such as *nap*, *nirK*, *nor,* and *nos* (Mesa et al., 2003, 2008; Bedmar et al., 2005; Robles et al., 2006). The denitrification regulator NnrR expands the downstream end of the FixLJ-FixK$_2$ cascade to compose the FixLJ-FixK$_2$-NnrR cascade (Mesa et al., 2003, Torres et al., 2011b). Induction of the RegSR-NifA pathway requires very low oxygen concentration (≤0.5%) and is mediated by the two-component regulatory system, RegSR. RegR induces expression of the *fixR-nifA* operon, which is preceded by two overlapping promoters, P1 and P2 (Barrios et al., 1995, 1998; Bauer et al., 1998). RegR activates transcription originating from P2 under all oxygen conditions via binding to a DNA element located around position −67 upstream of the transcription start site. Upon a switch to low-oxygen or anoxic conditions, the redox-responsive NifA protein in concert with RNA polymerase containing RpoN (σ^{54}) enhances its own synthesis via activation of the −24/−12-type P1 promoter. In *B. japonicum*, RpoN is encoded by the two highly similar and functionally equivalent genes (*rpoN*$_1$ and *rpoN*$_2$, Kullik *et al.*, 1991). Since *rpoN*$_1$ is under the control of FixK$_2$, this gene represents the link between the two regulatory cascades. Targets of NifA include *nif* and *fix* genes, which are directly or indirectly involved in nitrogen fixation, and also genes that have an unknown function in this process (Fischer, 1994; Nienaber *et al.*, 2000, Hauser et al., 2007). Recent results from our group showed that NifA is required for maximal expression of *nap*, *nirK*, and *nor* genes, suggesting a new role for the RegSR/NifA regulatory cascade in the control of the denitrification genes in *B. japonicum* (Bueno et al., 2010). In fact, we have recently demonstrated that expression of NorC is significantly lower in membranes from a *B. japonicum regR* mutant incubated under denitrifying conditions with succinate as carbon source (Torres et al., 2011b). However, the involvement of RegSR on *norCBQD* genes expression has not been investigated so far.

RegSR are members of the family of the two component regulatory systems present in a large number of Proteobacteria. These proteins are named RegBA in *Rhodobacter capsulatus, Rhodovulum sulfidophilum*, and *Roseobacter denitrificans* (Elsen et al., 2004; Wu and Bauer, 2008), PrrBA in *Rhodobacter sphaeroides* (Oh et al., 2001; Happ et al., 2005), ActSR in *Sinorhizobium meliloti* (Emmerich et al., 2000; Fenner et al., 2004) and *Agrobacterium tumefaciens* (Baek et al., 2008), and RoxSR in *Pseudomonas aeruginosa* (Comolli and Donohue, 2002). In *Rhodobacter* species, the RegBA/PrrBA regulon encodes proteins involved in numerous energy-generating and energy-utilizing processes such as photosynthesis, carbon fixation, nitrogen fixation, hydrogen utilization, aerobic respiration and denitrification, among others (reviewed by Swem et al., 2001; Elsen et al., 2004; Wu and Bauer, 2008; Bueno et al., 2012).

RegSR two-component regulatory system comprises the membrane associated RegS histidine protein kinase and its cognate RegR response regulator. *Rhodobacter* species RegS senses the cellular redox state via key elements such a highly conserved quinone binding site and the redox-active cysteine (Cys^{265}) (Malpica et al., 2004, 2006; Swem et al., 2003, 2006, Wu and Bauer, 2008; Bueno et al., 2012). However, the precise nature of the signal perceived and transduced by the *B. japonicum* RegSR is still unknown. In addition to *fixR-nifA*, a large number of novel members of the RegR regulon have been identified by transcriptomic analysis of a *B. japonicum regR* mutant under free-living oxic and microoxic coditions and during symbiosis (Lindemann et al., 2007). However, no data exist concerning the RegR regulon in cells grown under denitrifying conditions.

Here, we have performed a comparative transcriptomic analysis of wild-type and a *B. japonicum regR* mutant grown under denitrifying conditions. Among the novel RegR targets identified, we have demonstrated by using different approaches, the involvement of RegR on expression of the *B. japonicum nor* genes encoding the nitric oxide reductase.

4.1.3. Material and Methods.

4.1.3.1. Bacterial strains and growth conditions.

Bradyrhizobium japonicum 110*spc*4 (Regensburger and Hennecke, 1983), *regR 2426* (Bauer et al., 1998), *regS* 2409 (Bauer et al., 1998), and *fixK*$_2$ 9043 (Nellen-Anthamatten et al., 1998) strains were used in this study. Strain 2499 (Mesa et al., 2003) is *B. japonicum* 110*spc*4 containing a *norC–lacZ* fusion. In this work, plasmid pRJ2499 containing a *norC–lacZ* fusion (Mesa *et al.*, 2003), was integrated by homologous recombination into the chromosome of the *regR* and *regS* mutants strains resulting in strains 2499RR and 2499RS, respectively. *B. japonicum* strains were grown oxically in liquid batch cultures containing peptone-salts-yeast extract (PSY) medium (Regensburger and Hennecke 1983; Mesa et al., 2008) supplemented with 0.1% L-arabinose at 30°C. Growth under oxygen-limiting conditions was performed in Bergersen minimal medium (Bergersen 1977) with succinate as carbon source and supplemented (BSN) or not (BS) with 10 mM KNO_3. For comparison with previous experiments, yeast extract-mannitol (YEM) medium (Daniel and Appleby 1972) supplemented with 10 mM KNO_3 was used to grow anoxically wild-type and *fixK*$_2$ mutant in some primer extension experiments series. Microoxic conditions were reached by fluxing a gas mixture (2 % oxygen, 98 % argon) at incubation starting point into the gas atmosphere of rubber-stoppered 17 ml serum tubes or 500 ml flasks containing 5 or 200 ml medium, respectively. Anoxic conditions were obtained in completed filled 200 ml bottles or 17 ml tubes. Antibiotics were added to *B. japonicum* cultures at the following concentrations (mg ml^{-1}): cloramphenicol 20, spectinomycin 200, streptomycin 100 and tetracycline100. *Escherichia coli* strains were cultured in Luria–Bertani (LB) medium (Miller, 1972) at 37°C. *E. coli* S17-1 (Simon *et al.*, 1983) served as the donor in conjugative plasmid transfer. The antibiotic used was (mg ml^{-1}): tetracycline, 10.

4.1.3.2. RNA isolation, cDNA synthesis, and microarray analysis.

Cultures of B. japonicum wild type and regR mutant strains were grown anoxically in BSN medium to an optical density at 600 nm about 0.4. Cell harvest, isolation of total RNA, cDNA synthesis, fragmentation, labeling and conditions for

microarray hybridization were done as described previously (Hauser et al., 2006, 2007; Lindemann et al., 2007 and Pessi et al., 2007). Details of the custom designed Affymetrix B. japonicum gene chip BJAPETHa520090 and conditions for microarray hybridization have also been described previously (Hauser et al., 2007). For each strain, a minimum of four biological replicates was analyzed, respectively. Details on data processing, normalization, and further analysis are described elsewhere (Pessi et al., 2007). GeneSpring GX 7.3.1 software (Agilent Technologies) was used for comparative analyses. Only the probe sets that were called "present" or "marginal" in ≥75% of the replicates of each experiment were considered for further analysis. The student t-test with a P value threshold of 0.025 was applied for statistical comparisons. We considered genes passing the statistical tests as differentially expressed only if the relative change in expression (n-fold) was ≥ 2 or ≤ -2 when different two conditions were compared. Operon predictions were done as described in Hauser et al., 2007 and Mesa et al., 2008. An operon-like organization of genes (bicistronic or larger) was assumed if they were orientated in the same direction and separated by less than 32 bp. This distance was enlarged to 100 bp if the first three letters in the gene names were identical.

4.1.3.3. Quantitative real-time PCR.

The expression of genes *nosZ, nosY, norC, blr2808, napE, napA, cycA, copC, bll3466, bll4130* and *cy_2* were analyzed by quantitative reverse transcription-PCR using an iQ™5 Optical System (Bio-Rad). Primers for the PCR reactions (Table 3.S1 in supplemental material) were designed using the Primer3Web v.0.4.0 (http://frodo.wi.mit.edu/primer3/input.htm), to have a melting temperature of about 57ºC to 62ºC and to give a PCR product of about 50 to 100 bp. Each PCR reaction contained 9.5 µl of iQ™ SYBR® Green Supermix (Bio-Rad), 2 µM final concentration of individual primers and appropriate dilutions of different cDNA concentrations in a total volume of 19 µl. Reactions were run in triplicates. Melting curves were generated for verifying the specificity of the amplification. Relative changes in gene expression were calculated as described elsewhere (Pfaffl 2001). Expression of the primary sigma factor *sigA* was used as a reference for normalization (primers SigA-1069F and SigA-1155R (Lindemann et al., 2007).

4.1.3.4. Haem-staining analysis.

Cells of *B. japonicum* grown oxically in 150 ml PSY medium were harvested by centrifugation at 8000 g for 5 min, washed twice with BS or BSN, resuspended in 500 ml of the same medium, and finally incubated under microoxic or anoxic conditions in BS or BSN for 48 hours. Cells were disrupted using a French pressure cell (SLM Aminco, Jessup) and membranes were isolated as described earlier (Delgado et al., 2003). Membrane protein aliquots were diluted in sample buffer [124 mM Tris-HCl, pH 7.0, 20% glycerol, 4.6% sodium dodecyl sulfate (SDS) and 50 mM 2-mercaptoethanol], and incubated at room temperature for 10 min. Membrane proteins were separated at 4 ºC in 12%-SDS polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and stained for haem-dependent peroxidase activity as described previously (Vargas et al., 1993) using the chemiluminiscence detection kit 'SuperSignal' (Pierce, Thermo Fisher Scientific).

4.1.3.5. NO consumption activity.

Cells of *B. japonicum* were incubated anoxically during 24 and 48 hours in BSN. Then, cells were harvested by centrifugation at 8000 *g* for 10 min at 4 °C, and washed with 50 mM Tris/HCl buffer (pH 7.5). NO consumption rates were determined with a 2-mm ISONOP NO electrode APOLO 4000® (World Precision Inst.) in a 2 ml thermostatted and magnetically stirred reaction chamber (Zhang and Broderick 2000). The membrane-covered electrode was situated at the bottom of the chamber above the stirrer and reactants are injected with a Hamilton syringe through the port in the glass stopper. The chamber was filled with 760 µl of 25 mM phosphate buffer (pH 7.4), 900 µl (4-5 mg protein) of cellular solution, 100 µl of a enzymes mix of *Aspergillus niger* glucose oxidase (80 units/2ml) and bovine liver catalase (500 units/2 ml), 90 µl 1 M sodium succinate, and 100 µl of 320 mM glucose. Once a steady base line was observed, 50 µl of a saturated NO solution (1.91 mM at 20ºC) was added to the cuvette to begin the reaction. Each assay was run until the NO detection had dropped to zero, that is, when all NO was oxidized (except for *norC* mutant).

4.1.3.6. β-Galactosidase assays.

To measure β-galactosidase activity, strains 2499, 2499RR and 2499RS were grown oxically in PSY medium, collected by centrifugation at 8000 *g* for 10 min at 4°C, washed twice with BS or BSN medium and finally incubated microoxically or anoxically in the same medium. Cultures with an initial OD_{600} of about 0.2 were incubated for 2 days at 30°C. Activity was determined with permeabilized cells from at least three independently grown cultures assayed in triplicate for each strain and condition. β-Galactosidase assays were performed essentially as described previously (Miller, 1972).. The absorbance data for A_{420}, A_{550}, and A_{600} were determined for all samples in a plate reader (SUNRISE Absorbance Reader, TECAN) by using the software XFluor4 (TECAN). Data were then transferred to Microsoft Excel to calculate the specific activities in Miller units.

4.1.3.7. Fluorescently labeled oligonucleotide extension (FLOE).

Total RNA of *B. japonicum* wild type, *regR* and *fixK₂* mutant strains was isolated using the hot phenol extraction procedure described previously (Babst et al. 1996). To determine the transcription start site of the *norC* gene, the NorC53 reverse primer was synthesized and HPLC-purified by Eurofins NWG Operon and labeled with 6-FAM in the 5´-end. The sequence of NorC53 was 5´-GAGCCGCCGTAGAAGACGTTTC-3´ which was 31-53 bp downstream of the annotated translational start codon. The primer extension assay was performed using Avian Myeloblastosis Virus Reverse Transcriptase AMV RT (Promega). The reaction mixture (50 µl) contained $MgCl_2$ (5 mM), dNTP (2 mM each), AMV RT 1X buffer, 50 pmol FAM-labeled primer, 7-10 µg of ARN and water. Reactions were incubated at 65ºC for 15 minutes, then stopped by the addition of 2 µl of the mixture AMV/RNasin (Promega) (1:1). Next, the mixtures were treated at 15ºC for 10 minutes followed by incubation at 45ºC and 95ºC for 45 and 5 minutes, respectively. Finally, 2 µl of RNAse (2 mg/ml) was added to the mixtures and incubated at 37ºC for 90 minutes. The sequence of the reverse transcribed cDNA products was analysed by Newbiotechnic S.A. (NBT) in an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems) capillary electrophoresis instrument. GeneScan® version 3.1.2 (Applied

Biosystems) was used to screen the data, to identify the major peaks and to determine the lengths and amounts of DNA (the area under the curves in arbitrary units).

4.1.3.8. Eletromobility sihift assays (EMSAs).

Binding of RegR to putative target promoters was tested in electrophoretic mobility shift assays using radiolabeled PCR fragments obtained with primers showed in Table 3.S1. PCR fragments were end labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (MBI Fermentas), and subsequently purified over Micro Bio-Spin 6 chromatography columns (Bio-Rad). His-tagged RegR was overexpressed and purified as described previously (Emmerich et al., 1999). For *in vitro* phosphorylation, RegR protein (40 µM final concentration) was incubated with 25 mM acetyl phosphate (Sigma-Aldrich) in DNA binding buffer (Bauer et al., 1998) for 1 h at 30°C. Phosphorylated RegR (0 to 7.5 µM) was incubated with column-purified DNA fragments (0.5 to 1 µg) in DNA binding buffer in a total volume of 20 µl. After 15-min incubation at 30 ºC, samples were mixed with loading dye and separated on 8% nondenaturing polyacrylamide gels in 1X Tris-borate EDTA electrophoresis buffer for 2h at 70 V. Gels were dried, and radioactive bands were visualized with a phosphorimager (Bio-rad).

4.1.3.9. Microarray data accession number.

The microarray data are available in the NCBI Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo) under GEO Series accession number……….

4.1.4. Results.

4.1.4.1. Transcription profiling of a *B. japonicum* regR mutant grown under free-living denitrifying conditions.

Comparative analyses of *B. japonicum* wild-type and the *regR* mutant strain grown under anoxic conditions in BSN medium revealed that approximately 1700 genes were differentially expressed in the *regR* with a relative change of at least twofold (Fig. 3.1, GEO Series accession number…..). The main focus of this work was

the identification of genes upregulated in denitrifying conditions (in comparison with oxically-grown wild type, Hauser et al., 2007), and at the same regulated by RegR. The comparison of both regulons gave a total number of 620 genes (Fig. 3.1, Table 3.S2 in supplemental material). Within this group, we focused our attention on the genes positively controlled by RegR under denitrifying conditions (344 genes). Among them, we found genes involved in the denitrification process, such as *nosRZDFYLX* encoding the nitrous oxide reductase (Velasco et al., 2004), and *norECBQD* genes encoding the nitric oxide reductase (Mesa et al., 2002). We also identified *cycA* which encodes the cytochrome c_{550} implicated in electron delivery to the NirK reductase (Bueno et al., 2008). Concerning *napEDABC* genes encoding the periplasmic nitrate reductase (Delgado et al., 2003), only *napB* and *napC* genes could be identified as RegR targets, but the relative fold change (FC) in expression was only of -2.8 and -3.4, respectively (Table 3.S2). Surprisly, *nirK* coding for the Cu-containing nitrite reductase (Velasco et al. 2001) could not be identified amongRegR-controlled genes (Table 3.S2). We also found as RegR target, cy_2 gene that encodes the previously identified $FixK_2$-dependent cytochrome c_2 (Mesa et al., 2008), which suggests that this gene is possibly important for life under denitrifying conditions.

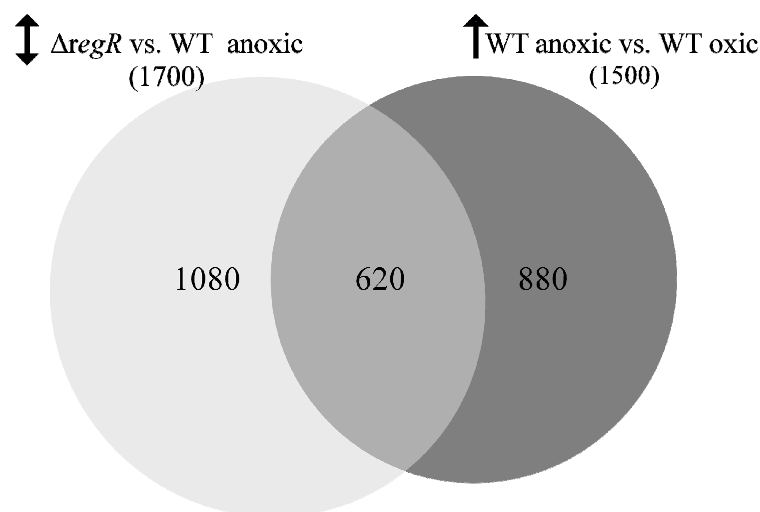


Figure 3.1. Venn diagram representing the number of differentially expressed genes in transcriptome comparisons of the *B. japonicum* wild type (WT) and the *regR* mutant grown under anoxic conditions with nitrate (light grey circle). Number of genes induced upon a switch from oxic to anoxic conditions in the WT are indicated in the dark grey circle. The overlap shows the proportion of genes anoxically induced in the wild type that are controlled by RegR. Strains and conditions are indicated alongside the circles. Up-down arrows reflect increased and decreased gene expression in microarray analyses. Numbers in parentheses indicate the total number of differentially expressed genes.

In addition to denitrification genes, new genes were identified as promising candidates for being RegR targets under anoxic conditions. Comments of few examples follow. (i) There are genes such as *blr2807* which has been reported to encode a single domain haemoglobin (Bjgb) implicated in NO detoxification (Cabrera et al., 2011). Other genes such as *blr2808* encoding a FAD and NAD(P)H-binding reductase (annotated as putative nitrite reducase) and *blr2809* encoding the large catalytic subunit, NasA, from the assimilatory nitrate reductase were also RegR targets. (ii) There are *copCAB* genes encoding proteins involved in copper homeostasis (Hernandez-Montes et al., 2012). (iii) There are genes coding for putative transcription factors that suggests that RegR might be integrated in a complex regulatory network as it has been previously proposed by Lindemann et al., 2007. Among them, we found *bll3466* encoding a FixK-like protein which has been proposed to be involved in the negative feed-back of $fixK_2$ expression (Reutimann et al., 2010). (iv) We also identified the *phyR* homologue (*bll7795*) and its associated ECF σ^{70} factor gene [*ecfG* (*blr7797*)] which are involved in stress resistance and symbiotic capacity of *B. japonicum* (Gourion et al., 2009). Curiously, *fixR* or *nifA* were not found as targets of RegR within this group, since they were not induced in wild-type cells grown under the conditions used in this work.

In order to identify potential direct targets of RegR, we made a selection of those genes under the control of RegR independently to the growth conditions. We found 37 genes differentially expressed in the *regR* mutant under oxic, microoxic (Lindemann et al., 2007) and anoxic conditions (this work, see Table 3.S3 in the supplemental material). As expected, the previously known RegR targets are included in this group. Expression levels of *fixR*decreased in *regR*-deficient cells under all conditions. Likewise, this group also includes *bll2087* (Hauser et al., 2006), the operon *blr1515-blr1516* that encodes the *B. japonicum* multidrug efflux system, BdeAB (Lindemann et al., 2010), and *bll4130* encoding a LysR-type transcriptional regulatory protein. Direct RegR-mediated control at promoter regions of these genes were previously demonstrated by DNA binding experiments (Lindemann et al., 2007).

To validate the microarrays results, we also performed qRT-PCR on several genes (Figure 2). As shown in Figure 2, we could clearly validate genes involved in Nos

(*nosZ*, *nosY*) and Nor (*norC*) synthesis, as well, as genes involved in electron transfer (*cycA, cy_2*). As expected, qRT-PCR results indicate that genes encoding the periplasmic nitrate reductase (*napE* and *napA*) were not significantly controlled by RegR (Fig. 3.2). In addition to denitrification genes, we also confirmed the RegR-dependent expression of *blr2808* which is part of a gene cluster (*blr2806-blr2809*) possibly involved in NO detoxification and nitrate assimilation en *B. japonicum* (Cabrera et al., 2011). For its putative involvement in in the assembly of Cu-containing denitrification enzymes (NirK and NosZ) we also considered of interest to validate RegR-dependent expression of *copC*. Finally, we confirmed by qRT-PCR RegR-dependent expression of *bll3466*, and *bll4130* encoding a FixK-like, and a LysR-like transcriptional regulators, respectively.

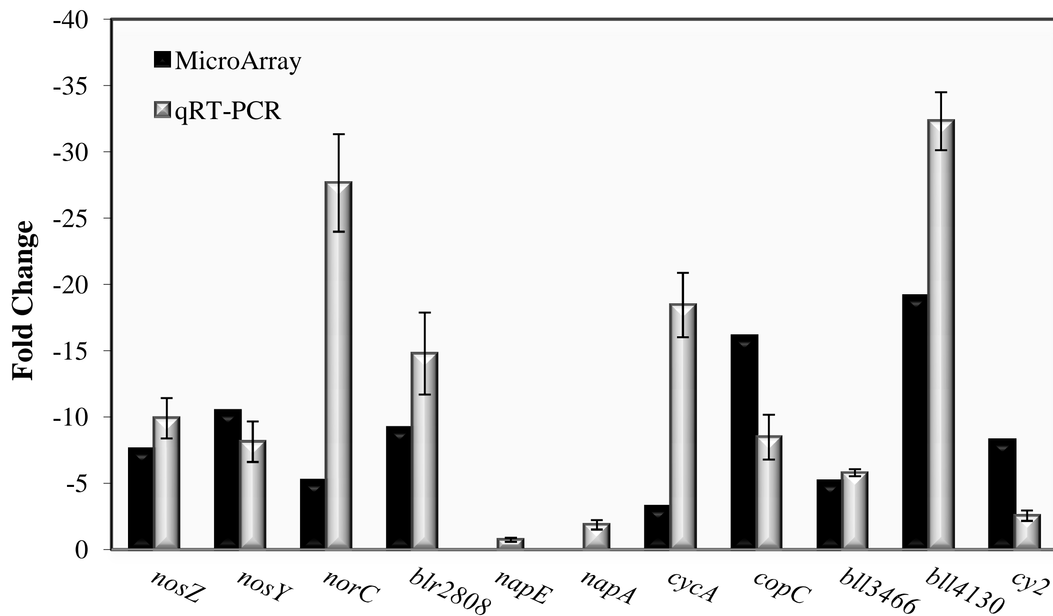


Figure 3.2. Validation of RegR-dependent genes by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Microarray data (black bars) were plotted against qRT-PCR data (grey bars).The qRT-PCR data were analyzed according to the method of Pfaffl 2001 and the expression levels were normalized against the constitutively expressed house-keeping sigma factor gene *sigA*. Efficiency of the primers was previously determined by using different cDNA concentrations .The values are means of three independent experiments and four amplification reactions with standard deviations.

4.1.4.2. RegR binding to the promoter region of new target genes.

Because microarray analysis does not allow to discriminate between directly and indirectly controlled genes, we performed DNA binding analyses by EMSA to

identify direct RegR target genes. We used a PCR fragment covering the promoter region of 6 candidates whose expression was found to be under positive control of RegR (Table 3.1). The ^{32}P-labeled amplification products were were incubated with increased concentrations of phosphorylated RegR covering from 0 (negative control) to 7.5 µM. As positive and negative control, we used amplification products covering the promoter and the coding region of the *bll2087* gene, respectively (data not shown). These fragments were successfully used by Lindemann and colleagues (2007) for the same purpose. As expected, RegR displayed a consistent binding to the promoter region of the *bll4130* gene (Fig. 3.3C), whose expression is controlled by RegR under oxic, microoxic and anoxic conditions. (Table 3.S3) In addition to *bl4130*, binding was observed to DNA probes originating from promoter regions of the positively controlled genes *bll3466* (Fig. 3.3D), *norC* (Fig. 3.3A), and *nosR* (Fig. 3.3B). By contrary, we were unable to detect a shifted band upon addition of different concentrations of phosphorylated RegR when we used DNA sequences from the promoter regions of *bll2388* (cytochrome c_2) and *blr2806* (nitrite extrusion protein) (Fig. 3.3E, F). Inspection of DNA sequences used in the EMSA assays allowed us to identify two putative RegR binding sites in all promoter sequences except for *bll2388* (Table 3.1). As observed previously (Lindemann et al., 2007), the half sites DNA consensus sequences found in the putative promoters used in this work are differently spaced and poorly conserved (Table 3.1). It is worth noting that RegR did not bind to the promoter region of *blr2806* despite of the presence of two putative RegR binding sites.. EMSAs of the *norC, nosR* and *bll3466* promoter regions showed a single retarded band. However, two bands which appeared at different concentrations were observed when the *bll4130* promoter was used. It might be possible that in the latter case, RegR first binds to a high-affinity Reg-box and afterwards, at higher concentration, binds to a low-affinity Reg-box, thus generating the second retardation. A hierarchical binding has also been already described for PhoR-PhoB two-component system that activates the transcription of several genes involved in phosphate uptake and assimilation (Blanco et al., 2012). By EMSA aessays, these authors demonstrated that two PhoB dimers bind to two consecutive *pho* boxes in a hierarchical and cooperative manner. The DNA-PhoB complex improves the interaction with the sigma^{70} factor of the RNA polymerase and hece PhoB effectively triggers the expression of the specific genes.

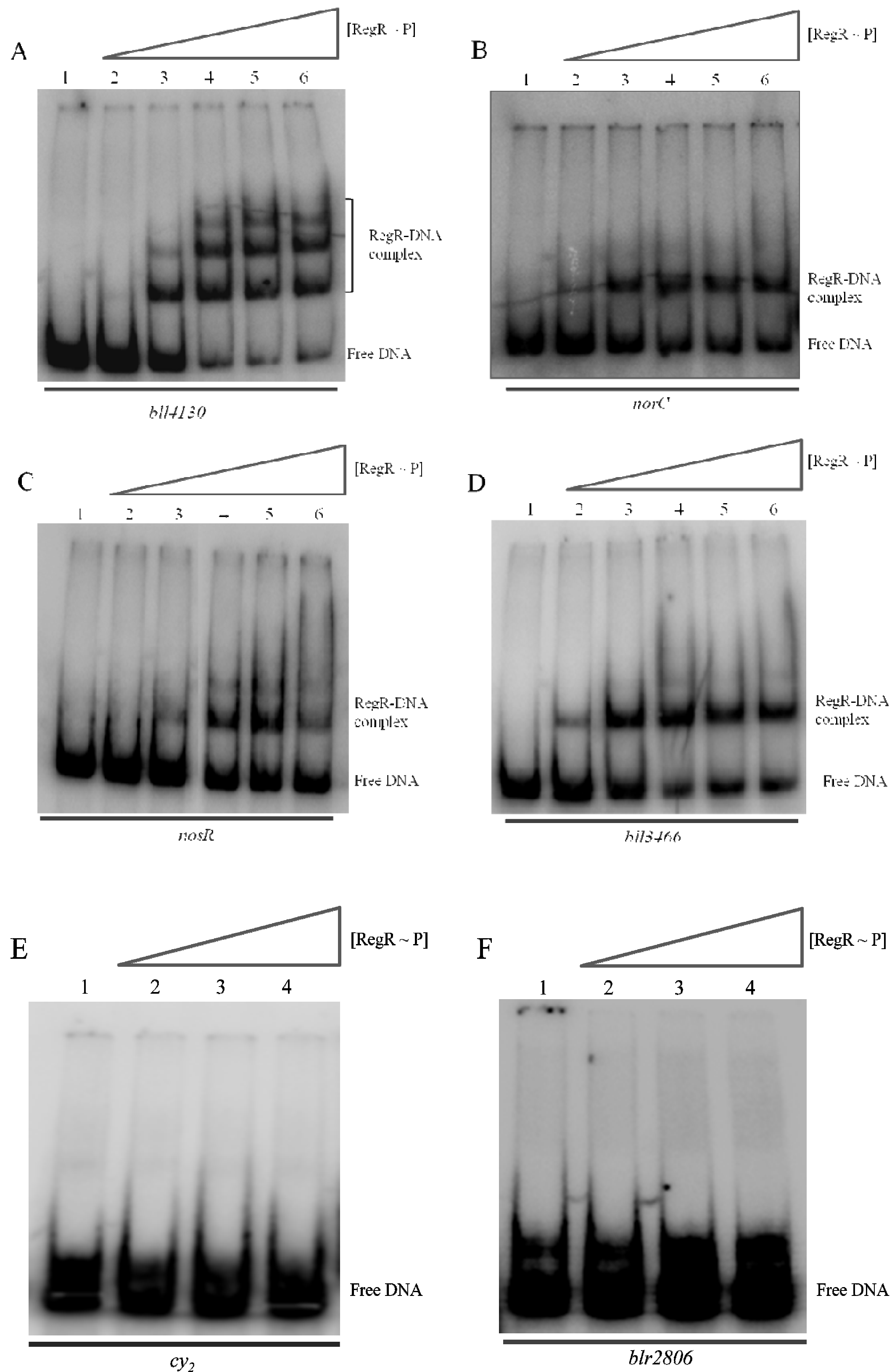


Figure 3.3. Analysis of RegR binding to promoter region of several putative target genes using EMSA. Increasing amounts of purified RegR~P were incubated with constant amounts (1 µg) of double-stranded ^{32}P-labeled PCR amplified products from the promoter region of *B. japonicum norC, nosR, bll4130*, *bll3466*, *cy*$_2$ and *blr2806* genes. From panels A to D, RegR concentrations were 1.5 µM (lanes 2), 3 µM (lanes 3), 4.5 µM (lanes 4), 6 µM (lanes 5) and 7.5 µM (lanes 6). From panels E and F, RegR

concentrations were 0.8 µM (lanes 2), 1.6 µM (lanes 3) and 3.2 µM (lanes 4). No RegR protein was added to the control reaction in lane 1 from any panel. Samples were run on 6% nondenaturing polyacrylamide gels and visualized with a phosphorimager.

Table 3.1. Putative RegR-controlled *B. japonicum* genes whose promoter region was tested for RegR binding in electrophoretic mobility shift assays.

Gene no.[a]	Gene name[b]	Description[c]	Genomic region[d]	Shift[e]	Putative RegR-box position[f]	Putative RegR-box sequence[g]
blr0314	*nosR*	Nitrous oxide reductase expression regulator	-195 to +46	+	-73 -39	TGCGTCAACGGCGA CGCGGCCCGGTCGG
bll2388	*cy_2*	Cytochrome c_2	-143 to +31	–		Not found
blr2806		Nitrite extrusion protein	-214 to +34	–	-64 -49	CGCGCCTCCGTGGCCG GGAGGCAGAGCCTG
blr3214	*norC*	Nitric oxide reductase subunit C	-149 to +53	+	-64 -123	CGCGCGAAGCGGC CGTGTCGGCCGTCGT
bll3466	*fixK*	Transcriptional regulator FixK-type	-160 to +58	+	-78 -88	TGCGACATCGGCGGC CGAGCCGGAGTGCGAC
bll4130		Transcriptional regulatory protein LysR-family	-114 to +61	+	-51 -98	TGCGGCTTTCGTGCC TGCGGCAAAGGAGCC

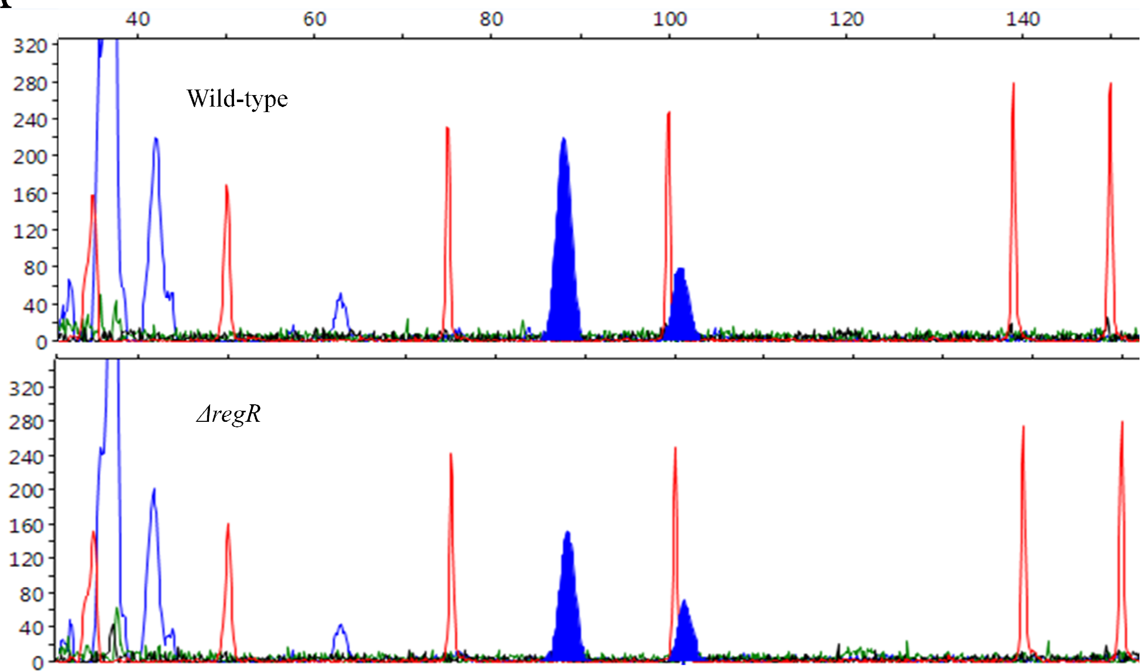
[a] Genes whose promoter regions were tested for RegR binding. All genes tested are differentially expressed by a factor < -2 in the comparison of the wild type with the *ΔregR* under the conditions analyzed in this work.
[b] Gene name as indicated in the EMBL-EBI database.
[c] Protein description according to Kaneko and coworkers., 2002.
[d] Genomic region included in the PCR fragment used for EMSAs. Coordinates refer to the first nucleotide position of the annotated translation start site of the genes listed in column 1.
[e] Indicates qualitatively whether RegR binding (+) or not (–) to the promoter region.
[f] Position of the 5´-end nucleotide of the putative RegR box relative to the annotated translational start site of the associated gene.
[g] Sequence of the putative RegR binding site as previously described by Lindemann et al., 2007.

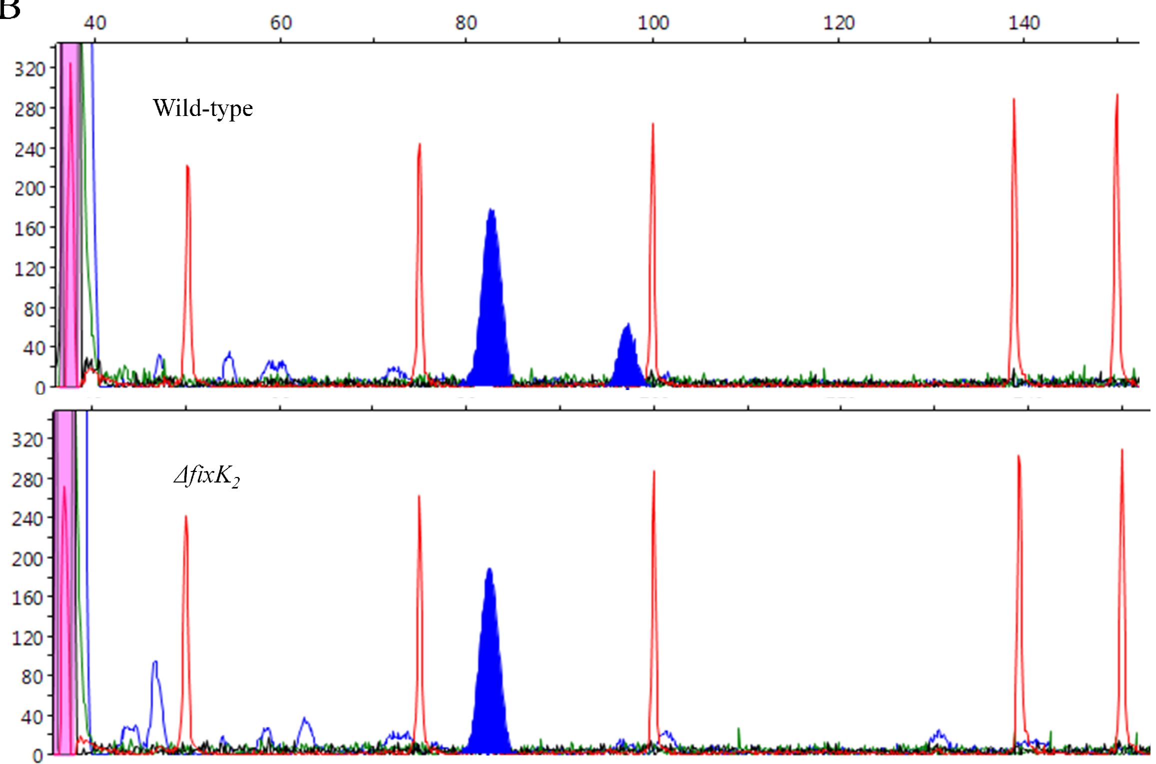
4.1.4.3. Primer extension analysis of *norC* mRNA.

In order to better understand RegR control on *nor* genes, we mapped the transcriptional start site of *norC* in wild-type and *regR* cells grown under dentrifying

conditions. We used the FLOE technique involving reverse transcription of mRNA with a sequence-specific fluorescently FAM-labeled primer (Fekete et al., 2003). The length of the FAM-labeled cDNA primer extension products were analyzed by sequencing resulting in fluorescent peaks corresponding to the transcriptional start sites of the *norC* mRNA. Using this technology, we were able to identify two FAM signals on wild-type mRNA (Fig. 3.4, panel A, upper electropherogram) suggesting the presence of two transcriptional start sites. The smaller signal (P1) corresponds to the previously proposed start site (Mesa et al., 2002) which maps to a guanine base 35 bp upstream of the translational start codon of NorC (Fig. 3.5). The higher signal (P2) corresponds to a second start site which maps to an adenine located at 14 bp from P1 and at 21 bp from the ATG (Fig. 3.5). By using this quantitative primer extension technique, the peak area is directly proportional to the number of cDNA molecules present in the sample (Fekete et al., 2003). As shown in Fig. 3.4A (upper electropherogram), the area of P2 obtained from wild-type RNA was around 2075 arbitrary units. However, the area of P2 obtained using *regR* RNA was around 1350 arbitrary units (Fig. 3.4A, lower electropherogram). The fact that the P2 transcriptional start site is significantly lower in the *regR* mutant than in the wild type suggests that P2 is modulated by RegR. Comparative analyses of P1 and P2 areas obtained from wild-type mRNA (Fig. 3.4, panel A, upper electropherogram) let us to conclude that P2 is the principal transcriptional start site under anoxic conditions.

The transcriptional start site is located at 45 pb from a $FixK_2$ box (Fig. 3.5). Thus, it has been previously proposed that depends on the $FixK_2$ transcription factor (Mesa el al., 2002). In order to demonstrate this hypothesis, in this work, we have performed primer extension experiments of the wild-type and a *fixK*$_2$ mutant in the same conditions used in previous experiments (Mesa et al., 2002). As shown in Fig. 3.4 (panel B, lower electropherogram) the primer extension signal corresponding to P1 was absent when mRNA isolated from the *fixK*$_2$ mutant was used. However, the signal corresponding to the product P1 was present when wild-type mRNA was used (Fig. 3.4B, upper electropherogram).


A
40 60 80 100 120 140
320 280 240 200 160 120 80 40 0
Wild-type
ΔregR


B
40 60 80 100 120 140
320 280 240 200 160 120 80 40 0
Wild-type
ΔfixK2

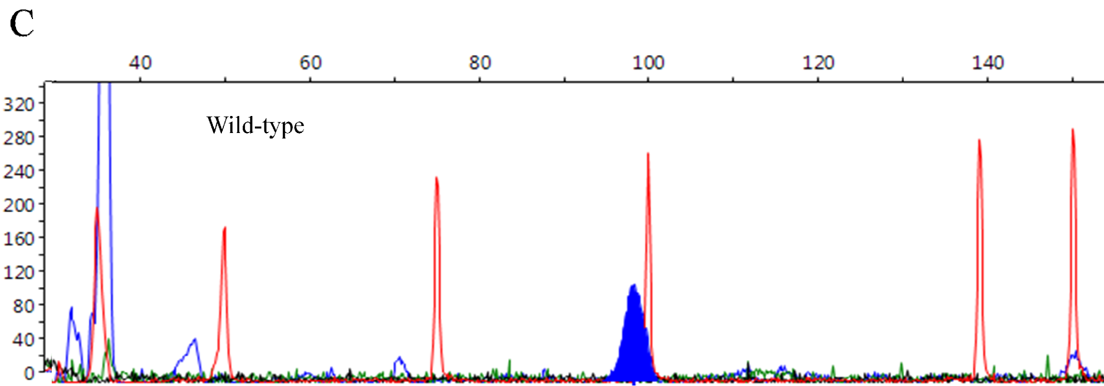


Figure 3.4. Transcription start site mapping of *B. japonicum norC* by fluorescent labeled oligonucleotide extension method (FLOE). Panel A corresponds to FLOE electropherograms obtained by using RNA from wild type (upper panel) and *regR* mutant (bottom panel) grown anoxically in BSN medium. In panel B, RNA was isolated from wild type (upper panel) and *fixK*$_2$ mutant (button panel) grown anoxically in YEM complete medium amended with 10 mM KNO_3. In panel C; RNA was isolated from wild type grown microoxically in BSN medium. The red peaks are the GeneScan®-500 ROXTM internal lane standards and the size of each peak is shown (in base pairs). The shaded blue peak in each panel is the primer extension products marked as P1 or P2.

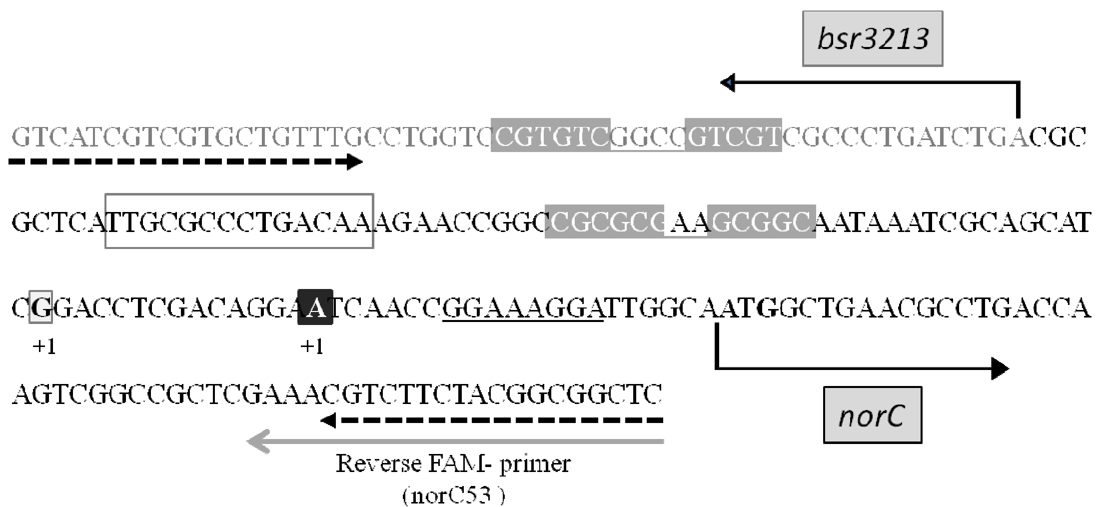


Figure 3.5. Sequence analysis of *B. japonicum norC* promoter. Forward and reverse primers used in EMSA assays are indicated with dashed narrows. The continuous grey narrow indicates the FAM-labeled reverse primer used in FLOE analyses. Continuous black narrows indicate the end of *bsr2313* and the beginning of *norC*. The putative translation start codon annotated in the *B. japonicum* genome database (http://kazusa.or.jp/rhizobase) is shown in bold. The putative FixK$_2$ binding site is indicated with a white box. A potencial Shine-Dalgarno (rbs) sequence is underlined. The nucleotide at which transcription initiates are marked +1 below and shown inside a white square (P1) or a black square (P2). The putative RegR biding sites according to Lindemann et al., (2007) are marked with a grey box.

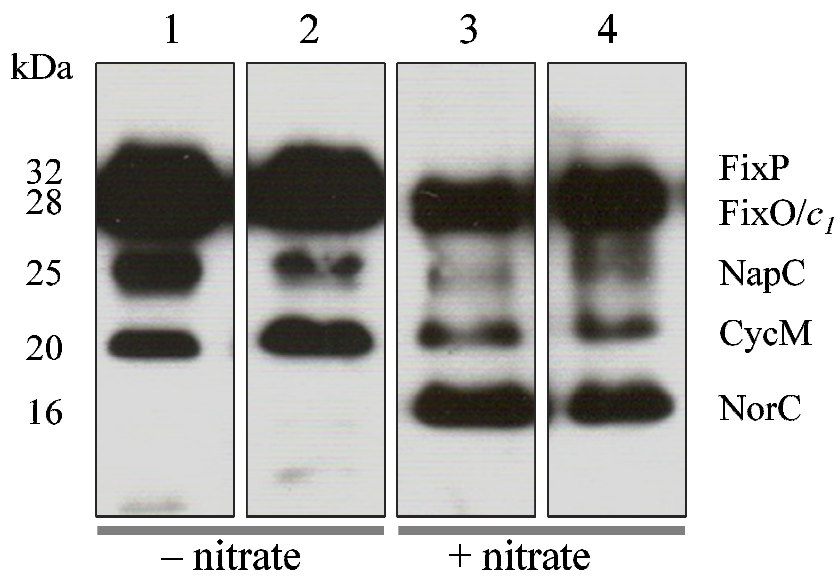
In other bacteria, conserved two component transcriptional regulators homologous to RegRS (known as RegAB, PrrAB or ActRS) respond to oxygen levels by perceiving the redox state of the environment and allow bacteria to adapt their aerobic respiration from oxic to microoxic conditions (Bueno et al., 2012). Therefore, we were interested to know whether the primer extension product P2 proposed to be RegR-dependent under anoxic conditions was also present under microoxic conditions. To achieve this objective, we performed primer extension experiments using mRNA from cells grown in BSN medium under microoxic conditions. As shown in Fig. 3.4 (panel C), the FAM signal corresponding to P2 was absent in mRNA isolated from microoxically grown wild-type cells. However, the signal P1 corresponding to the extension product from the $FixK_2$-dependent start site was present in such conditions (Fig. 3.4, panel C). Similar results were obtained when we used RNA from cells of the *regR* mutant grown under microoxic conditions (data not shown). These results suggest that the transcriptional start site proposed to be modulated by RegR is specific for anoxic conditions.

4.1.4.4. RegR control of *nor* genes requires anoxia and nitrate.

In order to investigate whether or not anoxic conditions with nitrate are required for RegR-dependent induction of *nor* genes, we analyzed expression of *nor* genes in wild-type and *regR* mutant cells incubated anoxically or microoxically in the absence or the presence of nitrate. Previous haem-staining analyses reported byfrom our group (Torres et al., 2011b) showed that expression of *B. japonicum* NorC under anoxic conditions in BSN medium is significantly decreased in the *regR* mutant. Conversely, NorC expression was not affected in the *regR* mutant when cells were incubated microoxically in BSN medium (Fig. 3.6A, lane 4). Interestingly, the presence of nitrate was required to induce NorC in wild-type cells grown under microoxic conditions (Fig. 3.6A, lane 3 in comparison to lane 1).Nevertheless, this induction did not depend onthe RegR protein (Fig. 3.6A, lane4). As previously reported by our group (Mesa et al. 2002), the presence of nitrate was required to fully induced expression of a *norC-lacZ* transcriptional fusion in wild-type cells incubated under microoxic or anoxic conditions (Fig. 3.6B). Futher, β-galactosidase activity of the *norC-lacZ* transcriptional fusion decreased about 54-fold in the *regR* mutant compared to wild-

type levels when cells were grown anoxically in BSN medium (Fig. 3.6B). However, *norC* expression was not decreased in the *regR* mutant when cells were incubated in the same medium but under microoxic conditions (Fig. 3.6B). By opposite, levels of β-galactosidase activity were slightly higher in the *regR* mutant compared to wild-type cells under microoxic conditions either in the presence or in the absence of nitrate., Altogether these results suggestthat both, the presence of nitrate and anoxic conditions, are required for RegR-dependent induction of *nor* genes.

A)



B)

Growth conditions Strain	Relevant genotype	β-Galactosidase activity (Miller units)	
Anoxic		**- nitrate**	**+ nitrate**
2499	*norC-lacZ*, wild type	76.20 (6.81)	921.68 (65.34)
2499RR	*norC-lacZ*, *regR*	143.99 (41.05)	17.61 (3.14)
Microoxic		**- nitrate**	**+ nitrate**
2499	*norC-lacZ*, wild type	39.83 (11.30)	347.31 (52.04)
2499RR	*norC-lacZ*, *regR*	169.57 (25.68)	499.49 (22.60)

Figure 3.6. A) Haem-stained membrane-bound proteins from cells of *B. japonicum* wild type (lanes 1 and 3) and *regR* mutant strain 2426 (lanes 2 and 4) incubated under microoxic conditions. Each lane contains about 20 mg membrane proteins. Haem-stained *c*-type cytochromes identified previously are specified at the right margin. Apparent masses of the proteins (kDa) are shown at the left margin. B) β-Galactosidase activity from a *norC–lacZ* fusion in wild-type *B. japonicum* strain 2499 and *regR* mutant derivative 2499RR incubated under anoxic or microoxic conditions. Cells were grown for 2 days in Bergersen minimal medium in the absence or in the presence of nitrate. In B, data are means with standard error from at least three different cultures, assayed in triplicate

The involvement of RegR on *nor* expression was also confirmed by performing Nor activity assays, by measuring NO consumption in wild-type and RegR cells incubated anoxically in BSN medium. As shown in Figure 3.7, after incubation during 24 or 48 h, NO reductase activity was significantly decreased in the *regR* mutant compared with that from wild-type cells. As expected, Nor activity in a *B. japonicum norC* mutant was almost zero independently of the incubation time.

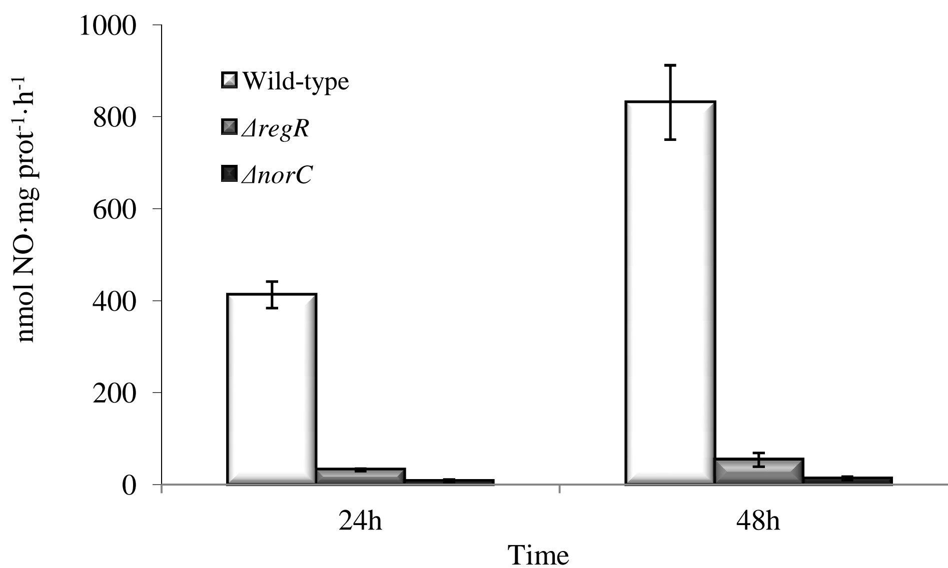
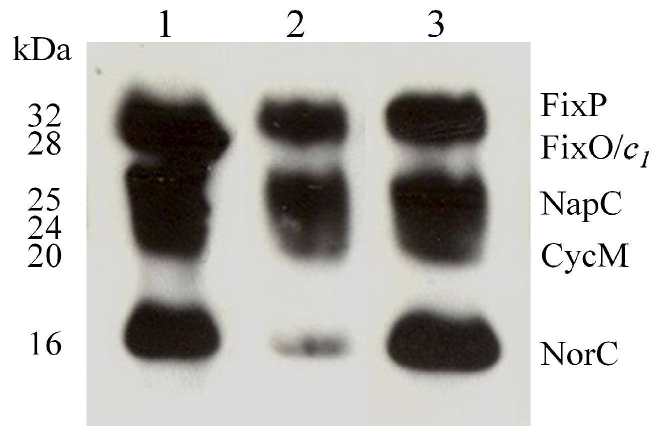


Figure 3.7. NO consumption activity by wild-type *B. japonicum*, *regR* and *norC* mutant strains incubated under anoxic conditions in BSN after 24 and 48 hours incubation. Data are means with the standard error from at least two different cultures, assayed in triplicate.

4.1.4.5. RegS dependent expression of *nor* genes?.

Finally, we investigated the involvement of RegS on RegR-dependent induction of *nor* genes under denitrifying conditions. To achieve this objective, we performed haem-*c* staining analyses in a *regS* mutant growing under denitrifying conditions. As previously reported (Torres et al., 2011b) a decreased expression of NorC was observed in the *regR* mutant compared to wild type incubated under anoxic conditions in BSN medium (Fig. 3.8A, lanes 1 and 2). However, NorC was present in the *regS* mutant at similar levels as in the wild-type (Fig. 3.8A, lane 3). Similar results were obtained by qRT-PCR analyses, where a very low effect of *regS* mutation was observed on *norC* expression (Fig. 3.8B). Whereas *norC* expression was 27-fold lower in the *regR* mutant compared to the wild-type strain incubated anoxically in BSN medium for 24 h (Fig. 3.8B, lane 2), only a slight decrease of *norC* expression of about 3-fold was observed in the *regS* mutant relative to the wild-type (Fig. 3.8B, lane 3). These results were also confirmed by β-galactosidase activity experiments of a *norC-lacZ* transcriptional fusion. As shown in Figure 8C, expression of *nor* genes in the *regS* mutant was very similar to that observed in the wild-type cells. However, induction of the *norC-lacZ* fusion was reduced about 30-fold in the *regR* mutant compared to the wild type (Fig. 3.8C). These results suggest that the control of RegR on *norC* expression is independent on the RegS sensor protein.

A)



B) 1(0) -27.65 (3.67) -3.69 (0.92)

C)

Strain	**Relevant genotype**	**β-Galactosidase activity (Miller units)**
2499	*norC-lacZ*, wild type	1237 (75.11)
2499RR	*norC-lacZ*, *regR*	39.62 (4.75)
2499RS	*norC-lacZ*, *regS*	1452 (87.34)

Figure 8. Haem-stained membrane-bound proteins (A) and relative change of *norC* expression by qRT-PCR (B) in cells of wild-type *B. japonicum* (lane 1), *regR* mutant strain 2426 (lane 2), and *regS* mutant strain 2409 (lane 3). In A, each lane contains about 20 mg membrane proteins. Haem-stained *c*-type cytochromes identified previously are specified at the right margin. Apparent masses of the proteins (kDa) are shown at the left margin. C) β-Galactosidase activity from a *norC–lacZ* fusion in wild-type *B. japonicum* strain 2499, *regR* 2499RR and *regS* 2499RS mutant derivatives. Cells were incubated for 2 days under anoxic conditions in BSN medium. In B and C, data are means with standard error from at least three different cultures, assayed in triplicate.

4.1.5. Discussion.

The involvement of *B. japonicum* RegSR two-component regulatory system in the transcriptional activation of the nitrogen fixation regulatory gene *nifA*, thus forming the RegSR-NifA cascade is well-established (Bauer et al., 1998). Further, transcriptional profiling of *regR* cells grown under oxic or microoxic conditions revealed that expression of almost 250 genes is controlled by RegR (Lindemann et al., 2007). Although the capability of *B. japonicum* to grow under denitrifying conditions is well-documented (Bedmar et al., 2005), no data exist so far concerning the identification of new target genes controlled by RegR under denitrifying conditions. Our transcriptome analyses showed that expression of approximately 1700 genes was affected in *regR* cells in comparison with wild-type cells, both grown anoxically in BSN medium. Among them, 620 RegR- were also induced in wild-type cells under denitrifying conditions, a result that underscores the relevant role of RegR in *B. japonicum* denitrification. If we consider those genes differentially expressed with a $-5 \geq FC \geq 5$ (about 20% from 1700 genes, Table 3.S4), the large majority (83%) of the diffentially expressed genes in the *regR* mutant are subjected to a positive control, which points out that this protein mainly acts as an activator in these conditions. Similarly, microarray analyses of *B. japonicum regR* mutant under free-living oxic, microoxic coditions or during symbiosis revealed that a large proportion of the differentially expressed genes were subjected to positive control by RegR (Lindemann et al., 2007). This meets the expectation that response regulators of this global family act predominantly as activators (Wu and Bauer, 2008; Bueno et al., 2012). Among this group of genes, we identified the previously demonstrated RegR targets such as *fixR*, *nifA*, or the operon (*blr1515-blr1516*) that encodes a predicted multidrug efflux system (Lindemann et al., 2007; Lindemann et al., 2010) among others, which confirms the validity of the approach used in this work. Interestingly, we were able not only to identify, but also to validate by qRT-PCR as RegR targets, structural denitrification genes (*nor*, *nos*) as well as genes participating in electron transport (*cycA*, c_2) through the denitrification pathway. In this context, it is worth mentioning the involvement of *R. sphaeroides* PrrAB system in *nirK* expression (Laratta et al., 2002). Similarly, insertional inactivation of the response regulatory gene *actR* significantly reduced *nirK*

expression as well as the expression of *paz* encoding a electron transport pseudoazurin (Baek et al., 2008). Recently, it has been reported that the NtrY/X two-component system of *Brucella* spp. acts as a redox sensor and regulates the expression of *nar*, *nir*, *nor* and *nos* operons in response to microoxic conditions (Carrica et al., 2012; Roop and Caswell, 2012). Results from our work indicate that, in contrast to *nor* or *nos* genes, *nirK* or *nap* denitrification genes were not under the control of RegR which impliesthat *B. japonicum* denitrification genes have a different behaviour with regard to their dependence on RegR. In this context, a disparate regulation of *nap*, *nirK* and *nor* genes by $FixK_2$ has also been observed in transcription profiling analyses of *B. japonicum* $fixK_2$ mutant strain grown under microoxic conditions. In the latter studies, *nap*, *nirK*, and *nnrR*, but not *nor* genes are the targets for $FixK_2$ (Mesa et al., 2008). We have also identified and validated as RegR targets *copCAB*, genes encoding proteins involved in the assembly of periplasmic and secreted cuproproteins (Hernandez-Montes et al., 2012) that might be involve in the denitrifying activity of NirK or Nos enzymes. Interestingly, genes (*blr2806-09*) involved in NO detoxification and nitrate assimilation (Cabrera et al., 2011), as well as genes encoding transcriptional regulators (*bll3466* and *bll4130* among others) were found to be controlled by RegR

By performing DNA binding experiments, we were able to show direct RegR-mediated control at promoter regions of *norC*, *nosR, bll3466* (encoding a FixK-like protein), and *bll4130* (encoding a LysR-type regulator) and . RegR binding to the latter gene was also observed by Lindemann and co-workers (2007) Since the relative change of *bll4130* expression in the *regR* mutant is more significant under anoxic conditions (this work, Table 3.S3), it might possible that its function is more relevant under these conditions.

Given the experimental evidences (Torres et al., 2011b, this work) that suggest that RegR is required for *nor* genes activation, we focused our research on the better understanding of the mechanism involved in such control. Firstly, we used the FLOE technique for to map the transcriptional start sites of *norC*. This methodology have a high level of precision and can allow quantification of the primer extension products obtained from a FAM-labeled primer, since the peak area is directly proportional to the number of cDNA molecules present in the sample (Fekete et al. 2003; Amanda et

al., 2005). Therefore, we demonstrated that *norC* has two major transcription start sites: G at 35 nt (P1) and A at 21 nt (P2) of the predicted translational start codon. The P1start site was previously proposed in our group (Mesa et al., 2002) to be the $FixK_2$-dependent. In this work, we have confirmed this hypothesis by performing FLOE with RNA isolated from *B. japonicum* $fixK_2$ mutant cells. The areas under the peaks let us to conclude that P2 is the principal transcription start site under our experimental conditions and it is modulated by RegR. Contrary to this work, previous primer extension analyses of *norC* only revealed the presence of the $FixK_2$-dependent start site but not the RegR-dependent start site (Mesa et al., 2002). The apparent discrepancy with the results presented here could be due to the different methodological approaches used, since in Mesa and colleagues (2002), primer extension was performed by using [γ-^{32}P]ATP and the subsequent extension products were run in denaturing polyacrylamide gels. Although FLOE is as sensitive as radioactive method, it extends the stretch of analyzable sequence, and simplifies quantification.

A comparative analysis of *norC* expression under microoxic or anoxic conditions with or without nitrate has demonstrated that anoxia and nitrate are the conditions required for RegR-dependent induction of *nor* genes. Supporting our observations, in microarray experiments performed previously to characterize the *B. japonicum* RegSR regulon, *nor* genes did not appeared as RegR targets in cells grown under microoxic conditions (Lindemann et al., 2007). These authors identified RegR-dependent genes under either oxic or microoxic (free-living and symbiotic) environments suggesting that RegSR system is somehow involved in sensing different oxygen conditions. As we showed here, RegR has also a regulatory role under anoxic conditions, and especially in the case of *nor* genes, the presence of nitrate or a nitrogen oxide resulted from nitrate reduction is also essential for RegR control. Supporting our findings, in *Bacillus subtillis*, the ResDE-dependent anaerobic induction of *nasDE* and *hmp* genes encoding a nitrite reductase and a NO-detoxifying flavohaemoglobin, requires the presence of nitric oxide (NO). In this bacterium, NO inactivates the NO-sensitive NsrR transcriptional repressor of *nasDE* and *hmp* (Kommineni et al., 2010), then anaerobic induction of *nasDE* and *hmp* by ResDE occurs. *B. japonicum* genome lacks of genes coding for a

NsrR homologue (Rodionov et al., 2005). Thus, another mechanism might be involved in the nitrate-dependent RegR induction of *nor* genes under anoxic conditions.

By analogy with the well-studied sensing mechanism of the orthologous two-component regulatory systems RegBA in *R. capsulatus* or the ArcBA system in *E. coli*, it seems attractive to speculate that the redox state of the membrane-localized quinone pool or the redox-active cysteine (Cys^{265}) present in the sensor protein RegS are important cues also for *B. japonicum* RegSR. However, an intriguing observation in this work was the different expression levels of *norC* in *regS* and *regR* mutants. While RegR certainly controlled *norC* expression, a mutation in *regS* showed wild-type expression levels. Supporting our observations, previous work reported that the *B. japonicum regR* mutant displayed a strong growth defect, while the *regS* mutant grew like the wild type under anaerobic conditions (Bauer et al., 1998). A similar phenomenon has been described for *regB* and *regA* mutants of *Rhodobacter capsulatus* (Mosley et al., 1994) or the RoxSR two-component system of *Pseudomonas aeruginosa* (Comolli et al., 2002). In *B. japonicum*, it might be possible that RegR is phosphorylated via cross-talk by an alternative sensor protein in the *regS* mutant. In fact, another two-component regulatory system with similar characteristics of RegRS encoded by *blr1154* and *blr1155* has been found in *B. japonicum* (H-M Fischer, personal communication). Alternatively, we can not exclude the possibility that RegR functions as a transcriptional activator in its non-phosphorylated form. In this context, it has been shown that both phosphorylated and unphosphorylated forms of RegA/PrrA are capable of binding DNA *in vitro* and activating transcription (Ranson-Olson et al., 2006). Further experiments are needed to dilucidate the mechanism of signal perception and regulation of *nor* genes by the RegSR system.

4.1.6. Supplemental material.

Table 3.S1: List of primers used for qRT-PCR analyses and EMSAs assays.

Experimental technique	Gene	Forward primer		Reverse primer	
qRT-PCR	*sigA*	SigA-1069F[1]	5’-GAGATCATCGTCGAGGTGAAG-3’	SigA-1155R[1]	5’-GCGCTTGTTGATGTCGTAGA-3’
	nosZ	nosZ_for_1	5'-TCAGGTCACCGTCTACATCAC-3'	nosZ_rev_1	5'-CCATCTGGATACCGTAGTTCAC-3'
	nosY	nosY_for_1	5'-ATGACGCTGCTCCTGAGTTA-3'	nosY_rev_1	5'-CCGTAGCCGATCACTGTC-3'
	norC	norC_3_for	5'-GCAGATGCCGCAGTTCAAC-3'	norC_3_rev	5'-TGATCGTGCTCACCCATTG-3'
	blr2808	bll2808_1_for	5'-TGGTCTCGACCAATCTGAAG-3'	blr2808_1_rev	5'-CGATGACGAGCTTCTTGTA-3'
	napE	bsr7036_for_4	5'-GCCTTCCTGTTCCTGAC-3'	bsr7036_rev_4	5'-CCGGCAAACATCTGGTAGA-3'
	napA	bsr7038_for_1	5'-GAGCATCCGCTGCAGAAGA-3'	bsr7038_rev_1	5'-CGTGTACTCCGAGACGAACTTG-3'
	cycA	cycA_for_1	5'-AACAAGAATTCCGGCATCAC-3'	cycA_rev_1	5'-TGATCTCGGTCTCGTTCTTG-3'
	copC	bll2209_1_for	5'-CAGGAATTCGTGGTCTTC-3'	bll2209_1_rev	5'-TACGAGCCGTCGAACATC-3'
	bll3466	bll3466_for_1	5'-ACGAGCGATTCAAATCCAA-3'	bll3466_rev_1	5'-ACCGTCCGACAGGAGTTTA-3'
	bll4130	bll4130_for	5'-ATATGGAGCGTCATGCCTTC-3'	bll4130_rev	5'-TCTTGCGATAGGTTTTCTGGA-3'
	cy_2	bll2388_for	5'-GAATGTCATCGACCGCAAG-3'	bll2388_rev	5'-TTGCATCAGAATAGGCGAAG-3'
EMSA	*bll2087*	2087-23F[2]	5´-AGCAAGCTCTGGTGTCCAAG-3´	2087-24R[2]	5´-TAAACGTCAACGCGACAAAG-3´
	bll2087	2087-11F[3]	5´-TACGCTGCCTACACCCAAT-3´	2087-12R[3]	5´-AGGAGGTAATGCCGTCTTGT-3´
	norC	EMSA_NorC_F	5'-GTCATCGTCGTGCTGTTTG-3'	EMSA_NorC_R	5'-GAGCCGCCGTAGAAGACG-3'
	nosR	EMSA_NosR_2F	5'-CGCTCATCAGCAGCGAAG-3'	EMSA_NosR_R	5'-CCGCTTGGGTTAGAAAATCC-3'
	bll3466	EMSA_3466_F	5'-CACCGACCTGTCCCTTGGTAC-3'	EMSA_3466_R	5'-CGAGGTCTTTGAGCGAATTG-3'
	bll4130	EMSA_4130_F	5'-CGTCGAAATCATGCCTTGC-3'	EMSA_4130_R	5'-CGTCCAGGGCTTCTTCAC-3'
	cy_2	EMSA_2388_F	5'-CGGTTGATGCAGGACAAAG-3'	EMSA_2388_R	5'-GCATGAGCACGAAGATCAGA-3'
	blr2806	EMSA_2806_F	5'-GCAATTCGAACAAGCCACTG-3'	EMSA_2806_R	5'-GCCAGTCTGAAATCCAGGTC-3'

[1] Oligos for the primary sigma factor sigA, used as reference for normalization (Lindemann et al., 2007).
[2] Oligos used as positive control to amplified a previous demonstrated region binding by RegR (Hauser et al., 2006).
[3] Oligos used as negative control previous demonstrated to be region not binding by RegR (Hauser et al., 2006).

Table 3.S2: Anoxically induced genes (as compared to oxic conditions) whose expression differed in the Δ*regR* strain relative to the wild-type[a].

Class and gene no.[b]	Putative operon member (gene no.)[c]	Gene name[d]	Description[e]	Relative change in expression (*n*-fold)[f]	
				WT_anoxic_vs_WT_oxic[g]	Δ*regR*_anoxic_vs_WT_anoxic[h]
Class 1 (downregulated in the Δ*regR* strain)					
bll0100			ferredoxin NADP+ reductase	2,0	-4,4
bsr0136			hypothetical protein	2,2	-2,8
bll0161			hypothetical protein	3,0	-2,2
blr0274			hypothetical protein	11,1	-12,7
bll0301		*ragC*	cation efflux protein	2,4	-3,2
	bll0300	*ragD*	RagD protein	2,4	-4,2
blr0305			hypothetical protein	8,9	-9,5
blr0306			hypothetical protein	6,4	-4,1
blr0314		*nosR*	nitrous oxide reductase expression regulator	122,8	-2,2
	blr0315	*nosZ*	nitrous-oxide reductase precursor	95,5	-7,6
	blr0316	*nosD*	periplasmic copper-binding precursor	38,7	-12,1
	blr0317	*nosF*	copper ABC transporter	37,1	-14,2
	blr0318	*nosY*	nitrous oxide metabolic protein	76,7	-10,5
	blr0319	*nosL*	NosL protein	39,1	-11,1
	blr0320	*nosX*	NosX protein	29,4	-10,5
bll0322		*otsA*	probable trehalose-6-phosphate synthase	6,4	-5,5
bll0342		*fah*	fumarylacetoacetase	3,9	-8,6
bll0346			hypothetical protein	–	–
	bsl0345		hypothetical protein	–	–
	bll0344		hypothetical protein	–	–
	bll0343		homogentisate 1,2-dioxygenase	7,0	-9,6
blr0401			hypothetical protein	14,4	-5,6
bsr0421		*rpmA*	50S ribosomal protein L27	2,2	-4,6
blr0444			hypothetical protein	4,5	-7,5
bll0464			hypothetical protein	4,2	-2,1
bll0465			hypothetical protein	2,7	-10,6
blr0468		*ccmB*	heme exporter protein B	–	–
	blr0469	*ccmC*	heme exporter protein C	–	–
	bsr0470	*ccmD*	heme exporter protein D	–	–
	blr0471	*ccmG*	thiol:disulfide interchange protein	3,9	-2,2
bll0506			hypothetical protein	5,0	-4,8
	bll0505		hypothetical protein	3,3	-5,4

bll0527			hypothetical oxidoreductase	2,1	-3,0
blr0536			transcriptional regulatory protein	9,1	-2,5
bll0556			hypothetical protein	2,1	-2,5
blr0806			hypothetical protein	3,5	-7,6
blr0807			succinate-semialdehyde dehydrogenase	7,3	-2,2
bll0818			hypothetical protein	106,8	-20,7
bsr0858			hypothetical protein	9,7	-11,8
bsr0859			hypothetical protein	4,5	-8,0
bsr0862			hypothetical protein	7,9	-2,6
bll0888			hypothetical protein	5,0	-3,8
bll0905		*regS*	two-component sensor histidine kinase	–	–
	bll0904	*regR*	two-component response regulator	5,3	-91,7
blr0908			hypothetical protein	2,0	-2,4
bsl0950			hypothetical protein	13,5	-5,3
blr1091		*pstS*	ABC transporter phosphate-binding protein	3,3	-2,1
bll1101		*apaG*	hypothetical protein	3,0	-2,2
bsl1208			hypothetical protein	3,8	-3,2
bll1231			hypothetical protein	–	–
	bll1230		3-oxoacyl-[acyl-carrier-protein] reductase	7,7	-4,1
blr1263			hypothetical protein	–	–
	blr1264		hypothetical protein	4,0	-6,4
	blr1265		hypothetical protein	4,7	-6,3
bll1285			hypothetical protein	6,3	-151,1
blr1289			hypothetical protein	174,1	-8,1
bll1299			hypothetical protein	2,2	-3,5
blr1311			outer membrane protein	7,2	-2,5
bsl1312			hypothetical protein	5,5	-3,3
bsl1363			hypothetical protein	14,5	-4,6
blr1429			hypothetical protein	4,0	-8,1
bll1465			hypothetical protein	4,1	-4,1
bll1467			hypothetical protein	10,4	-5,5
	bll1466		hypothetical protein	12,5	-11,9
blr1468			hypothetical protein	35,4	-26,1
	blr1469		hypothetical protein	35,6	-22,3
bsr1472			hypothetical protein	7,3	-2,1
bsl1473			hypothetical protein	62,0	-14,9
blr1482			ABC transporter sulfate-binding protein	11,1	-2,7
blr1483			sulfate ABC transporter permease protein	5,7	-2,8
	blr1484		sulfate ABC transporter permease protein	3,9	-3,1
	blr1485		sulfate ABC transporter ATP-binding protein	3,8	-4,4
	blr1486		hypothetical protein	–	–

bll1523		*gapA*	glyceraldehyde-3-phosphate dehydrogenase	2,3	-2,9
blr1601			ABC transporter substrate-binding protein	2,1	-2,7
	blr1602		ABC transporter permease protein	–	–
	blr1603		ABC transporter permease protein	–	–
	blr1604		ABC transporter ATP-binding protein	–	–
blr1617		*trbL*	conjugal transfer protein	–	–
	blr1618	*trbF*	probable conjugal transfer protein	–	–
	blr1619	*trbG*	conjugal transfer protein	–	–
	blr1620	*trbI*	conjugal transfer protein	2,4	-4,5
	bsr1621		hypothetical protein	2,8	-12,5
bll1766			outer membrane protein	8,0	-10,2
bll2007		$hemN_1$	coproporphyrinogen III oxidase	72,4	-3,7
bsl2064			hypothetical protein bsl2064	2,1	-5,3
bll2067		*nfeC*	nodulate formation efficiency C protein	4,1	-2,9
blr2177			hypothetical protein	2,0	-2,7
	blr2178		two-component hybrid sensor and regulator	–	–
bsl2212			hypothetical protein	27,1	-6,8
	bll2211	*copB*	copper tolerance protein	16,7	-15,2
	bll2210	*copA*	multicopper oxidase	9,8	-10,7
	bll2209	*copC*	copper tolerance protein	6,6	-16,1
	bll2208		hypothetical protein	8,2	-9,2
bll2213			hypothetical protein	9,2	-4,8
bll2388		cy_2	cytochrome c_2	232,9	-8,3
bsl2407			hypothetical protein	14,4	-7,8
blr2426			hypothetical protein	–	–
	blr2427		acetyl-CoA acetyltransferase	–	–
	blr2428		putative fatty acid oxidation complex alpha subunit	–	–
	blr2429		hypothetical protein	3,1	-2,2
bll2445			oxidoreductase	5,1	-3,2
bll2449			hypothetical protein	–	–
	bll2448		probable cellulose synthase catalytic subunit	5,5	-3,6
blr2451			hypothetical protein	12,0	-2,2
bll2462			hypothetical protein	4,5	-6,0
bll2465			MoxR family protein	10,6	-7,5
	bll2464		hypothetical protein	5,0	-4,6
	bll2463		hypothetical protein	6,3	-6,6
blr2487			hypothetical protein	3,3	-2,5
blr2501			hypothetical protein	4,3	-15,1
blr2505			hypothetical protein	10,7	-11,0
blr2511		*moeB*	molybdopterin biosynthesis protein B	2,7	-2,5
blr2557			RNA polymerase sigma-70 factor	5,9	-2,1

bsl2596			hypothetical protein	22,6	-7,8
bsl2602			hypothetical protein	5,3	-9,6
blr2603			hypothetical protein	10,5	-3,4
blr2668			hypothetical protein	15,0	-2,2
blr2694			VirG-like two component response regulator	5,3	-12,4
bll2734			sulfur oxidation protein SoxY	–	–
	bll2733		probable sulfur oxidation protein	2,6	-2,5
	bll2732		putative cytochrome *c*	–	–
	bll2731		probable ABC transporter substrate-binding protein	–	–
	bll2730		probable ABC transporter permease protein	–	–
	bll2729		probable ABC transporter permease protein	–	–
bll2737			oxidoreductase with iron-sulfur subunit	12,8	-14,2
	bll2736		putative aldehyde dehydrogenase protein	7,0	-10,3
bll2743			hypothetical protein	2,8	-3,6
bll2752			probable glycosyl transferase	12,5	-4,1
blr2753			ABC transporter HlyB/MsbA family	24,9	-4,5
blr2806[i]			nitrite extrusion protein	27,8	-10,3
	blr2807		probable bacterial hemoglobin	14,9	-4,0
	blr2808		putative FAD and NAD(P)H-binding reductase protein	21,4	-9,2
	blr2809	*nasA*	nitrate reductase large subunit	5,0	-5,5
bll2830			probable enoyl-CoA hydratase	2,3	-3,5
bll2850			probable 6-phosphofructokinase	–	–
	bll2849		hypothetical protein	14,3	-8,4
bll2851			hypothetical protein	3,0	-2,5
blr2852			hypothetical protein	19,4	-2,1
blr2932			hypothetical protein	2,7	-3,7
blr2943			enoyl-CoA hydratase	–	–
	blr2944		hypothetical protein	2,3	-2,7
	blr2945		hypothetical protein	–	–
blr2983			hypothetical oxidodeductase	2,2	-4,2
blr3017			hypothetical protein	4,0	-2,1
bll3037			hypothetical protein	4,8	-11,9
bll3087			transcriptional regulatory protein	3,8	-2,2
	bll3086		putative arsenate reductase	–	–
	bll3085		sodium bile acid symporter family protein	–	–
blr3130			serine protease DO-like precursor	2,3	-2,9
blr3169			hypothetical protein	10,8	-38,3
blr3212		*norE*	nitric oxide reductase subunit E	403,6	-13,6
	bsr3213		hypothetical protein	46,0	-10,8
blr3214		*norC*	nitric oxide reductase subunit C	340,9	-5,2
	blr3215	*norB*	nitric oxide reductase subunit B	291,4	-4,9

	blr3216	*norQ*	NorQ protein	335,1	-7,2
	blr3217	*norD*	NorD protein	65,8	-11,3
blr3218			putative hydrolase phosphatase protein	25,7	-5,8
	blr3219		probable transcriptional regulator	9,7	-4,5
blr3456			hypothetical protein	–	–
	blr3457	*pta*	phosphate acetyltransferase	–	–
	blr3458	*ackA2*	acetate/propionate kinase	–	–
	blr3459	*fabI*	enoyl-(acyl carrier protein) reductase	8,4	-2,3
bll3466		*fixK*	transcriptional regulator FixK	8,0	-5,2
bll3594			hypothetical protein	3,1	-4,1
	bll3593		hypothetical protein	3,0	-2,7
blr3607			hypothetical protein	2,3	-4,6
bll3611			hypothetical protein	4,5	-2,5
bll3717		*lipA*	lipoyl synthase	2,3	-5,8
bll3765			glutamine amidotransferase	2,2	-6,9
	bll3764		hypothetical protein	12,3	-6,1
blr3767			hypothetical protein	4,0	-2,1
bll3768			hypothetical protein	13,2	-6,6
blr3769			hypothetical protein	6,0	-7,7
	blr3770		hypothetical protein	6,4	-38,0
blr3860			hypothetical protein	17,4	-21,5
bsl3938			putative biotinylated protein	9,2	-10,6
bsl4014			hypothetical protein	12,1	-5,6
bll4065			hypothetical protein	3,1	-2,2
bsr4099			hypothetical protein	–	–
	blr4100		hypothetical protein	7,2	-16,4
bll4149			putative glutathione peroxidase	8,2	-4,1
bll4166			hypothetical protein	3,3	-2,2
bll4168			hypothetical protein	–	–
	bsl4167		putative glutamine synthetase translation inhibitor	8,9	-6,8
bsr4179			hypothetical protein	2,3	-2,8
blr4182			hypothetical protein	3,0	-28,2
bll4218			hypothetical protein	3,9	-26,0
blr4219			hypothetical protein	4,2	-4,1
bll4234			hypothetical protein	5,4	-2,8
blr4238			hypothetical protein	2,5	-3,1
bll4247			hypothetical protein	3,5	-5,2
bll4278			hypothetical protein	4,1	-2,9
bsl4407			hypothetical protein	7,0	-2,8
bsr4408			hypothetical protein	13,1	-9,5
bll4412			hypothetical protein	10,8	-7,6

bsl4437			hypothetical protein	7,7	-10,1
blr4463			probable ABC transporter substrate-binding protein	2,7	-2,3
	blr4464		probable ABC transporter ATP-binding/permease protein	–	–
blr4465			hypothetical protein	3,0	-2,1
bsr4491			RNA-binding protein Hfq	2,0	-2,5
blr4588			hypothetical protein	2,6	-2,9
bsl4622			hypothetical protein	3,6	-4,9
bsl4623			hypothetical protein	15,6	-4,8
bll4722			hypothetical protein	2,9	-3,2
blr4723			hypothetical protein	6,2	-5,5
bll4741			putative arylsulfatase protein	2,5	-2,7
blr4795			putative hydrolase	14,9	-2,9
bll4828			hypothetical protein bll4828	31,7	-3,1
blr4870			MFS permease	4,7	-2,6
bll4880			hypothetical protein	2,2	-3,0
	bll4879		hypothetical protein	2,8	-2,9
	bll4878		possible Copper export protein	–	–
blr4891			hypothetical protein	4,6	-3,8
bll4896			ABC transporter substrate-binding protein	2,1	-5,7
bll4919		*nuoA*	NADH dehydrogenase alpha subunit	2,3	-2,2
	bll4918	*nuoB*	NADH dehydrogenase beta subunit	–	–
	bll4917	*nuoC*	NADH dehydrogenase subunit C	–	–
	bll4916	*nouD*	NADH dehydrogenase delta subunit	–	–
	bll4915		hypothetical protein	–	–
	bll4914		ATP synthase subunit E	–	–
	bsl4913		hypothetical protein	–	–
	bll4912	*nuoF*	NADH ubiquinone oxidoreductase chain F	–	–
	bll4911	*nuoG*	NADH dehydrogenase gamma subunit	–	–
	bll4910	*nuoH*	NADH dehydrogenase subunit H	–	–
	bll4909	*nuoI*	NADH dehydrogenase subunit I	–	–
blr4930			hypothetical protein	26,9	-3,4
	blr4931		hypothetical protein	71,4	-13,4
	blr4932		putative cation efflux system protein	62,1	-22,0
blr4933			probable cation efflux system protein	37,0	-26,5
bll4983			hypothetical protein bll4983	2,8	-6,9
blr4984			transcriptional regulatory protein	2,1	-6,8
bll4985			hypothetical protein	7,0	-11,4
bsl5034			hypothetical protein	3,1	-5,1
bsl5035			hypothetical protein	7,9	-4,6
bll5043			hypothetical protein	–	–
	bll5042		hypothetical protein	6,5	-3,1

	bll5041	hypothetical protein	4,0	-4,8
	bll5040	hypothetical protein	2,0	-2,9
bll5081		putative multidrug resistance protein	–	–
	bll5080	AcrB/AcrD/AcrF family protein	9,3	-2,7
	bll5079	hypothetical protein	10,3	-5,6
bll5130		hypothetical protein	2,2	-2,2
	bll5129	NTP pyrophosphohydrolase MutT family	–	–
bll5205		hypothetical protein	3,0	-2,9
bsl5208		hypothetical protein	–	–
	bll5207	hypothetical protein	8,2	-4,9
blr5292		hypothetical protein	4,4	-7,6
bsl5321		hypothetical protein	2,3	-3,3
	bll5320	hypothetical protein	–	–
bll5323		hypothetical protein	9,8	-2,8
bll5324		hypothetical protein	10,0	-5,3
blr5341		hypothetical protein	17,2	-5,4
bll5373		probable short-chain dehydrogenase	2,3	-7,1
	bll5372	hypothetical protein	–	–
blr5441		hypothetical protein	4,1	-3,8
bll5475		putative formate dehydrogenase	13,4	-6,4
bll5477		similar to formate dehydrogenase	12,6	-8,4
	bll5476	formate dehydrogenase iron-sulfur subunit	22,0	-7,6
bll5480		putative chaperone	9,7	-3,1
	bsl5479	hypothetical protein	12,6	-3,3
	bll5478	similar to formate dehydrogenase	14,5	-3,5
bll5481		hypothetical protein	2,3	-2,0
blr5502		hypothetical protein	10,5	-10,5
bll5510		outer-membrane immunogenic protein precursor	7,2	-4,5
blr5512		hypothetical protein	2,5	-4,6
blr5554		hypothetical protein	7,0	-3,4
bll5555		hypothetical protein	37,3	-13,2
blr5556		hypothetical protein	4,6	-7,7
bll5570		hypothetical protein	24,0	-3,0
bsr5571		hypothetical protein	18,9	-2,7
bll5579		hypothetical protein	2,0	-2,8
blr5597		carboxypeptidase	2,0	-2,2
bll5643		hypothetical protein	3,1	-2,3
bsr5670		hypothetical protein	8,9	-8,1
blr5675		ABC transporter substrate-binding protein	2,1	-3,9
blr5693		probable substrate-binding protein	8,9	-7,7
bsl5717		hypothetical protein	4,2	-4,8

bsr5760			hypothetical protein	18,3	-4,5
bll5807			hypothetical protein	6,4	-4,9
bll5866			hypothetical protein	11,0	-4,4
bll5899			hypothetical protein	3,0	-4,0
blr5909			hypothetical protein	2,1	-4,1
bll6012			hypothetical protein	2,4	-2,2
blr6059			putative cyclase	4,6	-3,7
bll6065			ABC transporter permease protein	–	–
	bll6064		ABC transporter ATP-binding protein	–	–
	bll6063		ABC transporter substrate-binding protein	9,2	-2,2
bll6121			probable sulfite oxidase	3,7	-4,5
	bll6120		putative sulfite oxidase cytochrome subunit	3,4	-4,7
blr6123			hypothetical protein	42,0	-3,7
blr6167			hypothetical protein	2,8	-5,6
bll6168			hypothetical protein	3,4	-4,3
bsr6217			hypothetical protein	3,2	-3,6
	blr6218		putative oxidoreductase protein	–	–
	blr6219		putative aldehyde dehydrogenase	–	–
bll6221			Rieske iron-sulfur protein	3,0	-2,4
bll6222			probable Sec-independent protein translocase protein	44,5	-3,0
bll6223		*hbdA*	3-hydroxybutyryl-CoA dehydrogenase	2,1	-2,2
bll6252			transcriptional regulatory protein	4,8	-2,6
bll6262		*osmC*	probable osmotically inducible protein	2,6	-5,4
	bll6261		hypothetical protein	2,3	-5,7
	bll6260		methionine sulfoxide reductase A	–	–
blr6269			hypothetical protein	2,1	-2,3
bll6449			hypothetical protein	19,3	-4,7
bll6455			ABC transporter substrate-binding protein	195,0	-2,4
	bll6454		ABC transporter permease protein	111,9	-2,8
	bll6453		ABC transporter ATP-binding protein	53,1	-3,5
	bll6452	*acd*	acyl-CoA dehydrogenase	123,0	-5,8
	bll6451		probable alkanesulfonate monooxygenase	36,8	-9,5
	bll6450		probable substrate-binding protein	23,3	-10,2
bll6468			hypothetical protein	2,2	-2,5
blr6472			hypothetical protein	8,7	-2,7
bll6525			hypothetical protein	8,5	-3,1
bll6527			hypothetical protein	2,7	-3,2
bll6529			hypothetical protein	5,4	-4,3
	bsl6528		hypothetical protein	–	–
bll6540			putative oxidoreductase	15,5	-2,3
bsl6560			hypothetical protein	5,1	-2,5

blr6563			hypothetical protein	2,6	-8,3
	blr6564		putative dihydroflavonol-4-reductase	–	–
	blr6565		hypothetical protein	–	–
blr6582			hypothetical protein	3,5	-3,3
bsl6653			hypothetical protein	4,0	-4,4
blr6667			hypothetical protein	4,2	-2,2
bsr6700			hypothetical protein	5,2	-4,1
blr6718			hypothetical protein	2,8	-6,9
blr6729			putative decarboxylase	2,5	-3,3
blr6742			putative glutamate synthase small subunit	–	–
	blr6743		putative ferredoxin oxidoreductase alpha subunit	2,7	-2,4
	blr6744		ferrodoxin oxidoreductase beta subunit	3,1	-3,2
bll6746			hypothetical protein	–	–
	bll6745		hypothetical protein	3,9	-2,6
bll6756			hypothetical protein	–	–
	bll6755		hypothetical protein	5,2	-3,4
	bll6754		hypothetical protein	4,4	-5,7
blr6766			hypothetical protein	2,5	-3,0
	blr6767		trehalose synthase	2,1	-4,0
	blr6768	*glgB*	glycogen branching enzyme	–	–
	blr6769	*glgX*	glycogen debranching enzyme	–	–
	blr6770		alpha-amylase	–	–
	blr6771		probable glycosyl hydrolase	–	–
bll6799			hypothetical protein	10,0	-20,0
bll6995		*trmU*	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	–	–
	bll6994		putative phosphatidylethanolamine N-methyltransferase	–	–
	bll6993		hypothetical protein	8,9	-5,3
bll7010		*ssuD*	alkanesulfonate monooxygenase	–	–
	bll7009		aliphatic sulfonate ABC transporter permease protein	11,6	-6,5
	bll7008		aliphatic sulfonate ABC transporter ATP-binding protein	8,1	-3,5
	bll7007		putative oxidoreductase	–	–
bll7022			hypothetical protein	–	–
	bll7021		HlyD family secretion protein	–	–
	bll7020		efflux protein	–	–
	bll7019		AcrB/AcrD/AcrF family protein	–	–
	bll7018		hypothetical protein	9,5	-4,2
bsr7036		*napE*	periplasmic nitrate reductase protein	–	–
	blr7037	*napD*	periplasmic nitrate reductase	–	–
	blr7038	*napA*	periplasmic nitrate reductase large subunit precursor	–	–
	blr7039	*napB*	periplasmic nitrate reductase small subunit precursor	419,5	-2,8
	blr7040	*napC*	cytochrome *c*-type protein	130,4	-3,4

bsr7087			hypothetical protein	–	–
	blr7088		hypothetical protein	115,7	-3,4
blr7133			hypothetical protein	9,7	-3,7
bll7252			hypothetical protein	2,3	-2,2
bll7311			probable ArcD2 arginine/ornithine antiporter	22,5	-12,5
	bll7310		arginine deiminase	15,6	-15,2
bll7313			RND efflux membrane fusion protein	13,5	-5,0
	bll7312		AcrB/AcrD/AcrF family protein	15,3	-7,4
blr7314			hypothetical protein	547,7	-93,5
	blr7315		hypothetical protein	23,4	-14,1
	bsr7316		hypothetical protein	–	–
	bsr7317		hypothetical protein	4,6	-2,7
	blr7318		hypothetical protein	19,8	-2,1
	blr7319		hypothetical protein	–	–
	blr7320		hypothetical protein	5,6	-3,5
blr7321			hypothetical protein	321,1	-97,1
bll7322			hypothetical protein	57,7	-17,6
blr7323			probable ArcD1 arginine/ornithine antiporter	42,8	-8,3
blr7324			hypothetical protein	4,4	-4,8
	blr7325		hypothetical protein	23,4	-9,4
	blr7326		hypothetical protein	22,9	-7,9
blr7327			hypothetical protein	43,3	-17,2
bsr7328			hypothetical protein	97,6	-46,7
blr7329			putative multidrug resistance protein	5,5	-5,9
	blr7330		AcrB/AcrD/AcrF family protein	2,8	-2,9
bll7402		*serC*	phosphoserine aminotransferase	2,3	-4,7
bll7411			hypothetical protein	3,0	-6,4
bll7414			translation elongation factor EF-G	8,1	-7,0
blr7436			hypothetical protein	2,5	-5,9
bll7543		*glcD*	glycolate oxidase subunit	2,7	-2,7
blr7544		*cycA*	cytochrome c_{550}	5,5	-3,3
bll7559		*chrC*	probable Fe/Mn superoxide dismutase	8,6	-2,5
	bll7558		hypothetical protein	–	–
blr7560		*dhlB*	2-haloalkanoic acid dehalogenase	9,2	-19,1
	blr7561		hypothetical protein	–	–
bll7562			hypothetical protein	7,8	-9,1
bsr7564			hypothetical protein	97,6	-14,1
blr7621			hypothetical protein	3,2	-2,0
blr7625			probable mandelate racemase	3,4	-2,2
bll7626			hypothetical protein	234,8	-27,4
bll7628			hypothetical protein	49,6	-8,2

	bll7627		hypothetical protein	195,6	-10,7
blr7629			hypothetical protein	140,7	-26,7
	blr7630		probable decarboxylase	26,6	-6,8
blr7631			putative MutT/nudix family protein	5,3	-4,8
bsr7633			hypothetical protein bsr7633	9,1	-5,3
bll7635			hypothetical protein bll7635	3,2	-8,2
bll7638			putative cytochrome c_6 precursor	3,5	-6,8
	bll7637		hypothetical protein	–	–
	bll7636		hypothetical protein	–	–
bll7648			hypothetical protein	5,9	-4,6
bll7657			putative phosphoglycolate phosphatase	3,2	-2,4
bll7672			putative protease secretion ATP-binding protein	–	–
	bll7671		HlyD family secretion protein	–	–
	bll7670		hypothetical protein	4,6	-4,5
bll7763			acetoacetate decarboxylase	2,5	-2,7
bll7774		*sodF*	superoxide dismutase	2,6	-9,3
bll7790			hypothetical protein	30,6	-10,3
bll7795		*phyR*	two-component response regulator	4,1	-10,9
bsr7796		*nepR*	anti-sigma factor	5,7	-6,5
	blr7797	*ecfG*	RNA polymerase ECF sigma factor (σ^{EcfG})	5,4	-3,7
blr7887			hypothetical protein	4,3	-6,1
bll7908			hypothetical protein	–	–
	bll7907		hypothetical protein	2,1	-3,9
	bll7906		putative ferredoxin	–	–
blr7909			hypothetical protein	2,5	-2,1
bll7911			hypothetical protein	5,0	-2,9
bll7952			probable selenium-binding protein	4,2	-4,0
bll7960			hypothetical protein	20,9	-3,9
bll7982			hypothetical protein	–	–
	bll7981		putative dehydrogenase	41,4	-4,2
bll8011			putative hydrolase	2,7	-3,5
bll8024			hypothetical protein	–	–
	bsl8023		hypothetical protein	2,0	-2,4
bsr8030			hypothetical protein	4,9	-2,3
bll8048			hypothetical protein	17,9	-4,1
bll8143			hypothetical protein	33,7	-4,0
	bll8142		hypothetical protein	–	–

Class 2 (upregulated in the Δ*regR* strain)

bsl0098			hypothetical protein	2,3	4,3
bll0104		*gltP*	proton glutamate symport protein	2,9	2,9

	bll0102	*gltI*	glutamate/aspartate periplasmic binding protein precursor	–	–
bsl0170			hypothetical protein	6,0	5,4
	bsl0169		hypothetical protein	17,3	2,8
bsr0173			hypothetical protein	5,0	3,4
bll0182		*cisZ*	citrate synthase	2,3	2,0
blr0209		*comF*	competence protein F	2,2	2,4
bsr0210			glutaredoxin	7,9	2,2
	blr0211		amidohydrolase	–	–
blr0230			probable esterase	2,3	2,2
bll0307			transcriptional regulatory protein	2,4	4,9
bll0330			two-component response regulator	3,1	2,9
blr0366			hypothetical protein	18,1	2,9
trnS-CGA			tRNA-Ser(CGA)	3,7	3,1
bsr0431			hypothetical protein	10,2	3,4
blr0488		*leuC*	isopropylmalate isomerase large subunit	4,5	5,0
bll0531			hypothetical protein	11,6	3,1
bsl0578			hypothetical protein	9,8	2,3
blr0586			hypothetical protein	2,5	2,6
bll0661			hypothetical protein	2,4	4,3
blr0675		*hrcA*	heat-inducible transcription repressor	11,1	3,1
bll0688			hypothetical protein	2,0	2,9
blr0694			probable peptidase	2,0	2,3
blr0709			hypothetical protein	3,0	2,3
bsl0728			hypothetical protein	5,9	2,1
bll0729		*hspH*	small heat shock protein	7,4	2,0
bll0777			transcriptional regulatory protein	2,0	6,1
	bll0776		hypothetical protein	–	–
blr0850			hypothetical protein	4,4	3,5
	blr0851		hypothetical protein	–	–
	blr0852	*uppS*	undecaprenyl pyrophosphate synthase	–	–
blr0960			5'-methylthioadenosine phosphorylase	3,9	3,5
	blr0961		translation initiation factor IF-2B subunit alpha	5,3	5,7
bsl1006			hypothetical protein	6,4	2,3
blr1039			ABC transporter ATP-binding protein	–	–
	bsr1040		hypothetical protein	2,4	4,6
	blr1041		amidase	–	–
	blr1042		hypothetical protein	–	–
	blr1043		transcriptional regulatory protein	–	–
blr1072			hypothetical protein	11,4	8,1
blr1078			putative hydrolase	–	–
	blr1079		hypothetical protein	8,4	2,7

trnG-CCC			tRNA-Gly(CCC)	3,3	3,0
bll1134			hypothetical protein	3,7	2,5
bll1135			transcriptional regulatory protein	2,6	3,1
bll1150			transcriptional regulatory protein	14,6	7,3
blr1221		*phnG*	phosphonate metabolism protein	3,2	3,8
	blr1222	*phnH*	phosphonate metabolism protein	2,8	3,1
	blr1223	*phnI*	phosphonate metabolism protein	2,4	3,5
	blr1224	*phnJ*	phosphonate metabolism protein	–	–
	blr1225	*phnK*	phosphonate uptake transporter ATP-binding protein	–	–
	blr1226	*phnL*	phosphonate uptake transporter ATP-binding protein	–	–
	blr1227	*phnM*	phosphonate metabolism protein	–	–
	blr1228	*gmk*	guanylate kinase	–	–
	blr1229		hypothetical protein	–	–
blr1288			probable long-chain-fatty-acid-CoA ligase	12,2	2,1
bll1295			probable oxidoreductase	4,4	2,6
	bll1294		hypothetical protein	2,3	2,3
bll1367			hypothetical protein	8,5	2,5
	bll1366		hypothetical protein	2,1	3,7
blr1375			hypothetical protein	–	–
	blr1376		hypothetical protein blr1376	2,4	2,3
blr1399		*metX*	homoserine O-acetyltransferase	2,8	2,0
	blr1400		hypothetical protein	–	–
blr1404		*clpB*	ATP-dependent protease ATP-binding subunit	6,8	2,3
rrn16S			16S rRNA	8,6	3,1
trnA-UGC			tRNA-Ala(TGC)	2,9	2,3
rrn23S			23S rRNA	8,2	3,2
rrn5S			5S rRNA	8,4	3,3
blr1477			probable trifunctional enzyme subunit	16,4	2,0
	blr1478		hypothetical protein	–	–
	blr1479		ferredoxin-nitrite reductase	–	–
	blr1480		hypothetical protein	–	–
	blr1481	*cysH*	phosphoadenosine phosphosulfate reductase	–	–
blr1506			hypothetical protein	6,8	3,0
bll1688			probable suppressor protein	4,7	2,4
	bll1687		hypothetical protein	–	–
blr2071			similar to inosamine-phosphate amidinotransferas	9,1	5,5
bsr2110			hypothetical protein	8,5	5,1
	bsr2111		hypothetical protein	–	–
blr2113			hypothetical protein	–	–
	blr2114		hypothetical protein	2,5	5,5
	blr2115		hypothetical protein	–	–

bll2215			hypothetical protein	4,5	2,4
bll2284			hypothetical protein	3,9	3,3
blr2286			hypothetical protein	7,3	4,8
	blr2287		two-component hybrid sensor and regulator	–	–
	blr2288		two-component hybrid sensor and regulator	–	–
blr2325			transcriptional regulatory protein	2,3	2,9
bll2327		*pncB*	nicotinate phosphoribosyltransferase	2,6	2,1
bll2330			hypothetical protein	10,5	3,6
	bll2329		hypothetical protein	3,0	2,1
	bsl2328		hypothetical protein	–	–
bll2336			transcriptional regulatory protein	7,2	5,0
bll2417			hypothetical protein	4,3	2,7
bsl2435			hypothetical protein	2,5	3,3
	bll2434		plasmid stability protein	–	–
blr2456			hypothetical protein	6,6	2,2
bll2516			hypothetical protein	–	–
	bll2515		similar to pyruvate phosphate dikinase	–	–
	bll2514		hypothetical protein	–	–
	bll2513		hypothetical protein	2,3	2,2
bsr2531			hypothetical protein	4,3	2,4
	blr2533		hypothetical protein	–	–
bll2532		*ispB*	octaprenyl-diphosphate synthase	3,1	2,0
blr2579			hypothetical protein	6,2	4,1
bll2590			hypothetical protein	115,2	3,2
bll2604			transcriptional regulatory protein	2,5	4,8
blr2607			hypothetical protein	10,1	2,5
bll2628		*prtI*	ECF family sigma factor	2,7	3,8
trnQ-UUG			tRNA-Gln(TTG)	2,7	3,1
bsr2670			hypothetical protein	79,4	2,2
bsr2672			hypothetical protein	33,5	4,3
bll2683			hypothetical protein	–	–
	bll2682	*macA*	maleylacetate reductase	4,3	3,0
	bll2681		hypothetical protein	–	–
	bll2680		probable dehydrogenase	–	–
	bll2679		dioxygenase	–	–
blr2702			hypothetical protein	5,3	2,2
bll2757		$fixK_2$	transcriptional regulator FixK$_2$	17,9	2,2
bll2758			two-component response regulator	38,8	2,6
blr2761			hypothetical protein	77,3	3,4
blr2762			hypothetical protein	39,4	3,1
bll2855		*rocD*	ornithine--oxo-acid transaminase	10,5	3,7

bsr2878			hypothetical protein	2,6	4,2
	blr2879		hypothetical protein	–	–
blr2887			hypothetical protein	31,0	3,1
blr2912			probable ABC transporter permease protein	2,9	2,1
	blr2913		probable ABC transporter permease protein	–	–
blr2921			hypothetical protein	4,8	5,6
	blr2922		ABC transporter amino acid-binding protein	–	–
blr2987			hypothetical protein	45,3	2,2
blr2988			hypothetical protein	4,0	2,4
bsl3053			hypothetical protein	2,2	2,7
blr3067			hypothetical protein	2,5	2,3
bll3090			transcriptional regulatory protein	3,1	3,7
	bll3089		hypothetical protein	–	–
	bll3088		hypothetical protein	–	–
blr3091			transcriptional regulatory protein	8,3	2,4
blr3092			putative secreted protein	2,2	3,1
	blr3093		hypothetical protein	–	–
bll3117			putative thymidine phosphorylase	7,3	3,1
	bll3116		phosphoribosylpyrophosphate synthetase	–	–
	bll3115		hypothetical protein	–	–
bll3177			probable arginine/lysine/ornithine decarboxylase	–	–
	bll3176		acetyltransferase	7,7	2,4
	bsl3175		hypothetical protein	35,8	2,1
bll3434			transcriptional regulatory protein MarR family	3,0	2,2
blr3455			hypothetical protein	3,0	2,2
bll3471			hypothetical membrane protein	–	–
	bll3470		hypothetical protein	–	–
	bll3469		hypothetical protein	5,0	2,2
blr3479			hypothetical protein	9,6	4,4
bll3507			hypothetical protein	3,3	2,5
blr3521			hypothetical protein	12,8	4,9
blr3585			hypothetical protein	2,5	2,0
blr3741			hypothetical protein	2,1	5,8
	blr3742	*mrp*	probable multidrug-resistance related protein	–	–
bsl3746			hypothetical protein	2,1	3,4
bll3785		*coxM*	cytochrome *c* oxidase	27,2	5,7
	bll3784	*coxN*	cytochrome *c* oxidase	15,4	3,7
	bll3783	*coxO*	cytochrome *c* oxidase	8,9	3,0
	bll3782	*coxP*	cytochrome *c* oxidase	–	–
	bll3781		hypothetical protein	–	–
blr3787			hypothetical protein	22,3	5,0

blr3799			probable oxidoreductase	3,6	2,4
bll3835			hypothetical protein	40,5	2,2
blr3873			transcriptional regulatory protein	2,6	2,5
bll3877			transcriptional regulatory protein	3,2	3,8
	bll3876		aldehyde dehydrogenase	–	–
	bll3875		hypothetical metabolite transport protein	–	–
trnQ-CUG			tRNA-Gln(CTG)	6,5	2,8
blr3963			transcriptional regulatory protein	2,7	9,0
bll3994			hypothetical protein	–	–
	bll3993		hypothetical protein	18,5	2,4
blr3995			hypothetical protein	10,8	3,5
bsr3996			hypothetical protein	9,0	4,7
blr3997			hypothetical protein	2,1	2,6
blr4111			hypothetical protein	25,9	3,4
	blr4112		probale cation efflux system protein	2,5	2,3
	blr4113		hypothetical protein	–	–
blr4114			hypothetical protein	110,3	2,2
	blr4115	*actP*	acetate permease	–	–
blr4131			hypothetical protein	6,7	3,6
	blr4132		hypothetical protein	9,2	2,2
blr4162			hypothetical protein	47,8	2,6
bll4168			hypothetical protein	–	–
bsr4175			hypothetical protein	33,5	2,2
bll4189			putative acetyl-hydrolase	2,6	2,9
blr4224			hypothetical protein	54,4	2,7
bsr4236			hypothetical protein	63,7	2,3
blr4240			hypothetical protein	97,9	3,5
	blr4241		hypothetical protein	254,2	2,6
blr4242			hypothetical protein	12,7	3,0
bsr4244			hypothetical protein	4,2	4,2
blr4300			probable DNA-binding protein	3,0	2,4
bll4303			putative amidase	2,0	2,5
bll4574			hypothetical protein	3,3	2,3
blr4630			hypothetical protein	4,1	2,0
blr4637			probable HspC2 heat shock protein	143,1	2,3
blr4646			hypothetical protein	160,3	2,5
bll4651			hypothetical protein	180,5	2,3
blr4652			hypothetical protein	248,7	2,2
	blr4653	*dnaJ*	molecular chaperone DnaJ family	–	–
	blr4654		hypothetical protein	–	–
bsr4666			hypothetical protein	2,1	3,1

bsl4703			hypothetical protein	4,8	4,2
bll4718			hypothetical protein	56,0	3,5
bsr4726			hypothetical protein	35,5	2,9
bll4785			transcriptional regulatory protein	2,6	3,1
blr4890			hypothetical protein	4,2	2,7
trnD-GUC			tRNA-Asp(GTC)	5,2	2,8
bll4952			NfeD protein homolog	14,0	4,0
	bll4951		putative stomatin-like protein	22,2	4,0
blr4955			putative cytochrome b_{561}	48,0	3,1
bsr4956			hypothetical protein	2,8	5,6
bll4998			hypothetical protein	2,4	3,3
	bll4997		hypothetical protein bll4997	–	–
trnP-GGG			tRNA-Pro(GGG)	3,5	2,8
bll5019		*ihfA*	integration host factor alpha subunit	4,0	2,1
	bll5018		hypothetical protein	–	–
blr5037		*hemB*	delta-aminolevulinic acid dehydratase	4,9	2,4
bll5044		*mntH*	putative manganese transport protein MntH	2,2	2,2
blr5051			superoxide dismutase SodM-like protein	3,7	2,9
	blr5052		putative chromate transport protein	–	–
trnS-GCU			tRNA-Ser(GCT)	5,7	3,2
bsl5090		*gatC*	glutamyl-tRNA-Gln-amidotransferase chain C	3,7	2,9
blr5118			hypothetical protein	7,0	3,3
bll5146			hypothetical protein	3,1	3,6
	bll5145		probable mannose-6-phosphate isomerase	–	–
blr5150			hypothetical protein	30,4	3,0
blr5151			hypothetical transport protein	3,1	3,5
bll5155			hypothetical protein	8,9	2,5
bll5160			hypothetical protein	6,3	2,9
bll5164			hypothetical protein	2,1	5,8
bsl5165			hypothetical protein	6,9	2,6
bll5199			hypothetical protein	4,2	5,1
bll5219		*hspD*	small heat shock protein	2,6	2,1
blr5233		*hspB*	small heat shock protein	9,6	2,2
	blr5234	*hspC*	small heat shock protein	2,3	2,3
bll5249			oxidoreductase	3,5	4,3
bsr5273			hypothetical protein	810,9	3,5
bll5296			hypothetical protein	4,7	2,6
blr5308			anti-oxidant protein	8,5	2,2
bll5354			probable transmembrane protein	5,7	3,2
bsl5473			hypothetical protein	10,8	4,3
blr5525			hypothetical protein	5,3	2,1

bll5551			hypothetical protein	4,9	2,7
bll5589			hypothetical protein	3,6	2,4
	bll5588		hypothetical protein	–	–
trnN-GUU-2			tRNA-Asn(GTT)	3,3	6,4
trnF-GAA-2			tRNA-Phe(GAA)	3,6	5,4
bll5651			transcriptional regulatory protein	5,7	2,3
	bll5650		hypothetical protein	–	–
	bll5649		ABC transporter ATP-binding protein	–	–
	bll5648		nitrate ABC transporter permease protein	–	–
bll5662			hypothetical protein	–	–
	bll5661		hypothetical protein	3,8	2,1
	bll5660		hypothetical protein	–	–
	bll5659		hypothetical protein	–	–
bll5665		*cooxS*	putative carbon monoxide dehydrogenase small subunit	2,9	4,1
	bll5664	*cooxM*	putative carbon monoxide dehydrogenase medium subunit	–	–
blr5687			two-component sensor histidine kinase	4,7	2,7
bll5711		*grlA*	glutaredoxin-related protein	5,0	2,0
	bll5710		hypothetical protein	–	–
	bll5709		hypothetical protein	–	–
	bll5708		hypothetical protein	–	–
bll5729		*purC*	phosphoribosylaminoimidazole-succinocarboxamide synthase	–	–
	bsl5728		phosphoribosylformylglycinamidine synthase	3,7	2,1
blr5735			transcriptional regulatory protein	4,0	6,6
bll5764			hypothetical protein	7,5	3,9
bll5772			hypothetical protein	32,1	3,2
	bll5771		AcrB/AcrD/AcrF family cation efflux protein	–	–
	bll5770		hypothetical protein	–	–
bll5773			transcriptional regulatory protein	7,9	2,0
blr5774			probable sulfide-quinone reductase	12,3	3,7
	blr5775		putative thioredoxin	10,0	2,8
	bsr5776		hypothetical protein	28,0	2,6
blr5777			hypothetical protein	10,7	2,4
	blr5778	*fixG*	nitrogen fixation protein	–	–
bll5780			similar to FrnE protein	2,1	2,7
bsr5798			hypothetical protein	15,1	2,4
blr5858			hypothetical protein	3,1	2,4
blr5860			transcriptional regulatory protein	6,2	9,9
bll5941			putative partition protein	9,2	4,1
	bll5940		hypothetical protein	7,6	3,2
bll5959			hypothetical protein	–	–
	bll5958		hypothetical protein	–	–

	bll5957		hypothetical protein	7,0	2,3
bll6051			hypothetical protein	3,1	3,5
	bll6050		hypothetical protein	–	–
	bsl6049		hypothetical protein	–	–
bll6061		*fixK*$_1$	transcriptional regulatory protein	61,8	3,0
bll6069			hypothetical protein	97,4	2,3
bll6076			putative acetyl-CoA synthetase	2,4	2,1
	bll6075		hypothetical protein	–	–
bll6077			transcriptional regulatory protein	2,1	2,6
blr6078			probable substrate-binding protein	2,4	2,5
	blr6079		hypothetical protein	–	–
bll6110			hypothetical protein	2,7	8,2
bsr6229			hypothetical protein	3,0	2,0
blr6259			transcriptional regulatory protein	4,2	4,2
	bll6260		methionine sulfoxide reductase A	–	–
bll6264			putative hydrolase	–	–
	bll6263		putative enoyl-CoA hydratase	2,9	2,7
blr6277			transcriptional regulatory protein	3,6	2,9
blr6291			transcriptional regulatory protein	3,3	2,2
blr6408			transcriptional regulatory protein	2,7	3,1
ssrA2			tmRNA-coding_RNA	7,0	3,3
bll6552			hypothetical protein	12,4	2,2
blr6553			transcriptional regulatory protein	3,3	4,8
bll6613			hypothetical protein	3,5	2,2
	bll6612		hypothetical protein	–	–
rnpB			RNase P subunit B	7,6	3,0
bll6662		*rdh*	ribitol 2-dehydrogenase	2,4	2,6
bll6670			hypothetical protein	3,1	2,0
bll6673			hypothetical protein	17,7	2,6
blr6829			transcriptional regulatory protein	2,3	2,4
bll6893			hypothetical protein	29,8	3,9
blr6907			hypothetical protein	3,0	3,1
bll6937		*hupG*	HupG protein	12,7	2,3
	bll6936	*hupH*	HupH protein	–	–
	bsl6935	*hupI*	HupI protein	–	–
	bll6934	*hupJ*	HupJ protein	–	–
	bll6933	*hupK*	HupK protein	–	–
	bll6932	*hypA*	HypA protein	–	–
	bll6931	*hypB*	HypB protein	–	–
	bll6930	*hypF*	hydrogenase maturation protein	–	–
	bsl6929	*hypC*	hydrogenase expression/formation protein	–	–

	bll6928	*hypD'*	HypD' protein	–	–
	bll6927	*hypE*	HypE protein	–	–
	bll6926	*hoxX*	probable sensor protein	–	–
	bll6925		two-component response regulator	–	–
	bll6924		two-component hybrid sensor and regulator	–	–
bll7034			MDO-like protein	2,4	2,7
bll7035			transcriptional regulatory protein	3,8	3,4
bll7046			hypothetical protein	3,6	2,5
blr7054			hypothetical protein	16,0	3,3
bll7059			hypothetical protein	2,8	3,5
blr7084		*nnrR*	transcriptional regulatory protein	13,7	2,9
bsr7110			hypothetical protein	3,9	2,9
bll7113			hypothetical protein	2,6	2,3
bll7160			hypothetical protein	8,0	2,6
bll7164			hypothetical protein	9,0	2,9
blr7208			transcriptional regulatory protein	3,6	3,5
bsr7215			hypothetical protein	4,2	3,7
bll7217			probable site-specific integrase/recombinase	4,0	2,3
bll7221			hypothetical protein	7,7	4,3
blr7228			hypothetical protein	23,7	2,9
blr7261		*putA*	proline dehydrogenase	2,5	3,9
	blr7262		putative racemase	–	–
blr7345			hypothetical protein	72,0	3,1
bll7347			hypothetical protein	8,3	2,2
blr7381		*trxB*	thioredoxin reductase	3,6	2,6
bsr7426			hypothetical protein	3,0	2,6
bsl7442			hypothetical protein	3,2	3,5
bsr7468		*cspA*	cold shock protein	3,7	2,2
bll7513			hypothetical protein	2,8	2,0
blr7552			hypothetical protein	6,5	2,1
bll7565			transcriptional regulatory protein	3,3	2,1
bsr7705			hypothetical protein	3,2	2,1
	blr7706		hypothetical protein	–	–
bsr7707			hypothetical protein	3,9	2,2
blr7716			probable adenylate cyclase	4,3	2,1
	blr7717		putative adenylate cyclase	–	–
blr7740			small heat shock protein	22,9	3,4
bll7749			hypothetical protein	2,8	3,7
bll7750			hypothetical protein	3,1	3,9
bsl7781			hypothetical protein	34,2	3,1
bll7787			hypothetical protein	64,0	2,9

blr7813			transcriptional regulatory protein	2,7	2,1
	blr7814		putative L-proline 4-hydroxylase	–	–
bll7838		*pobA*	4-hydroxybenzoate 3-monooxygenase	3,0	2,1
blr7881			transcriptional regulatory protein	9,0	4,1
bll7941			aminopeptidase	4,6	2,4
blr7984			transcriptional regulatory protein	8,4	4,1
bsl7992			hypothetical protein	119,5	3,4
	bll7991		hypothetical protein	88,6	2,8
	bll7990		hypothetical protein	–	–
bll8020			putative ubiquinone/menaquinone biosynthesis methyltransferase	2,7	3,4
blr8039			hypothetical protein	4,2	2,0

[a] Δ*regR* and wild-type strains were grown under anoxic conditions (with nitrate as terminal electron acceptor) in Bergersen minimal medium with succinate as carbon source (BMS). Anoxically induced genes in the wild type were identified using as reference wild-type cells grown oxically in PSY medium (Pessi *et al*., 2007).

[b] Genes numbers are according to the Rhizobase (http://genome.kazusa.or.jp/rhizobase/).

[c] Operon predictions were performed as described in Hauser *et al*., 2007; Mesa *et al*., 2008. All putative operon members, although not controlled in the selected conditions, are included in the Table.

[d] Genes names as indicated in the EMBL-EBI database with modifications.

[e] Protein description according to Kaneko et al., 2002 with modifications.

[f] Gene expression changes (*n* fold) retrieved by microarray analyses. Negative values indicate decrease of expression; (–) indicates the gene was not differentially expressed.

[g] Fold change of expression of upregulated genes by comparison of anoxically-grown wild-type cells with oxically grown wild-type cells.

[h] Fold change of expression by comparison of Δ*regR* cells with wild type cells, both grown anoxically.

[i] *blr2806, blr2807, blr2808* and *blr2809* have been shown to belonging to an operon unit by J. Cabrera and M.J. Delgado (unpublished results), and *blr2807* has been recently named as bjgb (Cabrera et al., 2011).

Table 3.S3: Genes differentially expressed in the Δ*regR* strain in comparison with the wild type in oxic, microoxic and anoxic conditions[a]

Gene no.[b]	Putative operon member (gene no.)[c]	Gene name[d]	Description[e]	Fold change[f]		
				Δ*regR*_oxic_ vs_WT_oxic	Δ*regR*_microoxic_ vs_WT_microoxic	Δ*regR*_anoxic_ vs_WT_anoxic
bll0693			unknown protein	2,3	2,3	2,7
bll0905		*regS*	two-component sensor histidine kinase	–	–	–
	bll0904	*regR*	two-component response regulator	-111,6	-84,0	-91,7
bll1285			unknown protein	-11,4	-11,2	-151,1
bll1322			hypothetical protein	-2,5	-2,6	-4,3
blr1429			unknown protein	-3,5	-3,2	-8,1
blr1515		*acrA*	RND multidrug efflux membrane permease	-22,2	-21,2	-17,3
	blr1516	*acrB*	RND multidrug efflux transporter	-29,5	-21,6	-13,4
blr2036[g]		*fixR*	oxidoreductase	-13,0	-5,7	-6,3
	blr2037	*nifA*	nif-specific regulatory protein	-3,6	–	-7,7
bll2087			unknown protein	-24,3	-11,2	-5,2
blr2501			hypothetical protein	-12,2	-13,1	-15,1
bsl2596			unknown protein	3,9	4,0	-7,8
blr2614			hypothetical protein	-4,3	-3,0	-3,7
blr3161			hypothetical protein	-2,4	-3,5	-2,4
	blr3162		hypothetical protein	–	–	–
	blr3163		hypothetical protein	–	–	–
blr3769			hypothetical protein	-2,5	-2,5	-7,7
	blr3770		hypothetical protein	-7,6	-7,1	-38,0
blr3771			hypothetical protein	-3,3	-4,1	-16,0
bll4130			transcriptional regulatory protein LysR family	-2,5	-2,7	-19,1
bsl4168			unknown protein	–	–	–
	bsl4167		putative glutamine synthetase translation inhibitor	-3,1	-2,8	-6,8
blr4182			hypothetical protein	-3,3	-4,7	-28,2
blr4238			hypothetical protein	-3,4	-2,7	-3,1
blr4257			putative hydrolase	2,4	2,3	-3,9
	bsr4258		hypothetical protein	2,6	3,3	-3,8
	blr4259		hypothetical protein	2,4	2,0	-2,8
	blr4260		hypothetical protein	2,4	2,4	-12,5
	blr4261		hypothetical protein	–	–	–
	blr4262		hypothetical protein	–	–	–

Gene	Operon member	Description	Oxic	Microoxic	Anoxic
	blr4263	hypothetical protein	–	–	–
	blr4264	putative adenylate cyclase protein	–	–	–
bll5477		similar to formate dehydrogenase	-2,5	-5,2	-8,4
	bll5476	formate dehydrogenase iron-sulfur subunit	–	–	–
bll5480		putative chaperone	-4,6	-4,1	-3,1
	bsl5479	hypothetical protein	-2,2	-4,7	-3,3
	bll5478	similar to formate dehydrogenase	-2,2	-4,6	-3,5
blr5693		probable substrate-binding protein	-2,3	-2,9	-7,7
bll5806		putative glutamyl-tRNA(Gln) amidotransferase	-3,0	-2,1	-3,6
bll5807		hypothetical protein	-8,2	-5,7	-4,9
blr6210		hypothetical protein	-6,5	-6,1	-6,3
blr6267		transcriptional regulator	-3,1	-2,8	-3,4
bll6513		hypothetical protein	-30,2	-8,1	-8,7
bsl6653		unknown protein	-2,8	-3,0	-4,4
bll6844		unknown protein	-2,2	-5,3	-4,4
blr6918		probable substrate-binding protein	-2,8	-2,2	-3,7

[a]Both Δ*regR* and wild-type strains were grown under oxic (21% O_2) and microoxic (0.5% O_2) conditions in complete PSY medium (Lindemann *et al*., 2007) and under anoxic conditions in BMS medium.

[b]Gene numbers are according to the Rhizobase (http://genome.kazusa.or.jp/rhizobase/).

[c]Operon predictions were performed as described in Hauser et al., 2007; Mesa et al., 2008. Note that although not regulated by RegR, all putative operon members are included in this list.

[d]Genes names as indicated in the EMBL-EBI database.

[e]Protein description according to Kaneko et al., 2002

[f]Gene expression changes (*n*-fold) retrieved by microarray analysis of five biological replicates of *B. japonicum* wild type and Δ*regR* strain grown under oxic and microoxic conditions (Lindemann *et al*., 2007), and of four biological replicates of *B. japonicum* wild type and Δ*regR* strain grown under anoxic conditions (for details see above and Material and Methods). Negative values indicate decrease of expression, (–) indicates no change within the threshold fold change range between +2 and -2.

[g]*bll2036* and *bll3037* constitute an operon unit described by Thöny et al., 1987.

Table 3.S4: Differentially expressed genes by a factor of ≤ -5 or ≥ 5 in the Δ*regR* strain grown anoxically and their putative operon members[a]

Class and gene no.[b]	Putative operon member (gene no.)[c]	Gene name[d]	Description[e]	Fold change[f]
Class 1 (downregulated in the Δ*regR* strain)				**–**
bll0091			ABC transporter substrate-binding protein	–
	bll0090		ABC transporter ATP-binding protein	–
	bll0089		ABC transporter permease protein	–
	bll0088		glycerate dehydrogenase	-5,1
	bll0087		hypothetical protein	–
blr0149		*cyoA*	cytochrome *o* ubiquinol oxidase subunit II	-6,0
	blr0150	*cyoB*	cytochrome *o* ubiquinol oxidase subunit I	-4,4
	blr0151	*cyoC*	cytochrome *o* ubiquinol oxidase subunit III	-6,8
	blr0152	*cyoD*	cytochrome *o* ubiquinol oxidase subunit IV	-4,7
	blr0153		probable surfeit locus protein 1	-4,6
	blr0154		two-component sensor histidine kinase	–
	blr0155		two-component response regulator	–
bll0233			hypothetical protein	-18,1
bll0246		*bam*	amidase	-6,1
blr0274			hypothetical protein	-12,7
blr0305			hypothetical protein	-9,5
blr0314		*nosR*	nitrous oxide reductase expression regulator	-2,2
	blr0315	*nosZ*	nitrous-oxide reductase precursor	-7,6
	blr0316	*nosD*	periplasmic copper-binding precursor	-12,1
	blr0317	*nosF*	copper ABC transporter	-14,2
	blr0318	*nosY*	nitrous oxide metabolic protein	-10,5
	blr0319	*nosL*	NosL protein	-11,1
	blr0320	*nosX*	NosX protein	-10,5
bll0322		*otsA*	probable trehalose-6-phosphate synthase	-5,5
bll0342		*fah*	fumarylacetoacetase	-8,6
bll0346			putative oxidoreductase	–
	bsl0345		hypothetical protein	–
	bll0344		hypothetical protein	–
	bll0343		homogentisate 1,2-dioxygenase	-9,6
blr0401			hypothetical protein	-5,6
blr0420		*rplU*	50S ribosomal protein L21	-5,8
blr0444			-	-7,5
bll0465			hypothetical protein	-10,6
blr0495		*leuD*	isopropylmalate isomerase small subunit	-5,6

bll0506			hypothetical protein	-4,8
	bll0505		hypothetical protein	-5,4
blr0583		*ggt*	gamma-glutamyltranspeptidase	-5,9
bll0598			hypothetical protein	-11,0
bll0633		*gidA*	glucose-inhibited division protein A	-6,7
	bll0632	*gidB*	probable methyltransferase	–
	bll0631	*parA*	chromosome partitioning protein A	3,5
blr0651			hypothetical protein	–
	blr0652		glutamine amidotransferase	–
	blr0653		phosphoribosylformino-5-aminoimidazole carboxamide ribotide isomerase	–
	blr0654	*hisF*	imidazole glycerol phosphate synthase subunit HisF	-7,6
	blr0655	*hisE*	phosphoribosyl-ATP pyrophosphatase	-2,6
	blr0656		pantothenate kinase	–
bll0781			tRNA pseudouridine 55 synthase	–
	bsl0780	*rpsO*	30S ribosomal protein S15	-10,0
blr0806			hypothetical protein	-7,6
bll0816			hypothetical protein	-8,6
bll0818			hypothetical protein	-20,7
bsr0858			hypothetical protein	-11,8
bsr0859			hypothetical protein bsr0859	-8,0
bll0886			ABC transporter ATP-binding protein	-2,2
	bll0885		ABC transporter ATP-binding protein	–
	bll0884		ABC transporter permease protein	–
	bll0883		ABC transporter permease protein	-10,4
bll0887			ABC transporter substrate-binding protein	-8,5
bll0892			hypothetical protein	-5,2
bll0905		*regS*	two-component sensor histidine kinase	–
	bll0904	*regR*	two-component response regulator	-91,7
blr0918			ABC transporter permease protein	–
	blr0919		ABC transporter ATP-binding protein	–
	blr0920		hypothetical protein	-5,4
blr0925		*pcaF*	acetyl-CoA acetyltransferase	-6,4
bsr0948		*rpmF*	50S ribosomal protein L32	-7,4
bsl0950			hypothetical protein	-5,3
blr1170		*coxB*	cytochrome *c* oxidase subunit II	–
	blr1171	*coxA*	cytochrome *c* oxidase subunit I	–
	blr1172	*coxE*	putative heme *o* synthase	–
	bsr1173	*coxF*	CoxF protein	–
	blr1174	*coxG*	cytochrome *c* oxidase assembly protein	-3,0
	blr1175	*coxC*	cytochrome *c* oxidase subunit III	-5,5
bll1235		*cysD*	O-acetylhomoserine sulfhydrylase	-5,7

	bll1234		putative hydolase	–
blr1263			unknown protein	–
	blr1264		hypothetical protein	-6,4
	blr1265		hypothetical protein	-6,3
bll1285			hypothetical protein	-151,1
blr1289			hypothetical protein	-8,1
bll1320			probable penicillin-binding protein	-5,3
blr1377		*etfS*	electron transfer flavoprotein beta subunit	-4,7
	blr1378	*etfL*	electron transfer flavoprotein large subunit	-9,7
bll1385		*pcaC*	putative gamma carboxymuconolactone decarboxylase protein	-16,8
blr1424			ABC transporter substrate-binding protein	-2,6
	blr1425		ABC transporter substrate-binding protein	-4,1
	blr1426		ABC transporter permease protein	-5,1
	blr1427		ABC transporter permease protein	-3,0
blr1429			hypothetical protein	-8,1
bll1464			hypothetical protein	-11,8
bll1467			hypothetical protein	-5,5
	bll1466		hypothetical protein	-11,9
blr1468			hypothetical protein	-26,1
	blr1469		hypothetical protein	-22,3
bsl1473			hypothetical protein	-14,9
bsl1507		*rpmE*	50S ribosomal protein L31	-13,7
blr1515		*acrA*	RND multidrug efflux membrane permease	-17,3
	blr1516	*acrB*	RND multidrug efflux transporter	-13,4
bsl1589			hypothetical protein	-5,7
bsr1590			hypothetical protein	-6,7
blr1617			conjugal transfer protein	–
	blr1618		probable conjugal transfer protein	–
	blr1619		conjugal transfer protein	–
	blr1620		conjugal transfer protein	–
	bsr1621		hypothetical protein	-12,5
bsl1637			unknown protein	–
	bll1636		hypothetical protein	-6,0
bll1766			outer membrane protein	-10,2
bll1791			hypothetical protein	-5,5
blr1988			unknown protein	–
	blr1989		unknown protein	–
	blr1990		hypothetical protein	–
	blr1991		hypothetical protein	-5,4
blr2036[g]		*fixR*	oxidoreductase	-6,3
	blr2037	*nifA*	nif-specific regulatory protein	-7,7

bsl2064			hypothetical protein	-5,3
bll2087			hypothetical protein	-5,2
bsl2212			hypothetical protein	-6,8
	bll2211	*copB*	copper tolerance protein	-15,2
	bll2210	*copA*	multicopper oxidase	-10,7
	bll2209	*copC*	copper tolerance protein	-16,1
	bll2208		hypothetical protein	-9,2
blr2351			hypothetical protein	-7,0
bll2388		cy_2	cytochrome c_2	-8,3
blr2405		*fbp*	peptidylprolyl isomerase	-5,2
bsl2407			hypothetical protein	-7,8
bll2462			hypothetical protein	-6,0
bll2465			MoxR family protein	-7,5
	bll2464		hypothetical protein	-4,6
	bll2463		hypothetical protein	-6,6
blr2501			hypothetical protein	-15,1
blr2505			hypothetical protein	-11,0
bsl2596			hypothetical protein	-7,8
bsl2602			hypothetical protein	-9,6
blr2694			VirG-like two component response regulator	-12,4
bll2737			oxidoreductase with iron-sulfur subunit	-14,2
	bll2736		putative aldehyde dehydrogenase protein	-10,3
blr2787			hypothetical protein	-5,8
	bsr2788		unknown protein	–
blr2806[h]			nitrite extrusion protein	-10,3
	blr2807	*bjgb*	probable bacterial hemoglobin	-4,0
	blr2808		putative FAD and NAD(P)H-binding reductase protein	-9,2
	blr2809	*nasA*	nitrate reductase large subunit	-5,5
blr2811			hypothetical protein	-5,0
bll2850			probable 6-phosphofructokinase	–
	bll2849		hypothetical protein	-8,4
bll2876			ABC transporter permease protein	-7,9
	bll2875		ABC transporter permease protein	–
	bll2874		ABC transporter ATP-binding protein	–
	bll2873		ABC transporter ATP-binding protein	–
blr2928			oxidoreductase	–
	blr2929		hypothetical protein	-10,2
	blr2930		hypothetical protein	–
bll3037			hypothetical protein	-11,9
bll3108			hypothetical protein	-6,2
blr3169			hypothetical protein	-38,3

blr3212		*norE*	nitric oxide reductase subunit E	-13,6
	bsr3213		hypothetical protein	-10,8
blr3214		*norC*	nitric oxide reductase subunit C	-5,2
	blr3215	*norB*	nitric oxide reductase subunit B	-4,9
	blr3216	*norQ*	NorQ protein	-7,2
	blr3217	*norD*	NorD protein	-11,3
blr3218			putative hydrolase phosphatase protein	-5,8
	blr3219		probable transcriptional regulator	-4,5
blr3261		*cobW*	cobalamin synthesis protein	–
	blr3262		hypothetical protein	–
	blr3263	*cobN*	cobaltochelatase	-2,2
	blr3264		unknown protein	–
	blr3265	*cobH*	precorrin isomerase	–
	blr3266	*cobI*	precorrin-2 C20 methyltransferase	-16,4
	blr3267	*cobJ*	precorrin-3B C17-methyltransferase	–
bll3466		*fixK*	transcriptional regulator FixK	-5,2
blr3555			probable ferrichrome receptor precursor	–
	bsr3556		hypothetical protein	-9,2
bll3592			hypothetical protein	-11,1
bll3717		*lipA*	lipoyl synthase	-5,8
bll3765			glutamine amidotransferase	-6,9
	bll3764		hypothetical protein	-6,1
bll3768			hypothetical protein	-6,6
blr3769			hypothetical protein	-7,7
	blr3770		hypothetical protein	-38,0
blr3771			hypothetical protein	-16,0
bll3817			hypothetical protein	-7,6
	bll3816		putative sulfur-regulated protein	–
blr3860			hypothetical protein	-21,5
blr3904			probable iron transport protein	-12,9
	blr3905		putative hydroxylase	-12,4
	blr3906	*exbB*	biopolymer transport protein	-13,6
	blr3907		biopolymer transport protein	-26,6
	blr3908		hypothetical protein	-8,1
bsl3938			putative biotinylated protein	-10,6
bsl4014			hypothetical protein	-5,6
blr4046			hypothetical protein	-7,8
bsr4099			unknown protein	–
	blr4100		hypothetical protein	-16,4
blr4119		*ndk*	nucleoside diphosphate kinase	-9,9
bll4130			transcriptional regulatory protein	-19,1

blr4156			acetylornithine deacetylase	-6,0
bll4168			unknown protein	–
	bsl4167		putative glutamine synthetase translation inhibitor	-6,8
blr4182			hypothetical protein	-28,2
bll4218			hypothetical protein	-26,0
bll4228			putative ethidium resistance protein	-8,1
	bll4227		transcriptional regulatory protein TetR family	–
bll4247			hypothetical protein	-5,2
bll4252			putative hydrolase	-5,1
blr4257			putative hydrolase	-3,9
	bsr4258		hypothetical protein	–
	blr4259		hypothetical protein	-2,8
	blr4260		hypothetical protein	-12,5
	blr4261		hypothetical protein	-3,2
	blr4262		hypothetical protein	-2,0
	blr4263		hypothetical protein	–
	blr4264		putative adenylate cyclase protein	–
bll4291		*accB*	biotin carboxyl carrier protein subunit of acetyl-CoA carboxylasen	-5,6
	bll4290		biotin carboxylase subunit of acetyl-CoA carboxylase	–
bll4292		*aroQ*	3-dehydroquinate dehydratase	-19,6
bll4294			outer membrane protein	-5,5
blr4297			hypothetical protein	-6,0
bll4399		*gloA*	lactoylglutathione lyase	-5,1
bsr4408			hypothetical protein	-9,5
bll4412			hypothetical protein	-7,6
blr4416			hypothetical protein	-6,2
bsl4437			hypothetical protein	-10,1
blr4438			hypothetical protein	-5,9
	blr4439		penicillin binding protein	-4,4
blr4442			dolichol-phosphate mannosyltransferase	–
	blr4443		hypothetical protein	-5,3
blr4505			hypothetical protein	-6,0
bll4579			hypothetical protein	-13,4
bll4583		*qor*	quinone oxidoreductase	-5,2
bll4589			hypothetical protein	-7,4
blr4723			hypothetical protein	-5,5
bll4736			preprotein tranlocase protein	-5,5
bll4819			hypothetical protein	-5,1
	bll4818		hypothetical protein	–
	bll4817		hypothetical protein	-4,5
	bll4816		unknown protein	–

	bll4815		hypothetical protein	-4,7
	bll4814		unknown protein	–
blr4839		*gltA*	citrate synthase	-8,0
bll4867			putative outer-membrane immunogenic protein precursor	-13,0
bll4896			ABC transporter substrate-binding protein	-5,7
bll4906		*nuoL*	NADH dehydrogenase subunit L	-4,4
	bll4905	*nuoM*	NADH dehydrogenase subunit M	-4,0
	bll4904	*nuoN*	NADH dehydrogenase subunit N	-6,2
	bll4903		birA bifunctional protein	–
	bll4902		hypothetical protein	–
bll4908		*nuoJ*	NADH dehydrogenase subunit J	-11,8
	bll4907		NADH dehydrogenase kappa subunit	-9,8
bll4919		*nuoA*	NADH dehydrogenase alpha subunit	-2,2
	bll4918	*nuoB*	NADH ubiqionone oxidoreductase chain B	–
	bll4917	*nuoC*	NADH dehydrogenase subunit C	-3,1
	bll4916	*nouD*	NADH dehydrogenase delta subunit	-3,6
	bll4915		hypothetical protein	-3,0
	bll4914		ATP synthase subunit E	-8,4
	bsl4913		hypothetical protein	-2,4
	bll4912	*nuoF*	NADH ubiquinone oxidoreductase chain F	-14,7
	bll4911	*nuoG*	NADH dehydrogenase gamma subunit	-8,1
	bll4910	*nuoH*	NADH dehydrogenase subunit H	-10,5
	bll4909	*nuoI*	NADH dehydrogenase subunit I	-31,3
bll4920			ferrichrome iron receptor	-9,2
blr4930			hypothetical protein	-3,4
	blr4931		hypothetical protein	-13,4
	blr4932		putative cation efflux system protein	-22,0
	blr4933		probable cation efflux system protein	-26,5
blr4934			hypothetical protein	-8,4
blr4935			putative divalent cation resistant determinant protein C	-33,0
	blr4936		putative cation efflux system protein	-23,3
	blr4937		probable cation efflux system protein	-8,3
bll4983			hypothetical protein	-6,9
blr4984			transcriptional regulatory protein	-6,8
bll4985			hypothetical protein	-11,4
bsl5034			hypothetical protein	-5,1
bll5070		*smpB*	SsrA-binding protein	-7,6
	bll5069	*bcpB*	BcpB protein	-4,7
bll5081			putative multidrug resistance protein	–
	bll5080		AcrB/AcrD/AcrF family protein	-2,7
	bll5079		hypothetical protein	-5,6

bll5167			glutathione S-transferase	-8,9
	bll5166		hypothetical protein	–
blr5292			hypothetical protein	-7,6
bll5324			hypothetical protein	-5,3
blr5341			hypothetical protein	-5,4
bll5373			probable short-chain dehydrogenase	-7,1
	bll5372		hypothetical protein	-7,5
bll5412		*rplJ*	50S ribosomal Protein L10	-7,8
	bll5411		50S ribosomal protein L7/L12	–
blr5422			hypothetical protein	–
	blr5423		probable dTDP-glucose-4,6 dehydratase	–
	blr5424		hypothetical protein	–
	blr5425		hypothetical protein	–
	blr5426	*tktB*	transketolase	4,6
	blr5427		hypothetical transketolase family protein	–
	blr5428		hypothetical protein	–
	blr5429		hypothetical protein	–
	blr5430		hypothetical protein	-8,7
	blr5431		hypothetical protein	-3,1
	blr5432		hypothetical protein	–
bll5475			putative formate dehydrogenase	-6,4
bll5477			similar to formate dehydrogenase	-8,4
	bll5476		formate dehydrogenase iron-sulfur subunit	-7,6
blr5502			hypothetical protein	-10,5
bll5555			hypothetical protein	-13,2
blr5556			hypothetical protein	-7,7
bsl5585			hypothetical protein	-7,6
blr5601			hypothetical protein	–
	blr5602		hippurate hydrolase	-5,7
	blr5603		glutamyl-tRNA amidotransferase subunit A	–
bsr5670			hypothetical protein	-8,1
blr5693			probable substrate-binding protein	-7,7
blr5730			hypothetical protein	-9,3
bll5734			ABC transporter nitrate-binding protein	–
	bll5733		nitrate ABC transporter permease protein	–
	bll5732		ABC transporter ATP-binding protein	–
	bll5731		cyanate hydratase	-6,3
bll5918			putative dehydrogenase	–
	bll5917		DegT/DnrJ/EryC1/StrS family protein	-5,7
blr5933			hypothetical protein	-8,4
bll5984			unknown protein	-4,1

	bll5983		unknown protein	-3,5
	bll5982		hypothetical protein	-11,3
blr5985			unknown protein	-3,7
	blr5986		hypothetical protein	-6,5
blr5994			hypothetical protein	-7,8
	blr5995	*neuA*	hypothetical protein	-4,3
	blr5996	*ptmB*	posttranslational modification protein	-3,3
	blr5997		short chain dehydrogenase	–
	blr5998		putative membrane protein	–
	blr5999		unknown protein	-4,7
	blr6000		hypothetical protein	-8,5
blr6158			ABC transporter substrate-binding protein	-17,2
blr6167			hypothetical protein	-5,6
bll6198			hypothetical protein	-5,6
blr6210			hypothetical protein	-6,3
bll6262		*osmC*	probable osmotically inducible protein	-5,4
	bll6261		hypothetical protein	-5,7
	bll6260		peptide methionine sulfoxide reductase	–
bll6455			ABC transporter substrate-binding protein	-2,4
	bll6454		ABC transporter permease protein	-2,8
	bll6453		ABC transporter ATP-binding protein	-3,5
	bll6452	*acd*	acyl-CoA dehydrogenase	-5,8
	bll6451		probable alkanesulfonate monooxygenase	-9,5
	bll6450		probable substrate-binding protein	-10,2
bll6486			hypothetical protein	-5,6
	bll6485		hypothetical protein	–
bll6513			hypothetical protein	-8,7
blr6563			hypothetical protein	-8,3
	blr6564		putative dihydroflavonol-4-reductase	-3,5
	blr6565		hypothetical protein blr6565	-7,4
blr6659		*thiC*	thiamine biosynthesis protein ThiC	-8,3
blr6660			hypothetical protein	-7,7
bll6668			hypothetical protein	-13,1
blr6718			hypothetical protein	-6,9
bsl6734			hypothetical protein	-6,9
bll6756			hypothetical protein	–
	bll6755		hypothetical protein	-3,4
	bll6754		hypothetical protein	-5,7
blr6766			hypothetical protein	-3,0
	blr6767		trehalose synthase	-4,0
	blr6768	*glgB*	glycogen branching enzyme	-3,6

	blr6769	*glgX*	glycogen debranching enzyme	-5,4
	blr6770		alpha-amylase	–
	blr6771		probable glycosyl hydrolase	–
bll6799			hypothetical protein	-20,0
bll6995			tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	–
	bll6994		putative phosphatidylethanolamine N-methyltransferase	–
	bll6993		hypothetical protein	-5,3
bll7010		*ssuD*	sulfonate monooxygenase	–
	bll7009		aliphatic sulfonate ABC transporter permease protein	-6,5
	bll7008		aliphatic sulfonate ABC transporter ATP-binding protein	-3,5
	bll7007		putative oxidoreductase	–
bll7075			hypothetical protein	-18,2
	bll7074		hypothetical protein	-15,2
	bll7073	*exbB*	biopolymer transport protein	-9,5
	bll7072	*exbD*	biopolymer transport protein	-15,9
	bll7071	*tonB*	TonB protein	-5,8
bll7076		*hmuR*	hemin receptor precursor	-19,7
blr7077		*hmuT*	hemin ABC transporter hemin-binding protein	-7,3
	blr7078	*hmuU*	hemin ABC transporter permease protein	-16,5
	blr7079	*hmuV*	hemin ABC transporter ATP-binding protein	-4,0
blr7094			hypothetical protein blr7094	-6,5
bsr7117		*rpsU*	30S ribosomal protein S21	-11,2
blr7131			hypothetical protein blr7131	-7,1
blr7296			hypothetical protein blr7296	-8,0
blr7297			hypothetical protein blr7297	-12,2
bll7311			probable ArcD2 arginine/ornithine antiporter	-12,5
	bll7310		arginine deiminase	-15,2
bll7313			RND efflux membrane fusion protein	-5,0
	bll7312		AcrB/AcrD/AcrF family protein	-7,4
blr7314			hypothetical protein	-93,5
	blr7315		hypothetical protein	-14,1
	bsr7316		hypothetical protein	–
	bsr7317		hypothetical protein	–
	blr7318		unknown protein	-2,1
	blr7319		hypothetical protein	–
	blr7320		hypothetical protein	-3,5
blr7321			hypothetical protein	-97,1
bll7322			hypothetical protein	-17,6
blr7323			probable ArcD1 arginine/ornithine antiporter	-8,3
blr7324			hypothetical protein	-4,8
	blr7325		hypothetical protein	-9,4

	blr7326		hypothetical protein	-7,9
blr7327			hypothetical protein	-17,2
bsr7328			hypothetical protein	-46,7
blr7329			putative multidrag resistance protein	-5,9
	blr7330		AcrB/AcrD/AcrF family protein	-2,9
bll7411			hypothetical protein	-6,4
bll7414			translation elongation factor EF-G	-7,0
blr7436			hypothetical protein	-5,9
blr7465			hypothetical protein	-6,9
blr7471		*pgsA*	phosphatidylglycerophosphate synthase	-3,1
	bsr7472	*moaD*	molybdopterin converting factor small subunit	-18,9
	blr7473	*moaE*	molybdopterin converting factor, large subunit	–
	blr7474		hypothetical adenine-specific methylase	–
blr7484			hypothetical zinc protease	-3,0
	blr7485		hypothetical zinc protease	-7,6
blr7560		*dhlB*	2-haloalkanoic acid dehalogenase	-19,1
	blr7561		hypothetical protein	-3,3
bll7562			hypothetical protein	-9,1
bsr7564			hypothetical protein	-14,1
bll7626			hypothetical protein	-27,4
bll7628			hypothetical protein	-8,2
	bll7627		hypothetical protein	-10,7
blr7629			hypothetical protein	-26,7
	blr7630		probable decarboxylase	-6,8
bsr7633			hypothetical protein	-5,3
bll7635			hypothetical protein	-8,2
bll7638			putative cytochrome c_6 precursor	-6,8
	bll7637		unknown protein	–
	bll7636		hypothetical protein	–
blr7694			hypothetical protein	-6,8
bll7774		*sodF*	superoxide dismutase	-9,3
bll7790			hypothetical protein	-10,3
bll7795		*phyR*	two-component response regulator	-10,9
bsr7796		*nepR*	anti-sigma factor	-6,5
	blr7797	*ecfG*	RNA polymerase ECF sigma factor (σ^{EcfG})	-3,7
blr7887			hypothetical protein	-6,1
bll7908			hypothetical protein	–
	bll7907		hypothetical protein	-3,9
	bll7906		putative ferredoxin	-10,3
bll7938			hypothetical protein	-5,3
blr8111			hypothetical protein	-9,2

blr8132			RhtB family transporter	-5,2
trnE-CUC			tRNA-Glu(CTC)	-8,5
trnH-GUG			tRNA-His(GTG)	-7,5

Class 2 (upregulated the Δ*regR* strain)

bsl0170			hypothetical protein	5,4
	bsl0169		hypothetical protein	2,8
blr0624			hypothetical protein	7,1
bll0777			transcriptional regulatory protein	6,1
	bll0776		hypothetical protein	3,4
blr0903			hypothetical protein	6,3
blr0960			5'-methylthioadenosine phosphorylase	3,5
	blr0961		translation initiation factor IF-2B subunit alpha	5,7
blr1072			hypothetical protein	8,1
bll1113			methylated-DNA--protein-cysteine methyltransferase	7,5
	bll1112		transcriptional regulatory protein	–
bll1150			transcriptional regulatory protein	7,3
blr2071			similar to inosamine-phosphate amidinotransferas	5,5
bsr2110			hypothetical protein	5,1
	bsr2111		hypothetical protein	4,0
blr2113			hypothetical protein	4,4
	blr2114		hypothetical protein	5,5
	blr2115		hypothetical protein	2,8
bll2512			transcriptional regulatory protein	6,7
blr2605			putative short chain dehydrogenase	5,4
bsl2907			probable ferredoxin	6,7
blr2921			hypothetical protein	5,6
	blr2922		ABC transporter amino acid-binding protein	–
bll3077			transcriptional regulatory protein	7,7
bll3426			ABC transporter substrate-binding protein	7,8
bll3668			transcriptional regulatory protein	7,3
blr3741			hypothetical protein	5,8
	blr3742		probable multidrug-resistance related protein	2,8
bll3785		*coxM*	cytochrome *c* oxidase	5,7
	bll3784	*coxN*	cytochrome *c* oxidase	3,7
	bll3783	*coxO*	cytochrome *c* oxidase	3,0
	bll3782	*coxP*	cytochrome *c* oxidase	–
	bll3781		hypothetical protein	–
blr3787			hypothetical protein	5,0
blr3963			transcriptional regulatory protein	9,0
bll4010			transcriptional regulatory protein PadR-like	12,5

blr4080			transcriptional regulatory protein	3,6
	blr4081		hypothetical protein	6,2
bll4221			transcriptional regulatory protein	6,6
blr4222			phenol 2-monooxygenase	6,0
bll4347			hypothetical protein	6,0
blr4499			hypothetical protein	5,8
blr4673			hypothetical protein	6,5
bll4873			hypothetical protein	6,0
bsr4956			hypothetical protein	5,6
bll5010			putative resolvase	5,1
bll5164			hypothetical protein	5,8
bll5199			hypothetical protein	5,1
bll5353			hypothetical protein	5,3
	bll5352		hypothetical protein	6,5
blr5497			transcriptional regulatory protein	6,2
bll5501			hypothetical protein	7,0
blr5658			putative avidin	6,7
blr5735			transcriptional regulatory protein	6,6
blr5860			transcriptional regulatory protein	9,9
bll5900			hypothetical protein	7,0
bll6110			hypothetical protein	8,2
bll6243		*hutI*	imidazolone-5-propionate hydrolase	11,2
	bll6242	*hutH*	histidine ammonia-lyase	5,1
	bll6241	*hutU*	urocanate hydratase	3,7
blr6244			atrazine chlorohydrolase	6,7
	blr6245		transcriptional regulatory protein	3,2
blr6338			hypothetical protein	5,1
	blr6339	*hyfB*	NADH dehydrogenase subunit N	4,4
	blr6340	*hycC*	probable hydrogenlyase component	–
	blr6341		hypothetical protein	–
	blr6342	*hyfF*	probable hydrogenlyase component	–
	blr6343		probable hydrogenlyase component	–
	blr6344	*hycG*	probable hydrogenase-3 subunit G	–
bll6512		*thyA*	thymidylate synthase	3,4
	bll6511		acetyltransferase	5,6
	bll6510	*folA*	dihydrofolate reductase	–
bll6537			putative cytochrome P_{450}	7,2
blr7050			hypothetical protein	11,2
blr7098			transcriptional regulatory protein	6,5
bll7214			hypothetical protein	7,1
bsr7390			hypothetical protein	5,5

bsr7727	hypothetical protein	5,8
blr7895	hypothetical protein	6,3
trnN-GUU-2	tRNA-Asn(GTT)	6,4
trnF-GAA-2	tRNA-Phe(GAA)	5,4

[a] The list is a subset of genes differentially expressed in the Δ*regR* strain compared with the wild type, both grown in anoxically in BMS medium.

[b] Gene numbers are according to the Rhizobase (http://genome.kazusa.or.jp/rhizobase/).

[c] Operon predictions were performed as described by Hauser et al., 2007; Mesa et al., 2008.

[d] Genes names as indicated in the EMBL-EBI database with modifications.

[e] Protein description according to Kaneko et al., 2002 with modifications.

[f] Fold change of expression in the Δ*regR* strain in comparison with the wild type, both grown anoxically. (–) indicates no change within the threshold fold change range between +5 and -5.

[g] *bll2036* and *bl2037* constitute an operon unit described by Thöny and coworkers (Thöny et al., 1987).

[h] *blr2806, blr2807, blr2808* and *blr2809* have been shown to belonging to an operon unit by J. Cabrera and M.J. Delgado (unpublished results), and *blr2807* has been recently named as bjgb (Cabrera et al., 2011).

CHAPTER II

4.2. Functional analysis of the copy 1 of the *fixNOQP* operon of *E. meliloti* under free-living microoxic and symbiotic conditions.

4.2.1. Abstract.

Aim: In this work, phenotypic analyses of a *E. meliloti fixN1* mutant under free-living and symbiotic conditions have been carried out.

Methods and Results: *E. meliloti fixN1* mutant showed a defect in growth as well as in TMPD-dependent oxidase activity when cells were incubated under micro-oxic conditions. Furthermore, haem *c* staining analyses of a *fixN1* and a *fixP1* mutant identified two membrane-bound *c*-type cytochromes of 27- and 32-kDa, present in microaerobically grown cells and in bacteroids, as the FixO and FixP components of the *E. meliloti* cbb_3 oxidase. Under symbiotic conditions, *fixN1* mutant showed a clear nitrogen fixation defect in alfalfa plants that were grown in an N-free nutrient solution during 3 weeks. However, in plants grown for a longer period, *fixNOQP1* copy was not indispensable for symbiotic nitrogen fixation.

Conclusions: The copy 1 of the *fixNOQP* operon is involved in *E. meliloti* respiration and growth under micro-oxic conditions as well as in the expression of the FixO and FixP components of the cbb_3 oxidase present in free-living microaerobic cultures and in bacteroids. This copy is important for nitrogen fixation during the early steps of the symbiosis.

Significance and Impact of the Study: It is the first time that a functional analysis of the *E. meliloti* copy 1 of the *fixNOQP* operon is performed. In this work, the cytochromes *c* which constitutes the cbb_3 oxidase operating in free-living micro-oxic cultures and in bacteroids of *E. meliloti* have been identified.

4.2.2. Introduction.

Soil bacteria, collectively known as rhizobia, form nitrogen-fixing nodules on the roots of leguminous plants. They have been intensively studied because of their agronomic importance and the inherent biological interest of their complex interactions with their host plants. The establishment of an effective symbiotic association between rhizobia and legumes is a highly specific and complex developmental process, in which both partners undergo differentiation in a concerted way (reviewed by Jones *et al*. 2007; Oldroyd and Downie 2008; Oldroyd *et al*. 2011). Following invasion of the plant cells via a complex signalling pathway between bacteria and plant, rhizobia stop dividing and undergo differentiation into nitrogen-fixing bacteroids. The activity of the nitrogen-reducing enzyme nitrogenase requires a high rate of oxygen respiration to supply the energy demands of the nitrogen reduction process. However, oxygen irreversibly inactivates the nitrogenase complex. These conflicting demands are met by controlling oxygen flux to the infected plant cells through an oxygen diffusion barrier, which greatly limits permeability to oxygen (Minchin *et al*. 2008). Oxygen is then delivered to the bacteroids by the plant oxygen carrier, leghaemoglobin, present exclusively in the nodule (Downie, 2005). To cope with the low ambient oxygen concentration in the nodule (10–50 nM O_2), nitrogen fixing bacteroids induce a high-affinity cytochrome *cbb*$_3$-type oxidase (Delgado *et al*. 1998). Genes encoding the *cbb*$_3$ complex were initially isolated from rhizobia and named *fixNOQP* due to its requirement for symbiotic nitrogen fixation (Preisig *et al*. 1996). Since then, orthologous genes called *ccoNOQP* were identified in other Proteobacteria including photosynthetic and pathogenic bacteria (reviewed in Cosseau and Batut, 2004; Bueno *et al*. 2012; Ekici *et al*. 2012).

Cytochrome *cbb*$_3$ oxidases have been purified from several organisms, including *Paracoccus denitrificans*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus* and *Bradyrhizobium japonicum* (reviewed in Pitcher and Watmough, 2004). Subunit I (FixN or CcoN) is a membrane-integral *b*- type and copper-containing cytochrome. Electrons are delivered to the heme-Cu_B site on subunit I via the membrane-anchored mono-heme *c* and diheme *c*-type cytochromes FixO/CcoO and FixP/CcoP wich constitute subunits II and III, respectively (Buschmann *et al*. 2010). FixQ or CcoQ are required for

optimal oxidase activity, because it stabilizes the interaction of CcoP with the CcoNO core complex, leading subsequently to the formation of the active 230-kDa complex (Peters *et al*. 2008). The biogenesis of this oxidase depends on the *ccoGHIS* gene products, which are proposed to be specifically required for cofactor insertion and maturation of *cbb*$_3$-type cytochrome c oxidases (Kulajta *et al*. 2006; Pawlik *et al*. 2010). Several additional proteins including SenC (Swem *et al*. 2005), PCu$_A$C (Banci *et al*. 2005; Abriata *et al*. 2008; Serventi *et al*. 2012); DsbA (Deshmukh *et al*. 2003) and CcoA (Ekici *et al*. 2012) might be also involved in *cbb*$_3$ biogenesis.

Ensifer meliloti is an aerobic soil bacterium which establishes symbiotic N_2-fixing associations with plants of the genera *Medicago*, *Melilotus* and *Trigonella*. The expression of *E. meliloti* genes required for nitrogen fixation and for microaerobic respiration is coordinated by *fixLJ* and *fixK* genes which are conserved among rhizobia (Fischer, 1994; Dixon and Kahn 2004). Under oxygen limiting conditions, FixL autophosphorylates and transmits phosphate to the FixJ response regulator. Once phosphorylated, FixJ activates transcription of the *nifA* and *fixK* genes, which induce expression of *nif* and *fix* genes, respectively (Reyrat *et al*. 1993). A set of publications has demonstrated that *fixT* and *fixM* are also targets of FixJ (Ampe *et al*. 2003; Barnett *et al*. 2004; Becker *et al*. 2004; Bobik *et al*. 2006; Meilhoc *et al*. 2010)**.** While *fixT* negatively affects expression of FixLJ-dependent genes by inhibiting FixL autophosphorylation (Garnerone *et al*. 1999), *fixM* encodes a flavoprotein that modulates inhibition by 5-aminoimidazole-4-carboxamide nucleotide (AICAR) or 5'adenosine monophosphate (5'AMP) of respiratory and nitrogen fixation genes expression in *E. meliloti* (Cosseau *et al*. 2002).

Inspection of the *E. meliloti* 1021 genome sequence shows a composite architecture, consisting of three replicons with distinctive structure and function: a 3.65 Mb chromosome and two megaplasmids, pSymA (1.35 Mb) and pSymB (1.68 Mb) (Galibert *et al*. 2001). pSymA contains a large fraction of genes known to be specifically involved in symbiosis such as genes involved in nodulation or in nitrogen- fixation process, as well as genes involved in microaerobic metabolism or in denitrification (Barnett *et al*. 2001). A 290-kilobase (kb) region of pSymA contains nodulation genes as well as genes involved in nitrogen fixation (*nif* and *fix*) and it carries repeated

sequences (Renalier *et al*. 1987). One of these reiterated sequences had been identified as part of a cluster of *fix* genes located 220 kb downstream of *nifHDK* genes and contains regulatory genes (*fixLJ*, *fixT1*, *fixK1*, *fixM*), *fixGHIS* genes, as well as a copy of the *fixNOQP* operon (*fixNOQP1*) (Fig. 4.1). The second *fix* cluster maps 40 kb upstream of the *nifHDK* genes and carries another copy of the *fixNOQP* operon (*fixNOQP2*), regulatory genes (*fixT2*, *fixK2*) as well as a *nod* locus (Renalier *et al*. 1987). Both *fixNOQP* copies are closely related since they encode for proteins having a 95 % homology in their respective sequences. In the pSymA genome there is a third copy of the *fixNOQP* operon (*fixNOQP3*) which only presents a 61 % homology with the other two copies. Neither genes related with nodulation nor nitrogen fixation are located in the *fixNOQP3* genomic context (Fig. 4.1, http://genome.kazusa.or.jp/rhizobase/).

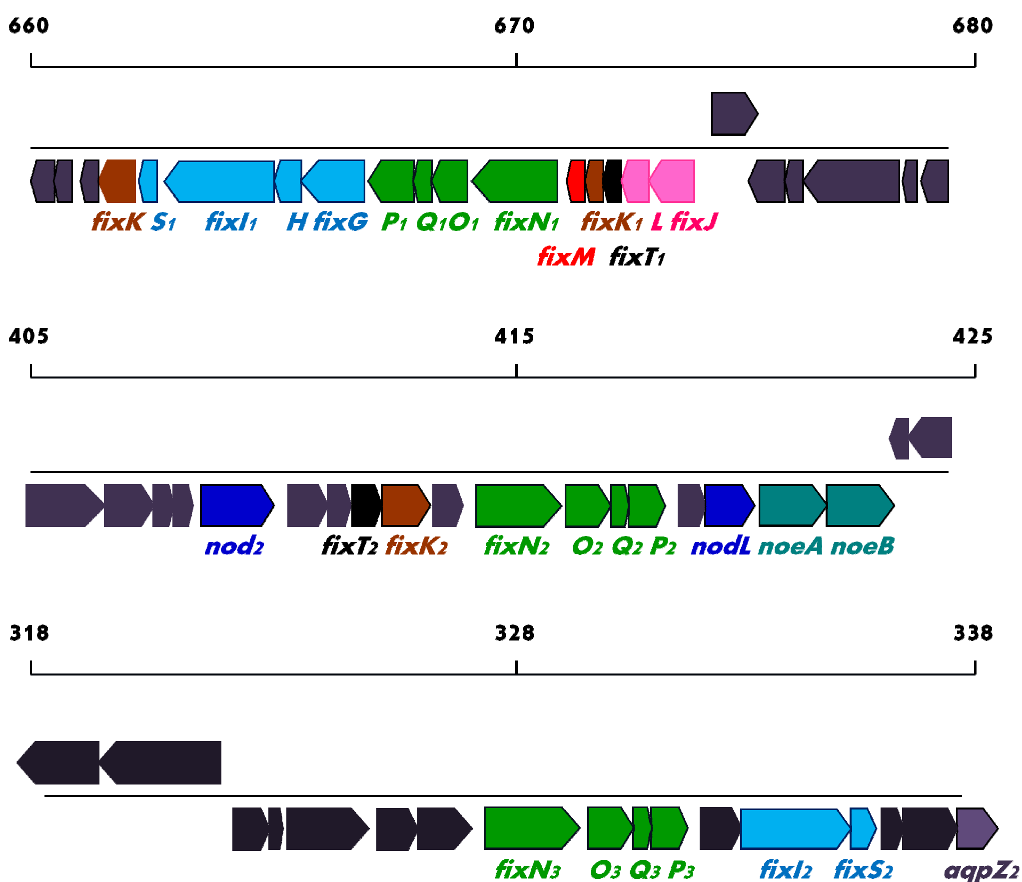


Figure 4.1. Genomic context of the three copies of the *fixNOQP* operon in the symbiotic plasmid pSymA of *E. meliloti*

Recent transcriptomic analyses have shown that *fixNOQP1* genes of *E. meliloti* are induced under microaerobic free-living and symbiotic conditions (Becker *et al*. 2004). Furthermore, *fixNOQP1* genes have been identified as targets of FixK and FixJ in response to low-oxygen conditions (Bobik *et al*. 2006; Meilhoc *et al*. 2010). However, up today functional analyses of these genes are missing. The involvement of *fixNOQP1* genes in free-living respiration and symbiotic nitrogen fixation has been investigated in this work.

4.2.3. Material and Methods.

4.2.3.1. Bacterial strains, growth conditions and recombinant DNA methods.

Bacterial strains used in this work are *E. meliloti* wild type strain 1021 (Meade *et al*. 1982), *fixP1* mutant strain G1PELR32C12 (RhizoGATE, (Becker *et al*. 2009) and *fixN1* mutant strain 2104 (this work). The *fixN1* gene was mutated by performing gene-directed mutagenesis by marker exchange. A PCR fragment of 1.5 kb containing the *fixN1* coding region of Sm1021 was subcloned into pK18 mobsac (Schäfer *et al*. 1994) to obtain plasmid pBG2104. Finally, the 2 kb fragment (Ω Spc/Sm interposon) of pHP45Ω (Prentki and Krisch, 1984) was inserted at the unique *NruI* site located 683 bp downstream of the FixN1 start codon in the 1.5 kb PCR fragment. The resulting plasmid pBG2104Ω was transferred via conjugation into *E. meliloti* 1021 using *E. coli* S17-1 carrying pBG2104Ω as donor. Double recombination events were favoured by growth on agar plates containing sucrose. Mutant strains resistant to spectinomycin/streptomycin but sensitive to kanamycin were checked by Southern hybridization experiments (data not shown) for correct replacement of the wild-type fragment by the Ω interposon. The mutant derivative 2104, used in this study, was obtained. Total and plasmid DNA isolation, digestion with restriction enzymes, cloning, agarose gel electrophoresis and *E. coli* transformation were performed using standard protocols (Sambrook and Russell, 2004) . Enzymes used for DNA restriction and modification were purchased from Fermentas (Vilnius, Lithuania) and were used according to the instructions of the manufacturer. For Southern hybridizations, DNA was digested with appropriate restriction enzymes, electrophoresed in 1 % (wt/vol)

agarose gels, and blotted onto nylon (Hybond N+). Hybridization was carried out under high stringency conditions using Rapidhyb buffer (Amersham, Bucks, U.K.**)**. Specific probes were normally obtained by PCR and were labeled with α^{32}P-CTP by random priming, using Amersham's Rediprime system.

E. meliloti strains were routinely grown in medium TY (Tryptone Yeast, Beringer, 1974) at 30°C. For determinations of growth rates, respiratory activity and heme *c* staining in free-living conditions, cells were grown in minimal medium (Robertsen *et al.* 1981). Cell culture under micro-oxic conditions was performed by fluxing a gas mixture of 2 % O_2 and 98 % Ar into de cultures. Initial optical density at 600 nm of the cultures was about 0.1. Antibiotics were added to *E. meliloti* cultures at the following concentrations (µg ml^{-1}): spectinomycin, 200; streptomycin, 200, kanamycin, 100. *E. coli* strains were cultured in Luria–Bertani medium (Miller, 1972) at 37°C. *Escherichia coli* DH5α (Stratagene, Heidelberg, Germany) was used as host in standard cloning procedures and *E. coli* S17-1 (Simon *et al*. 1983) served as the donor in conjugative plasmid transfer. The antibiotics used were (µg ml^{-1}): ampicillin, 200; streptomycin, 20; spectinomycin, 20; and kanamycin, 25.

4.2.3.2. Plants growth conditions.

Alfalfa (*Medicago sativa,* var. Aragón) seeds were surface-sterilized by immersing in 2.5 % $HgCl_2$ for 9 minutes. Then, seeds were washed with sterile water and germinated on wet filter paper in petri dishes in darkness at 28°C for 36 hours. Selected seedlings were planted in 1litre autoclaved Leonard jars (Leonard 1943) filled with vermiculite and containing nitrogen-free mineral solution (Rigaud and Puppo 1975). Seeds (eight per jar) were inoculated at sowing with 1 ml of a single bacterial strain (10^8 cells per ml). Plants were grown in controlled environmental chambers (night/day temperature 19/25 ºC, photoperiod 16/8 h, PPF 400 µmol m^{-2} s^{-1} and relative humidity 60 to 70 %). For nodulation kinetics assays germinated seeds were transferred into autoclaved glass tubes containing 5 ml of the N-free nutrient solution and inoculated with approximately $1x10^8$ of a single bacterial strain. Each tube, covered with a cotton stopper, was incubated in a growth chamber. After inoculation,

the number of nodulated plants and the number of nodules per plant were recorded daily.

4.2.3.3. Plants assays.

Shoots (separated from roots at the cotyledonary node) were dried to a constant weight at 60 ºC. Dry weight on shoots (SDW), height on shoots (SH) and roots (RH) and nodule fresh weight (NFW) were determined per plant. Nodules were harvested from 7-weeks-old plants and were frozen into liquid nitrogen and stored at –80°C. Total nitrogen was measured in oven-dried shoots weighed and grounded in an IKA A 11 basic analytical mill (Rose Scientific Ltd., Alberta, Canada). Total nitrogen was determined using a LECO TruSpec CN Elemental Analyzer.

4.2.3.4. Bacterial respiratory capacity.

Oxygen uptake was determined as described by Marroqui *et al*. (2001). Cells were harvested after 48h of growth at 30 ºC in minimum medium, washed and resuspended in 1 ml 25 mM potassium phosphate buffer (pH 7.0). The oxygen uptake at 21 ºC was measured using a Clark type oxygen electrode (Hansatech, Norkfolk, England) after addition of 2 mM N,N,N´,N´-tetramethyl-*p*-phenylenediamine (TMPD) and 4 mM sodium ascorbate to the cellular suspension (0,15 - 0,25 mg protein). The time taken to consume the oxygen present in the system was used to calculate the rate of TMPD-dependent oxygen consumption.

4.2.3.5. Membrane extraction, cells fractionation and haem *c* staining.

Cells of *E. meliloti* grown aerobically in 150 ml TY medium were harvested by centrifugation at 12,000 g for 5 min, washed twice with minimal medium, resuspended in 500 ml of the same medium and finally incubated under low-oxygen conditions for 2 days. Bacteroids from nodules were prepared as previously described by Mesa *et al*. (2004). Briefly, 1 g of fresh nodules was ground in 7.5 ml TRIS/HCl (pH 7.5) supplemented with 250 mM mannitol. The homogenate was filtered through four

layers of cheesecloth and was centrifuged at 250 g at 4 ºC for 5 min to remove nodule debris. The resulting supernatant was centrifuged twice at 12,000 g at 4 ºC for 10 min and was washed twice in 50 mM potassium phosphate buffer (pH 7). Free-living cells and bacteroids were resuspended in 3 ml of 50 mM potassium phosphate buffer (pH 7) containing 100 µM 4-(2-aminoethyl) benzene-sulfonyl flouride hydrochloride (ABSF), RNAse (20 µg ml^{-1}), and DNAse I (20 µg ml^{-1}). Cells were disrupted using a French pressure cell (SLM Aminco, Jessup, MD, USA). The cell extract was centrifuged at 20,000 g for 20 min to remove unbroken cells and the supernatant was then centrifuged at 140,000 g for 1 h. The membrane pellet was resuspended in 100 µl of the same buffer. Membrane protein aliquots (from free-living cells or bacteroids) were diluted in sample buffer [124mM Tris-HCl, pH 7.0, 20 % glycerol, 4.6 % sodium dodecyl sulfate (SDS) and 50 mM 2-mercaptoethanol], and incubated at room temperature for 10 min. Membrane proteins were separated at 4 ºC in SDS-12 % polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and stained for haem-dependent peroxidase activity as described previously (Vargas *et al*. 1993) using the chemiluminiscence detection kit 'SuperSignal' (Pierce, Thermo Fisher Scientific, IL, USA).

4.2.3.6. Analytical methods.

The protein concentration was estimated using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) with a standard curve of varying bovine serum albumin concentrations.

4.2.4. Results.

4.2.4.1. Free-living growth rates and respiratory capacity.

To investigate the involvement of the cbb_3 oxidase encoded by the *fixNOQP1* operon in free-living growth, a *fixN1* mutant was incubated aerobically and microaerobically (2 % O_2) in minimum medium. Growth was determined by monitoring the optical density at 600 nm (OD_{600}). After incubation under aerobic conditions no significant differences were observed in growth rates of the wild type and the *fixN1* mutant (Fig. 4.2). However, the *fixN1* mutant showed a defect in growth,

reaching an OD_{600} of only 0.5 compared to that of 0.9 determined in wild-type (WT) cells after 72 h incubation under micro-oxic conditions (Fig. 4.2). Cytochrome *c*-dependent oxygen consumption was measured in the WT strain and the *fixN1* deficient mutant using ascorbate-reduced TMPD as a nonphysiological electron donor (Fig. 3.3). Independently of the strain, TMPD oxidase activity observed in cells grown aerobically was lower than that observed in cells grown under oxygen-limiting conditions. This difference could be due to the induction under oxygen-limiting conditions of high-affinity cytochrome *c* oxidases which have greater activity than those induced in aerobic cultures that have low-affinity for oxygen. Alternatively, it might be possible that the affinity for TMPD-ascorbate is higher in micro-oxic cultures than in oxically grown cells. Under aerobic conditions, the *fixN1* mutant showed levels of TMPD oxidase activity similar to those of the WT strain (Fig. 4.3). However, when cells were incubated under micro-oxic conditions, oxygen consumption rates of *fixN1* cells were approximately about 37 % lower than those of WT cells after 48 hours growth (Fig. 4.3). The decrease in TMPD-dependent oxidase activity observed in the *fixN1* mutant under oxygen limiting conditions compared to that observed in the wild type strain could explain the defect of *fixN1* growth under these conditions (Figs 2, 3).

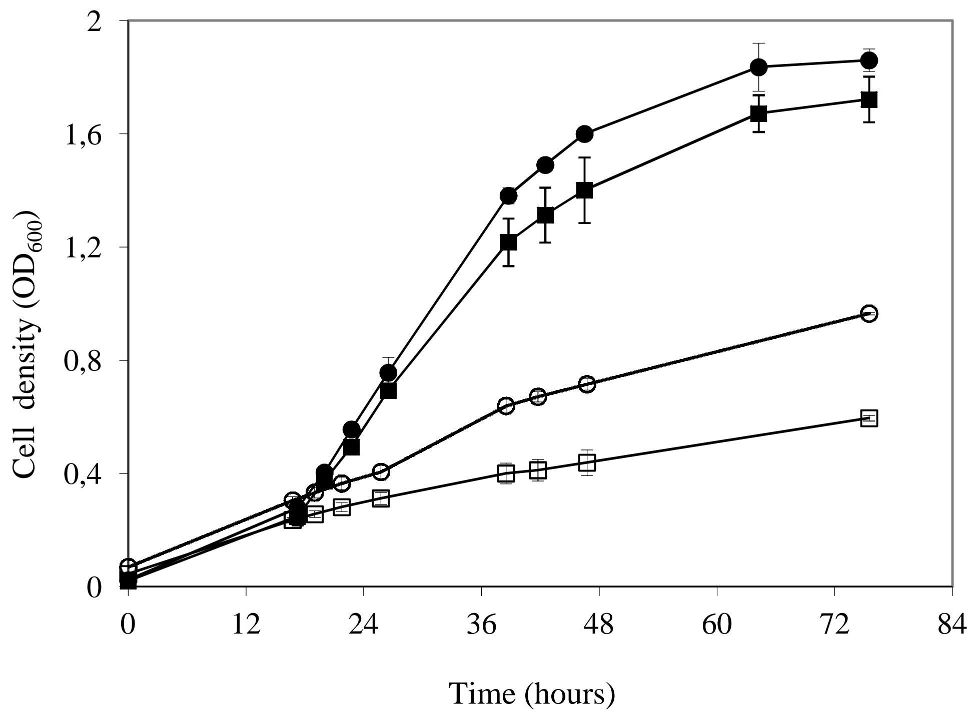


Figure 4.2. Growth of wild type *E. meliloti* 1021 (●, ○) and *fixN1* mutant (■, □) strains in minimal medium under aerobic conditions (closed symbols) or micro-oxic conditions (open symbols). Error bars represent standard deviation of data from at least two different cultures assayed in triplicate.

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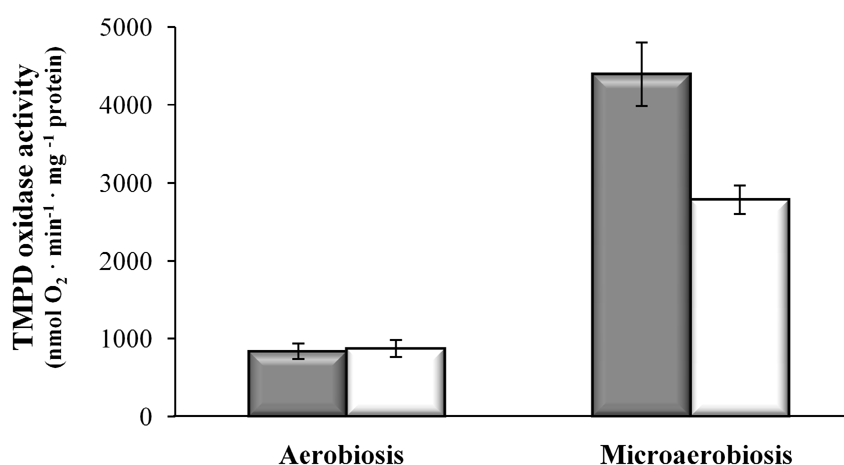


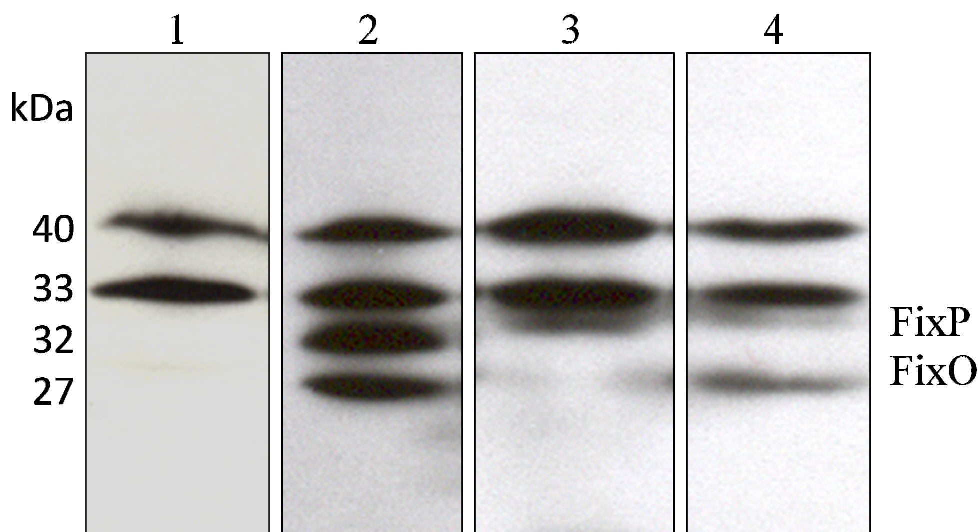
Figure 4.3. TMPD-dependent oxygen consumption capacity by whole cells of wild type *E. meliloti* 1021 (grey bars) and *fixN1* mutant (white bars) after 48 hours growth in minimal medium under aerobic or micro-oxic conditions. Error bars represent standard deviation of data from at least two different cultures assayed in triplicate.

4.2.4.2. Haem *c* staining analyses.

Iron present in haem groups that are covalently bound to proteins, such as *c*-type cytochromes, can be visualized by using a sensitive chemiluminescence assay (Vargas *et al*. 1993). Haem *c* staining of electrophoretically fractionated membrane preparations of *E. meliloti* 1021 grown under aerobic conditions revealed two bands of about 40 and 33 kDa (Fig. 4.4A, lane 1). In the membrane fractions of WT cells grown under micro-oxic conditions two additional stained bands at kDa values of 32 and 27 could be detected (Fig. 4.4A, lane 2). Profiles from the membrane fraction of microaerobically grown cells of the *fixN1* mutant showed that it lacked the *c*-type cytochromes of about 32 and 27 kDa (Fig. 4.4A, lane 3). Similarly, we could not detect the 32 kDa band in membrane fractions of a *fixP1* mutant (Fig. 4.4A, lane 4). However, the *c*-type cytochrome of about 27 kDa was present in membranes of the *fixP1* mutant

(Fig. 4.4A, lane 4). These results indicate that the 27 kDa and the 32 kDa *c*-type cytochromes only appear in *E. meliloti* cells grown under low oxygen conditions and they correspond to the *E. meliloti* FixP1 and FixO1 components, respectively, of the *cbb*$_3$-type cytochrome oxidase encoded by the *fixNOQP1* operon. The identification of the haem-stainable bands of approximately 40 and 33 kDa present in membranes of wild type cells grown under both aerobic and microaerobic conditions as well as in those of *fixN1* and *fixP1* cells grown under microaerobic conditions is at the moment unknown.

A)



B)

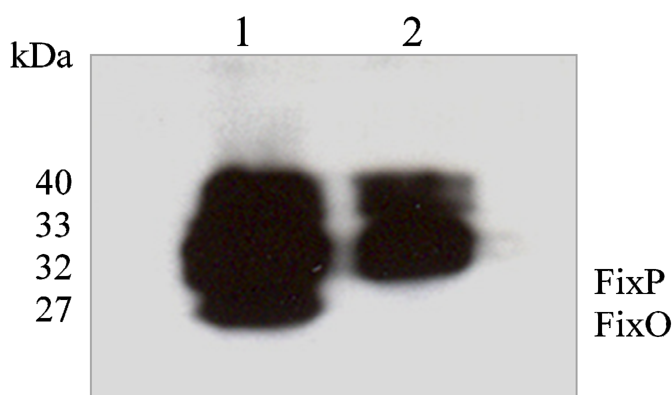


Figure 4.4. A) Heme *c* stained proteins in membranes prepared from wild type *E. meliloti* 1021 (lanes 1 and 2), *fixN1* mutant (lane 3) and *fixP1* mutant (lane 4). Cells were incubated aerobically (lane 1) or microaerobically (lanes 2, 3 and 4) in minimal medium. B) Heme *c* stained proteins in membranes of bacteroids of wild type *E. meliloti* 1021 (lane 1) and *fixN1* mutant (lane 2). Nodules were collected from 7 weeks grown alfalfa plants. In A and B, each lane contains about 20 mg membrane proteins. Apparent masses of the proteins (kDa) are shown at the left margin.

In the membrane fraction of bacteroids, the total amount of haem-stained proteins was considerably higher than that seen with membranes from free-living cells (Fig. 4.4A and B). Apparent masses of these haem-stained proteins suggest that they correspond to the 40, 33, 32 and 27 *c*-type cytochromes detected in membranes of free-living microaerobically grown cells of the WT strain (Fig. 4.4A lane 2 and B, lane 1). These results indicate that, as observed in microaerobically grown cells, bacteroids from *E. meliloti* 1021 produce the 32- and 27-kDa *c*-type cytochromes, corresponding to FixP and FixO components of the cbb_3 cytochrome oxidase. By contrast to free-living conditions, the band of about 33-kDa migrated together with FixP in membranes of bacteroids. Consequently, we could not demonstrate the absence of the 32 kDa FixP *c*-type cytochrome in membranes of *fixN1* bacteroids (Fig. 4.4A and B). However, the *c*-type cytochrome of 27 kDa corresponding to FixO was not detected in membranes from bacteroids of the *fixN1* mutant (Fig. 4.4B, lane 2).

4.2.4.3. Symbiotic phenotype of the *fixN1* mutant.

The *fixN1* mutant was used to inoculate alfalfa plants that were grown in N-free nutrient solution. After 3 weeks, the alfalfa plants that were not inoculated with any *E. meliloti* strains were short and turning yellow. The alfalfa plants that were inoculated with *E. meliloti* 1021 strain were tall and green and therefore had established an efficient symbiosis. However, most of the alfalfa plants inoculated with the *fixN1* mutant were shorter and lighter green than those inoculated with the wild type strain showing signs of nitrogen deficiency (Fig. 4.5). To further confirm the symbiotic deficiency of the *fixN1* mutant, physiological parameters, including nodulation capacity, shoot dry weight (SDW), total nitrogen content (N), and shoot and roots height (SH and RH) were measured in plants inoculated with the wild type or the *fixN1* mutant (Table 4.1). Non-inoculated alfalfa plants had no nodules (data not shown). Plants inoculated with either the wild type or the *fixN1* mutant had an average of four nodules per plant after 3 weeks (Table 4.1). Similarly, 100 % of the plants were nodulated by strains 1021 or *fixN1* (Table 4.1). However, plants inoculated with the *fixN1* mutant displayed significant decreases in SDW and [N] compared to those

inoculated with the wild type strain (38 % and 30 %, respectively) (Table 4.1). Similarly, SH and RH of plants inoculated with the *fixN1* mutant were significantly lower than those of plants inoculated with *E. meliloti* 1021 (46 % and 52 %, respectively) (Table 4.1). Taken together, these results demonstrate that *E. meliloti fixN1* mutant has not any defect in nodulation efficiency. However, this mutant shows a clear defect in symbiotic nitrogen fixation.

	SDW (mg plant^{-1})	[N] (mg N plant^{-1})	SH (cm plant^{-1})	RH (cm plant^{-1})	NNP	NP
WT	**13.9** (1.6) a	**0.570** (0.040) a	**9.31** (1.24) a	**32.95** (5.74) a	**4.36** (0.42) a	**100 % a**
fixN	**8.7** (0.7) b	**0.399** (0.057) b	**5.07** (0.96) b	**15.99** (2.34) b	**3.82** (0.52) a	**100 % a**

Table 4.1. Shoot dry weight (SDW), nitrogen content [N], shoot height (SH), root height (RH), nodules number per plant (NNP) and percentage of nodulated plants (NP) inoculated with the wild-type *E. meliloti* 1021 strain and the *fixN1* mutant derivative. Plants were grown for 3 weeks after inoculation. Data are means with the standard error in parentheses from at least one hundred different plants assayed in at least three independent experiments. In the columns, values followed by the different lower-case letter are significantly different as determined by the Tukey HSD test at $P \leq 0.05$.

To confirm further the symbiotic nitrogen fixation deficiency of the *fixN1* mutant, alfalfa plants inoculated with either the wild type or the *fixN1* mutant were grown over a longer period. Surprisingly, after seven weeks, alfalfa plants inoculated with *fixN1* were tall and green showing a similar aspect as those inoculated with the wild type strain (Fig. 4.6). To confirm these observations, SDW, [N], and nodulation capacity measured as nodule fresh weight (NFW) were determined in alfalfa plants after 7 weeks growth. As shown in Table 4.2, plants inoculated with the *fixN1* mutant had similar SDW, [N], and NFW than plants inoculated with the WT strain.

	SDW (mg plant^{-1})	[N] (mg N plant^{-1})	NFW (mg plant^{-1})
WT	281.1 (47.9) a	11.585 (0.564) a	35.5 (3.5) a
fixN	264.6 (52.0) a	11.616 (1.186) a	42.0 (4.0) a

Table 4.2. Shoot dry weight (SDW), nitrogen content [N] and nodule fresh weight (NFW) of plants inoculated with the wild-type *E. meliloti* 1021 and *fixN1* mutant derivative. Plants were grown for 7 weeks after inoculation. Data are means with the standard error in parentheses from at least seventy different plants, assayed in at least three independent experiments. In the columns, values followed by the same lower-case letter are not significantly different as determined by the Tukey HSD test at $P \leq 0.05$.

Figure 4.5. Nodulation kinetics of alfalfa plants inoculated with *E. meliloti* 1021 (●) or *fixN1* mutant (■) expressed as number of nodules for plant (A) or as a percentage of nodulated plants (B). Values are the mean from at least seventy different plants assayed in at least three independent experiments. Error bars represent standard deviations.

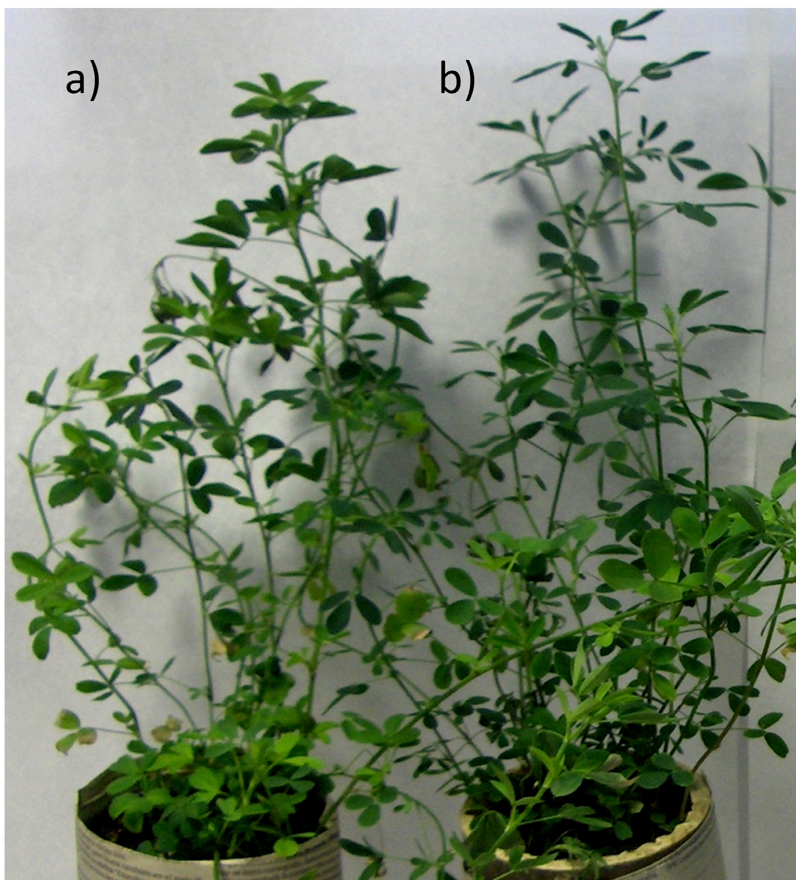


Figure 4.6. Nitrogen-fixation dependent growth of alfalfa plants inoculated with *E. meliloti* wild type (a) or *fixN*1 mutant (b) 49 days after inoculation.

4.2.5. Discussion.

The actual physiological role of the high affinity *cbb*$_3$ oxidase encoded by the *fixNOQP* operon in symbiotic nitrogen fixation has been investigated in many rhizobial species such as *B. japonicum* (Preisig *et al*. 1993; Preisig *et al*. 1996), *Azorhizobium caulinodans* (Mandon *et al*. 1993; Mandon *et al*. 1994), *Rhizobium leguminosarum* (Schlüter *et al*. 1997), and *Rhizobium etli* (Girard *et al*. 2000; Granados-Baeza *et al*. 2007). While *B. japonicum* and *A. caulinodans* have only one copy of the *fixNOQP* operon, reiteration of these genes has been reported in *R. leguminosarum* bv. *viciae* (Schlüter *et al*. 1997), *R. etli* (Girard *et al*. 2000) and in *Mesorhizobium loti* (Uchiumi *et al*. 2004). In *E. meliloti*, three copies of the *fixNOQP* operon have been identified (Fig. 4.1, http://genome.kazusa.or.jp/rhizobase/). The first copy is located in a DNA region containing also the whole set of regulatory genes (*FixLJ, fixK, fixT and fixM*) required for microaerobic respiration and nitrogen fixation. These observations suggest that copy 1 of *E. meliloti fixNOQP* genes is the potential candidate to support respiration under free-living and symbiotic conditions.

Free-living experiments have demonstrated the involvement of *fixNOQP1* genes in respiration and growth of *E. meliloti* cells under low oxygen conditions. Furthermore, chemiluminescent staining analyses used to visualize proteins that contain *c*-type cytochromes have demonstrated by the first time that the two membrane-bound *c*-type cytochromes, with molecular masses of 27 and 32 kDa, detected in microaerobically grown cells, correspond to the FixO and FixP components of the *E. meliloti cbb*$_3$ oxidase. The absence of these cytochromes in the *fixN1* mutant suggests that copy 1 of *fixNOQP* operon is the sole functional copy required to express the cytochrome *cbb*$_3$ terminal oxidase under free-living micro-oxic conditions. Supporting our findings, it has been recently proposed that *fixNOQP1* genes are regulated by FixJ under both microoxic free-living and symbiotic conditions, whereas copy 2, which is located next to the NifA regulon, was only detectable in bacteroids (Bobik *et al*. 2006). With respect to *fixNOQP3* operon, it was not induced under either microaerobic or symbiotic conditions (Bobik *et al*. 2006). Two genes from the *fixNOQP*$_3$ operon have been showed partially *phoB* regulated (Krol and Becker 2004). It has been proposed that the three copies of *fixNOQP* operon undergo differential regulation in *E.*

meliloti (Bobik *et al*. 2006) suggesting a different physiological role for them. It has been shown in *M. loti* that the *fixNOQP* copy located out of the symbiotic island functions preferentially in microaerobic environments, whereas bacteroids likely use two copies for symbiotic respiration (Uchiumi *et al*. 2004).
The haem-stained band of about 33 kDa also present in membranes of *E. meliloti* microaerobically grown cells is the predicted size for cytochrome c_1 that is a component of bc_1 complex. In *B. japonicum* (Thony-Meyer *et al*. 1989) and in *R. leguminosarum* (Wu *et al*. 1996) it has been demonstrated that bc_1 complex transfers electrons to the cbb_3 oxidase and is essential for symbiotic nitrogen fixation. The 40 kDa protein band had been previously detected in *E. meliloti* membranes by (Yurgel *et al*. 2007). A search for *E. meliloti* genes predicted to produce proteins that contain the CXXCH haem-binding motif and are in this molecular mass range allowed these authors to propose SMb21367 (*cycA*) or SMc02858 (a 41 kDa DnaJ-type protein) as potential candidates.

In this work we have also investigated the function of the *fixNOQP1* operon under symbiotic conditions. Nodulation kinetics, plant dry weigh and total nitrogen results in plants inoculated with the *fixN1* mutant and grown for 21 days in N-free nutrient solution clearly suggest that this copy is required for optimal fixation of nitrogen. However, symbiotic performance of alfalfa plants inoculated with the *fixN1* mutant and grown for 49 days was very similar to the plants inoculated with the wild type strain. It might be possible that for longer growth periods the other copies of *fixNOQP* are functional in symbiotic conditions. These results agree with those published previously by (Trzebiatowski *et al*. 2001) where a *E. meliloti* strain carrying a Tn*5-1063* insertion within *fixN* was symbiotically proficient suggesting that a second functional copy of *fixN* could be involved in symbiotic nitrogen fixation. In *R. leguminosarum* bv. *viciae*, both copies of *fixNOQP* genes are required for optimal nitrogen fixation (Schlüter *et al*. 1997). However, in *R. etli* a mutation in the *fixN* of plasmid d (but not in that of plasmid f) was severely affected, indicating a differential role for these reiterations in nitrogen fixation (Granados-Baeza *et al*. 2007).

The absence of the 27-kDa *c*-type cytochrome corresponding to FixO in membranes of bacteroids from nodules of seven weeks old plants inoculated with the *fixN1* mutant suggest that the copy one of *fixNOQP* genes is the sole functional copy responsible for expression of FixP and FixO proteins in bacteroids. By contrary to our observations, transcriptomic analyses have demonstrated expression of the copy 2 of *E. meliloti fixNOQP* operon in bacteroids (Bobik et al. 2006). However, these authors found that levels of induction of *fixNOQP2* genes by low oxygen conditions under free-living conditions is only 2-fold compared to 5-fold induction of *fixNOQP1* genes relative to expression levels under oxic conditions (Bobik *et al*. 2006). Hence, we do not exclude that the lack of detection of FixO in *fixN1* bacteroids where *fixNOQP2* genes might be expressed could actually be due to a technical limitation, given that overall sensitivity of the arrays is better than the haem-staining protein detection. Therefore on the long term, other copies could replace copy one through lower expression rates. Alternatively, after seven weeks some recombitation could take place with the remaining copies that complement a native-like *fixNOQP1* 1 operon. It might be also possible that other terminal oxidases such as the high-affinity *bd*-type oxidase or the *cyo* quinol oxidase are also involved in supporting nitrogenase activity in 7 weeks old plants. In this context, the *E. meliloti* chromosome contains *smc02254* and *smc02255* genes (http://genome.kazusa.or.jp/rhizobase/) which encode a high-affinity quinol oxidase that is likely to contribute to microoxic respiration in addition to or in the absence of the *cbb*$_3$ oxidase. Furthermore, recent transcriptional studies have reported that *cyo* genes encoding a cytochrome *o* ubiquinol oxidase were induced under microaerobic conditions (Bobik *et al*. 2006).

CHAPTER III

4.3. **Denitrification in *E. meliloti*.**

4.2.1. Abstract.

Denitrification is the complete reduction of nitrate or nitrite to N_2, via the intermediates nitric oxide (NO) and nitrous oxide (N_2O), and is coupled to energy conservation and growth under oxygen limiting conditions. In *Bradyrhizobium japonicum*, this process occurs through the action of the *napEDABC*, *nirK*, *norCBQD* and *nosRZDFYLX* gene products. DNA sequences showing homology with *nap*, *nirK*, *nor* and *nos* genes have been found in the genome of the symbiotic plasmid pSymA of *Ensifer meliloti* strain 1021. Whole genome transcriptomic analyses have demonstrated that *E. meliloti* denitrification genes are induced under microoxic conditions. Furthermore, *E. meliloti* has also been shown to possess denitrifying activities in both free-living and symbiotic forms. Despite possessing and expressing the complete set of denitrification genes, *E. meliloti* is considered as a partial denitrifier since it does not grow under anaerobic conditions with nitrate or nitrite as terminal electron acceptors. In this manuscript, we show that under microoxic conditions, *E. meliloti* is able to grow by using nitrate or nitrite as respiratory substrates, which indicates that, in contrast to anaerobic denitrifiers, oxygen is necessary for denitrification by *E. meliloti*. Current knowledge on the regulation of *E. meliloti* denitrification genes is also included.

4.2.2. Introduction.

The bacterial order *Rhizobiales* of the Alphaprotebacteria includes the genera *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Ensifer (Sinorhizobium*), among others. They are gram-negative soil bacteria collectively referred to as rhizobia with the unique ability to establish a N_2-fixing symbiotic association with legumes plants through the formation of a new differentiated organ called nodule on the roots and on the stems of some aquatic species (Willems, 2006; Zakhia and de Lajudie, 2006). The initiation of the rhizobia-legume symbiosis is a highly specific and complex developmental process, in which both partners undergo differentiation in a concerted way (Oldroyd and Downie, 2008). Within the nodules,

rhizobia transform into specialized cells, the so-called bacteroids, which synthesize the enzyme nitrogenase, which fixes atmospheric nitrogen (N_2) through its reduction to ammonia. Rhizobia have developed mechanisms to sense and adapt to changes in O_2 concentrations prevailing in the environment. Hence, many rhizobia species possess a branched electron transport chain, in which the terminal oxidases have different affinities for O_2 in order to survive under different environmental O_2 conditions (Delgado et al., 1998). In the microaerobic environment of the root nodule, rhizobia use the high affinity cytochrome *cbb₃* terminal oxidase to support the highly ATP-demanding nitrogen-fixation process (Delgado et al., 1998). When O_2 is limiting, some rhizobia species are able to switch from O_2-respiration to using nitrate to support ATP production by denitrification. During this process, nitrate (NO_3^-) is reduced into nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and N_2 through the action of the nitrate-, nitrite-, nitric oxide- and nitrous oxide reductase enzymes, encoded by *nar*/*nap*, *nir*, *nor* and *nos* genes, respectively (Van Spanning et al., 2007).

Many rhizobia species have genes for enzymes of some or all of the four reductase reactions for denitrification. In fact, denitrification can be readily observed in many rhizobia species in their free-living forms, in legume root nodules, or in isolated bacteroids (Delgado et al., 2007; Sánchez et al., 2011). *Bradyrhizobium japonicum*, the soybean symbiont, is considered the model organisms for studying rhizobial denitrification as it is the only rhizobial species able to denitrify under both free-living and symbiotic conditions. In *B. japonicum*, denitrification is dependent on the *napEDABC*, *nirK*, *norCBQD* and *nosRZDYFLX* genes that encode a periplasmic nitrate reductase, a Cu-containing nitrite reductase, a *c*-type nitric oxide-reductase and a nitrous oxide-reductase enzymes, respectively (Bedmar et al., 2005). In *B. japonicum*, a sophisticated regulatory network, consisting of two linked regulatory cascades, the FixLJ/$FixK_2$-NnrR and the RegSR/NifA systems, coordinates expression of denitrification genes in response to low oxygen conditions and the presence of nitrate (Torres et al., 2011). Under symbiotic conditions, *B. japonicum* denitrification contributes to NO and nitrosylleghaemoglobin (LbNO) production within soybean nodules in response to hypoxia (Meakin et al., 2007; Sanchez et al., 2010).

Ensifer meliloti is a rhizobia species which establishes symbiotic N_2-fixing associations with plants of the genera *Medicago*, *Melilotus* and *Trigonella*. *E. meliloti* had been shown previously to possess denitrifying activities in both free-living and symbiotic forms (O'Hara et al., 1983; Garcia-Plazaola et al., 1993; García-Plazaola, 1996). In fact, genes of the denitrification pathway, enabling the complete reduction of NO_3^- to N_2 are present in the *E. meliloti* genome. Furthermore, transcriptomic analyses have shown that *E. meliloti* denitrification genes were induced in response to oxygen limitation (Becker et al., 2004). However, and despite possessing the complete set of denitrification genes, *E. meliloti* has been considered a partial denitrifier due to its inability to grow under anaerobic conditions with nitrate or nitrite as terminal electron acceptors. In this work, we demonstrate by the first time that *E. meliloti* is able to grow under microoxic conditions using nitrate or nitrite as respiratory substrates.

4.2.3. Denitrification genes in *E. meliloti*.

Inspection of the *E. meliloti* 1021 genome sequence shows a composite architecture, consisting of three replicons with distinctive structural and functional: a 3.65-Mb chromosome and two megaplasmids, pSymA (1.35 Mb) and pSymB (1.68 Mb) (Galibert et al., 2001). pSymA contains a large fraction of the genes known to be specifically involved in symbiosis and genes likely to be involved in nitrogen and carbon metabolism, transport, stress and resistance responses that give *E. meliloti* an advantage in its specialized niche (Barnett et al., 2001). A 53-kb segment of pSymA is particularly rich in genes encoding proteins related to nitrogen metabolism, including a complete pathway for denitrification (Barnett et al., 2001). Among them, the *napEFDABC*-type (*sma1232, sma1233, sma1236* and *sma1239-41*) periplasmic nitrate reductase is present in this region (Fig. 5.1). The gene *sma1250* encodes a Cu-containing nitrite reductase, NirK, and is associated with a NirV-type protein which is encoded by *nirV* gene (*sma1247*) (Fig. 5.1). A nitric oxide reductase and nitrous oxide reductase encoded by *norECBQD* genes (*sma1269*, *sma1272*, *sma1273*, *sma1276* and *sma1279*) and *nosRZDFYLX* genes (*sma1179, sma1182-86* and *sma1188*), respectively, are also located in the pSymA (Fig. 5.1). The *nos* genes have been previously characterized by Chan and colleagues (Chan et al., 1997) and Holloway and colleagues (Holloway et al., 1996). Inoculation of alfalfa plants with *E. meliloti nos* mutants did not

affect N_2-fixing ability of the nodules, which demonstrates that *nos* genes are not essential for N_2 fixation (Holloway et al., 1996; Chan et al., 1997). The *hmp* gene is located downstream the *nos* genes and encodes a flavohaemoglobin which has recently been shown to be involved in NO detoxification in *E. meliloti* under free-living and symbiotic conditions (Meilhoc et al., 2010). Genes such as *nnrU* (*sma1283*), encoding a protein which is required for expression of *nir* and *nor* genes; *azu1 (sma1243*), that encode a blue copper protein associated with periplasmic nitrite reductase; *hemN* (*sma1266*), that encode a protein which is involved in haem maturation; *nnrR* (*sma1245*), encoding the CRP/FNR-like regulatory protein NnrR and the regulator NnrS encoded by *nnrS* gene (*sma1252*) are located on the 53-kb segment of pSymA (Fig. 5.1). Putative genes encoding the nitrate transport proteins NtrAB (SMa0583 and SMa0585) are located elsewhere on pSymA (Barnett et al., 2001).

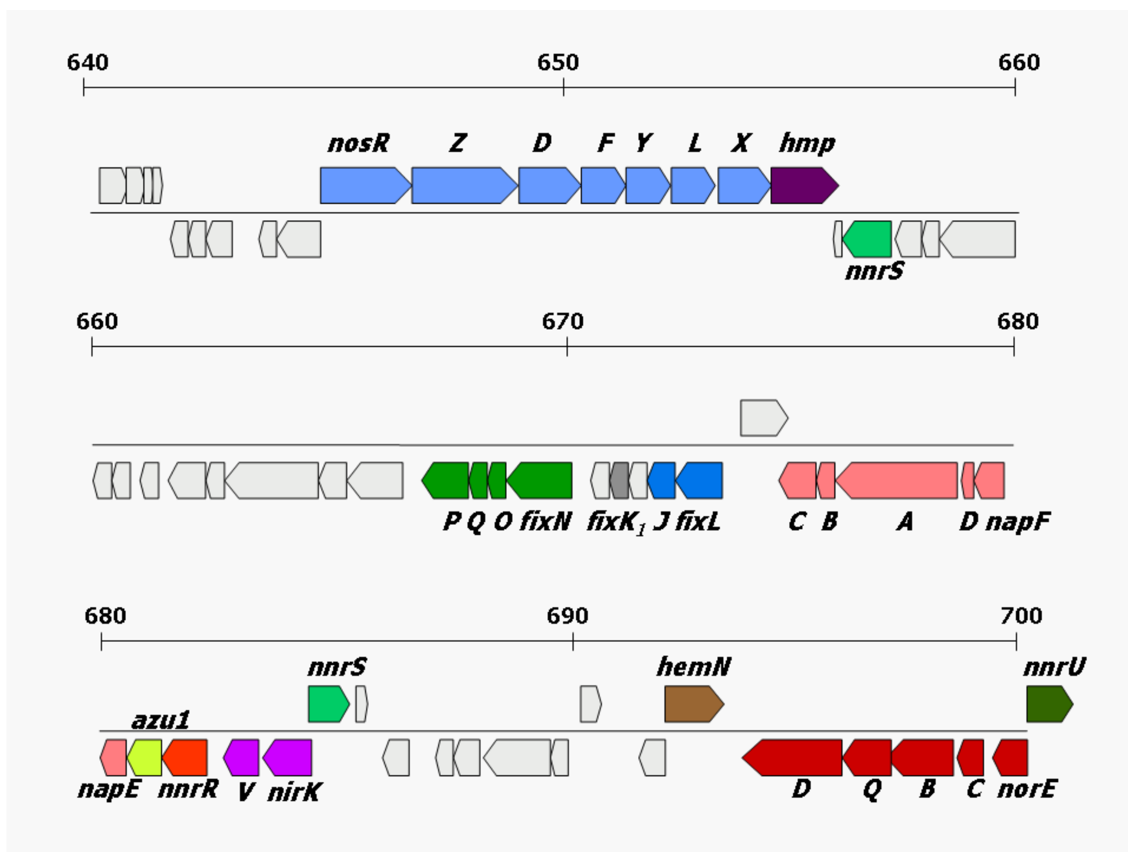


Figure 5.1: Genomic context of denitrification genes in the symbiotic plasmid pSymA of *S. meliloti*.

Transcriptomic studies have shown that oxygen limitation appears to be a key factor driving expression of *E. meliloti* denitrification genes (Becker et al., 2004). In *E. meliloti*, expression of genes in response to low oxygen conditions is coordinated via a two-component regulatory system, FixL/FixJ. Under microaerobic conditions, FixL autophosphorylates and transmits phosphate to the FixJ response regulator. Once phosphorylated, FixJ activates transcription of the *nifA* and *fixK* genes, encoding two intermediate regulators which induce expression of *nif* and *fix* genes involved in respiration and nitrogen fixation, respectively (Bobik et al., 2006). FixLJ, FixK and NifA are conserved among rhizobia, but they differ in connectivity and targets. In *E. meliloti*, microaerobic transcription of denitrification genes depends on FixLJ and FixK (Bobik et al., 2006). In contrast to *B. japonicum* where $FixK_2$ activates *nnrR*, the gene encoding the FNR/CRP-type regulator NnrR (Mesa et al., 2003), in *E. meliloti*, *nnrR* is not a target of the FixLJ/FixK regulatory cascade in response to low oxygen conditions (Bobik et al., 2006). *E. meliloti* NnrR-like transcriptional regulator controls *nirK* and *nor* genes as well as other genes related to denitrification (*nap*, *nos*, *azu1*, *hemN*, *nnrU*, *nnrS*) in response to nitric oxide (NO) (de Bruijn et al., 2006; Meilhoc et al., 2010). Whereas NnrR expands the FixLJ/$FixK_2$ regulatory cascade in *B. japonicum* (Torres et al., 2011) NnrR and FixK, are part of two different NO-responsive signaling pathways in *E. meliloti* (Meilhoc et al., 2010).

Recent findings have demonstrated that *E. meliloti napA* and *nirK* denitrification genes contribute about one-third to the nitric oxide generated in *Medicago truncatula* nitrogen-fixing nodules (Horchani et al., 2011).

4.2.4. Oxygen requirement for dentrification by *E. meliloti*.

In this work, we have investigated the denitrification ability of *E. meliloti* under microoxic and anoxic conditions. Results in Figure 5.2 confirmed that *E. meliloti* is unable to grow under anoxic conditions with nitrate or nitrite as terminal electron acceptors. When the cells were incubated under microoxic conditions (2% O_2 in the gas phase) with nitrate or nitrite in the culture medium, an increase in optical density at 600 nm (OD_{600}) was observed reaching a turbidity of 1.0 and 0.8, respectively, after 48

h growth. However, in cells grown without addition of nitrate or nitrite a slight increase in OD_{600} up to 0.4 was observed after 12 h incubation and it remained constant during the growth period. Figure 5.3 shows that nitrate was consumed during incubation under microoxic conditions, and nitrite was produced and accumulated in the incubation medium being further consumed by the cells. Maximal rates of nitrate consumption and nitrite productions were observed after incubation for 24 hours (Fig. 5.3). These results suggest that, after 24 h incubation under 2% O_2, activation of denitrification process in *E. meliloti* might take place (Fig. 5.2 and 3). When cells were incubated under anoxic conditions with nitrate, nitrite was not detected in the growth medium (Fig. 5.3), which confirms the inability of *E. meliloti* to denitrify under those conditions.

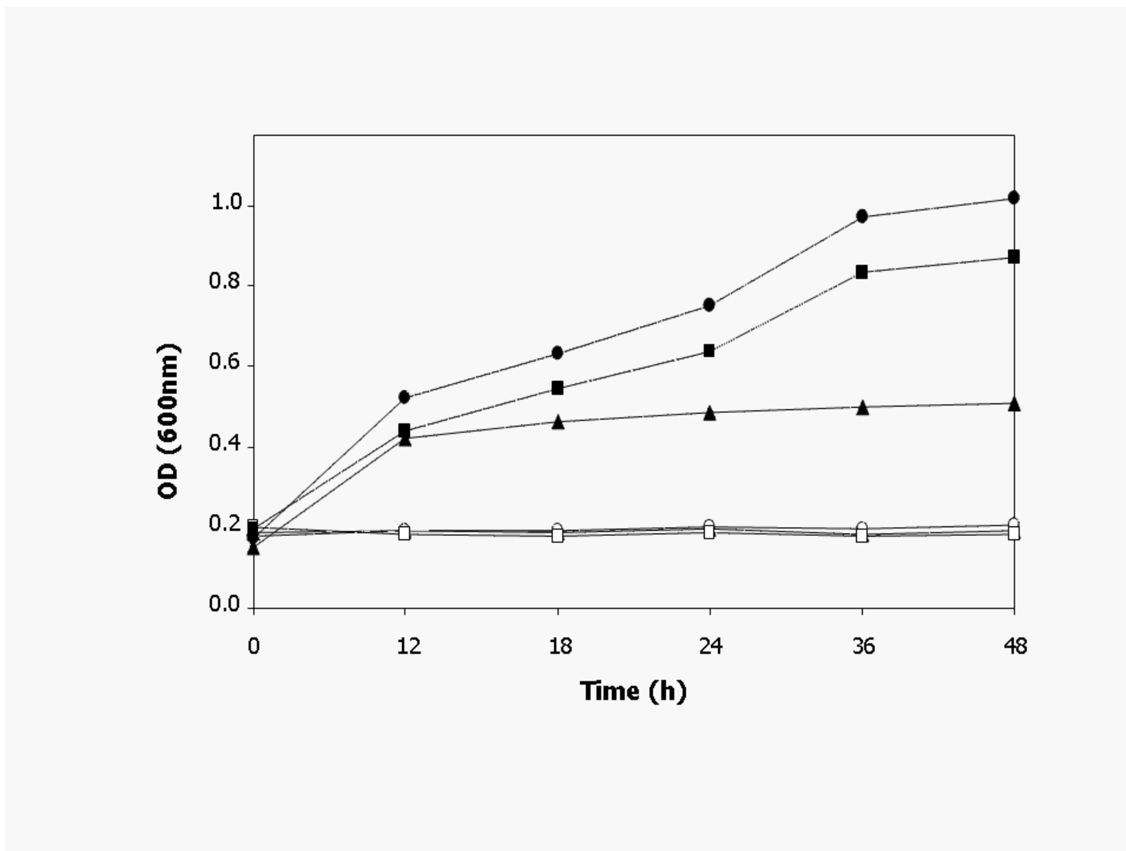


Figure 5.2: Growth of *S. meliloti* 1021 cells under microoxic (2% oxygen) (black symbols) or anoxic (white symbols) conditions without nitrate or nitrite (triangles), and with nitrate (circles) or nitrite (squares).

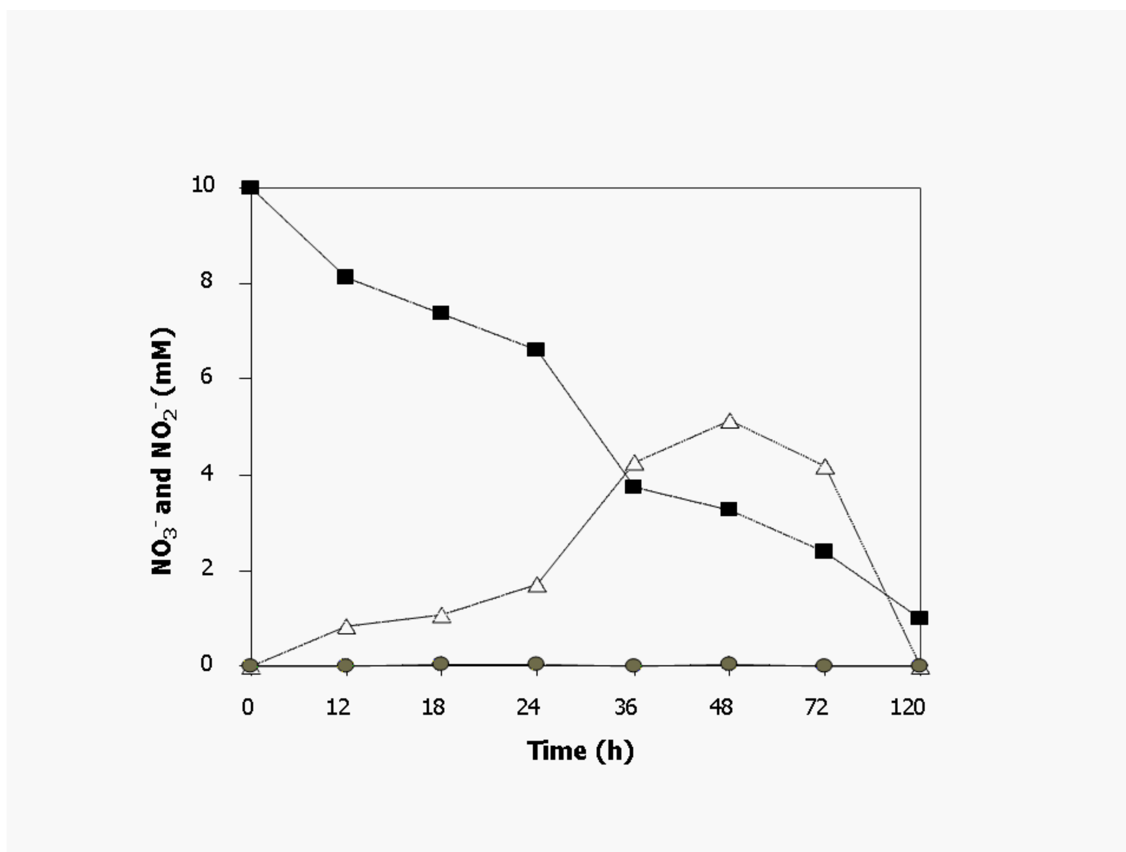


Figure 5.3: Nitrate uptake (black squares), and nitrite production (white triangles) under microoxic conditions. Nitrite production under anoxic conditions (grey circles). *S. meliloti* 1021 cells were cultured in minimal medium with 10 mM nitrate

E. meliloti was able to produce N_2O (28 μmol N_2O x mg protein^{-1} x min^{-1}) when grown under microoxic conditions with nitrate, indicating that this bacterium is able not only to reduce nitrate to nitrite but also to form N_2O under microoxic conditions. The nitrous oxide reductase enzyme is oxygen sensitive in many species. Therefore, aerobic denitrification is often incomplete, and an increase in N_2O formation has been observed when conditions switch from anaerobic to aerobic (Frette et al., 1997). However, in the case of *E. meliloti*, N_2 analyses are necessary to establish the ratio of N_2O/N_2 produced by cells grown under microoxic conditions with nitrate. It has been suggested that aerobic denitrification mainly occurs in environments of alternating oxic/anoxic conditions (Frette et al., 1997). Microorganisms capable of both aerobic and anaerobic denitrification would have the best chances of survival in those habitats (Gao et al., 2010).

Denitrification is generally accepted as an adaptative mechanism that works in anoxic environments. Usually, expression of the genes encoding the denitrifying enzymes is strictly repressed under oxic conditions, in which the bacteria can obtain sufficient energy through oxygen respiration (Van Spanning et al., 2007). Furthermore, post-translational inhibition by oxygen on denitrification enzymes and nitrate transport have also been reported (Hernandez et al., 1991; Zumft, 1997). However, by contrast to the strict requirement of anaerobic conditions for the expression of denitrification genes, in *E. meliloti* some O_2 is required for the induction of denitrification. Similarly, a minimal oxygen concentration is required for denitrification by the fungus *Fusarium oxysporum* (Zhou et al., 2001). *Pseudomonas aeruginosa* has also been shown to perform aerobic denitrification, this is, the simultaneous respiration using oxygen, nitrate and nitrite as terminal electron acceptors under aerobic conditions (Chen et al., 2006). Other aerobic denitrifiers belonging to the genera *Pseudomonas*, *Alcaligenes*, *Paracoccus*, and *Bacillus* have also been reported (Ozeki et al., 2001; Su et al., 2001; Kim et al., 2005; Wan et al., 2011).

No direct evidence can explain why some O_2 is required for induction of denitrification in *E. meliloti*. It might be possible that O_2 is necessary for oxygenase reactions involved in the biosyntheses of essential cell components, such as sterols, haems or unsaturated fatty acids. In this context, *Paracoccus denitrificans* requires cobalamin to express a cobalamin-dependent ribonucleotide reductase, which is essential for growth only under anaerobic conditions (Shearer et al., 1999). Since denitrification and oxygen respiration are two respiratory processes associated to the membrane, then they may share some components of the respiratory chain. Thus, it might be possible that both processes have to proceed simultaneously. However, the reason why the reduction of nitrate must be accompanied by O_2 respiration remains to be elucidated.

CHAPTER IV

4.4. **Functional characterization of *E. meliloti* denitrification genes.**

4.4.1. Abstract.

Denitrification is the complete reduction of nitrate or nitrite to N_2, via the intermediates nitric oxide (NO) and nitrous oxide (N_2O) and is coupled to energy conservation and growth under micro-oxic or anoxic conditions. *Ensifer meliloti* 1021 possesses the complete set of denitrification genes. However, a functional characterization of *E. meliloti nap*, *nirK*, *nor* and *nos* genes has not been reported so far. In this work, we have demonstrated the involvement of *nap*, *nirK* and *norC* genes in the ability of *E. meliloti* to grow by using nitrate as respiratory substrate under micro-oxic conditions. Haem-staining analyses have identified a membrane-bound *c*-type cytochrome of 16 kDa as the NorC component of the *E. meliloti* nitric oxide reductase. *E. meliloti napA*, *nirK* and *norC* mutants showed a significant defect in MV^+-dependent nitrate reductase (MV^+-NR), MV^+-dependent nitrite reductase (MV^+-Nir) and nitric oxide reductase (Nor) activity, respectively. A *nosZ* mutant accumulated N_2O when cultured under micro-oxic conditions in the presence of nitrate. These results demonstrate the involvement of *E. meliloti* denitrification genes in nitrate respiration and denitrification under micro-oxic conditions. In this work, we have also shown that *napA, nirK*, *norC* and *nosZ* were expressed not only under micro-oxic but also under anoxic conditions with nitrate. Furthermore, anoxically incubated cells also expressed MV^+-NR, MV^+-Nir, Nor and Nos activities. Thus, the inability of *E. meliloti* to grow under anoxic conditions with nitrate is not due to a defect on the expression of denitrification genes.

4.4.2. Introduction.

Denitrification is the respiratory reduction of N-oxides which enables facultative aerobic bacteria to survive and multiply under oxygen-limiting conditions. During this process the water-soluble nitrate (NO_3^-) is converted into molecular nitrogen (N_2) via nitrite and the gaseous intermediates nitric oxide (NO) and nitrous oxide (N_2O). N_2O is a powerful greenhouse gas (GHG) that has a 300-fold greater global warming

potential than CO_2, based on its radiative capacity, and can persist for up to 150 years in the atmosphere IPCC 2007, (Bates et al., 2008). The denitrification process requires four separate enzymatically catalyzed reactions. The first reaction of denitrification is catalyzed by a membrane-bound (Nar) or a periplasmic nitrate reductase (Nap) (reviewed in (Richardson et al., 2001; Gonzalez et al., 2006; Richardson et al., 2007; Kraft et al., 2011; Richardson, 2011). Nar proteins are encoded by *narGHJI* genes that are conserved in most species that express Nar. Eight different genes (*napDEABCFKL*) have been identified as components for operons that encode Naps in different organisms. Most of the operons comprise the *napABC* genes that encode the structural subunits where *napA* encodes the catalytic subunit containing the molybdopterin guanine-dinucleotide cofactor (MGD) and a 4Fe-4S cluster, *napB* an electron-transfer subunit, dihaem cytochrome *c*, and *napC* a membrane-bound *c*-type tetrahaem cytochrome. The remaining *napDEFKL* genes encode for different proteins that are not directly involved in nitrate reduction. In denitrifying bacteria, two types of respiratory Nir have been described: the NirS cd_1 nitrite reductase, a homodimeric enzyme with hemes *c* and d_1, and the NirK, a copper-containing Nir (van Spanning, 2005; Rinaldo and Cutruzzola, 2007; van Spanning, 2007; Rinaldo et al., 2008; van Spanning, 2011). Both are located in the periplasmic space, and receive electrons from cytochrome *c* and/or a blue copper protein, pseudoazurin, via the cytochrome bc_1 complex. They catalyze the one-electron reduction of nitrite to nitric oxide. While the *nirS* gene encoding the cd_1-Nir polypeptide is part of a *nirSMCFDLGHJEN* gene cluster, the *nirK* gene encodes the CuNir. Up to date, three types of Nor have been characterized and they are classified according to the nature of their electron donor as *c*-type nitric oxidoreductase (cNor), qNor and qCuANor (reviewed in (van Spanning, 2005; de Vries et al., 2007; van Spanning, 2007; Zumft and Kroneck, 2007; Richardson, 2011). The best-studied Nor is the cNor, an integral membrane enzyme harboring two subunits, NorC with a heme *c* group and NorB containing haems *b* and a non-haem iron. cNor is encoded by the *norCBQD* operon. The *norC* and *norB* genes encode subunit II and subunit I, respectively. The *norQ* and *norD* genes encode proteins essential for activation of cNor. Some more specialized denitrifiers have additional *norEF* genes, the products of which are involved in maturation and/or stability of Nor activity (Hartsock and Shapleigh, 2010). The final step in denitrification consists of the two-electron

reduction of nitrous oxide to dinitrogen gas. This reaction is performed by a copper containing homodimeric soluble protein located in the periplasmic space, the nitrous oxide reductase (Nos) (reviewed in (van Spanning, 2005, 2007; Zumft and Kroneck, 2007; van Spanning, 2011; Thomson et al., 2012). Nos is a homo-dimer of a 65 kDa copper-containing subunit. Each monomer is made up of two domains: the ''CuA domain'' and the '''CuZ domain''. The *nos* gene clusters often comprise the *nosRZDFYLX* genes. The *nosZ* gene encodes the monomers of Nos. The *nosDFYL* genes encode proteins that are apparently required for copper assemblage into Nos, although their specific role still remains unknown. The NosRX proteins have roles in transcription regulation, activation and electron transport to NosZ.

Bacteria of the order *Rhizobiales*, collectively referred to as rhizobia, are best characterized by their ability to establish a N_2-fixing symbiosis on legume roots and on the stems of some aquatic leguminous plants. In addition to fix N_2, many rhizobia species have genes for enzymes of some or all of the four reductase reactions for denitrification. However, up to date only *Bradyrhizobium japonicum* (Bedmar et al., 2005), *Azorhizobium caulinodans* (Raju et al., 1997) and *Ensifer meliloti* (Torres et al., 2011) have been reported to be able to grow using nitrate as electron acceptor to support respiration under low-oxygen conditions by performing complete denitrification. In *B. japonicum*, considered the model organism for studying rhizobial denitrification, this process is dependent on the *napEDABC*, *nirK*, *norCBQD* and *nosRZDYFLX* genes (Bedmar et al., 2005; Delgado, 2007; Sánchez, 2011). Denitrification can be observed in rhizobial species in their free-living forms and also in legume root nodules or in isolated bacteroids (García-Plazaola, 1996; Mesa et al., 2004; Meakin et al., 2007; Sanchez et al., 2010).

Ensifer (formerly *Sinorhizobium*) *meliloti* is a rhizobial species which establishes symbiotic N_2-fixing associations with plants of the genera *Medicago*, *Melilotus* and *Trigonella*. Genes of the complete denitrification pathway are present in the *E. meliloti* pSymA megaplasmid (Barnett et al., 2001; Torres et al., 2011). Furthermore, transcriptomic analyses have shown that *E. meliloti nap, nir, nor* and *nos* genes are induced in response to O_2 limitation (Becker et al., 2004). Under these conditions, denitrification genes expression is coordinated via a two-component regulatory system

FixLJ and via a transcriptional regulator, FixK (Bobik et al., 2006). Recent transcriptomic studies demonstrated that denitrification genes (*nirK* and *norC*), as well as other genes related to denitrification (*azu1*, *hemN*, *nnrU* and *nnrS*) are also induced in response to nitric oxide (NO) and that the regulatory protein NnrR is involved in such control (Meilhoc et al., 2010). In symbiotic association with *M. truncatula plants,* recent findings have demonstrated that *E. meliloti napA* and *nirK* denitrification genes contribute to nitric oxide production (Horchani et al., 2011). In contrast to all that has been carried out about regulation and symbiotic characterization of *E. meliloti* denitrification genes, the role of these genes under free-living conditions is not known.

Recent results from our group (Torres et al., 2011) have demonstrated that *E. meliloti* is able to grow by using nitrate or nitrite as respiratory substrates under micro-oxic conditions. In this work, phenotypic analyses of *E. meliloti* mutants lacking the *napA*, *nirK*, *norC* and *nosZ* denitrification genes have shown the involvement of those genes in nitrate respiration and denitrification under micro-oxic conditions.

4.4.3. Material and methods.

4.4.3.1. Bacterial strains and growth conditions.

Bacterial strains used in this study are listed in Table 6.1. *E. meliloti* strains were routinely grown aerobically at 30 ºC in Tryptone Yeast (TY) complete medium (Beringer, 1974) to obtain cellular mass. Cell incubation under oxygen-limiting conditions was performed in minimal medium (MM) (Robertsen et al., 1981) or in MM medium supplemented with 10 mM KNO_3 (MMN). Micro-oxic conditions were reached by fluxing a gas mixture (2 % oxygen, 98 % Ar) at incubation starting point into the gas atmosphere of rubber-stoppered 17 ml serum tubes or 500 ml flasks containing 5 or 200 ml medium, respectively. Anoxic conditions were obtained in completed filled 200 ml bottles or 17 ml tubes. Initial optical density at 600 nm of the cultures was about 0.1-0.15. Antibiotics were added to the cultures at the following concentrations (μg · ml^{-1}): streptomycin, 200; kanamycin, 200.

Table 6.1. Bacterial strains

Strain	Relevant characteristics	Reference
Ensifer meliloti		
1021	Wild type; Sm^r	Meade *et al.,* 1982
2011mTn5STM.3.02.F08	*napA*::mini-Tn5 Sm^r, Km^r	Pobigaylo *et al.,* 2006
2011mTn5STM.3.13.D09	*napC*::mini-Tn5; Sm^r, Km^r	Pobigaylo *et al.,* 2006
2011mTn5STM.1.13.B08	*nirK*::mini-Tn5; Sm^r, Km^r	Pobigaylo *et al.,* 2006
SmPl.1021.G1PELR32E8	*norC*::Pl.G1PELR32E8; Sm^r, Km^r	Becker *et al.,* 2009
2011mTn5STM.5.07.B03	*nosZ*::mini-Tn5; Sm^r, Km^r	Pobigaylo *et al*, 2006

4.4.3.2. Determination of nitrate reductase and nitrite reductase activity.

Cells of *E. meliloti* were incubated micro-oxically or anoxically during 18 hours in MM or MMN medium. Then, cells were harvested by centrifugation at 8000 *g* for 10 min at 4 °C, and washed with 50 mM Tris/HCl buffer (pH 7.5) until no nitrite was detected, and then resuspended in 0.5 ml of the same buffer. MV^+-NR activity was analyzed essentially as described by Delgado and colleagues (2003) (Delgado et al., 2003). For determination of MV^+-Nir activity, the reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 100 mM $NaNO_2$, 800 mM methyl viologen and 100 µl of cell suspension (0.02–0.04 mg of protein). The reaction was started by the addition of 50 µl of freshly prepared sodium dithionite solution (30 mg·ml^{-1} in 300 mM $NaHCO_3$). After incubation for 20 min at 30 °C, the reaction was stopped by vigorous shaking until the samples had lost their blue colour.

4.4.3.3. Haem-*c* analyses.

Cells of *E. meliloti* grown aerobically in 150 ml TY medium were harvested by centrifugation at 8000 g for 5 min, washed twice with MM, resuspended in 200 ml of MM or MMN at $O.D_{600}$ of 0.15-0.2 and finally incubated under micro-oxic (2 % O_2, 98 %

Ar) or anoxic (filled bottles) conditions for 24 hours. Cell pellets were resuspended in 3 ml of 50 mM potassium phosphate buffer (pH 7) containing 100 μM 4-(2-aminoethyl) benzene-sulfonyl flouride hydrochloride (ABSF), RNAse (20 $\mu g \cdot ml^{-1}$) and DNAse I (20 $\mu g \cdot ml^{-1}$). Cells were disrupted using a French pressure cell at a constant pressure of about 1000 psi (SLM Aminco, Jessup, MD, USA). The cell extract was centrifuged at 10000 g for 20 min to remove unbroken cells and the supernatant was then centrifuged at 140000 g for 1 h. The membrane pellet was resuspended in 100 µl of the same buffer. Membrane protein aliquots were diluted in sample buffer [124mM Tris-HCl, pH 7.0, 20 % glycerol, 4.6 % sodium dodecyl sulfate (SDS) and 50 mM 2-mercaptoethanol] and incubated at room temperature for 10 min. Membrane proteins were separated at 4 ºC in 12 % SDS--polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and stained for haem-dependent peroxidase activity as described previously (Vargas et al., 1993) using the chemiluminiscence detection kit 'SuperSignal' (Pierce, Thermo Fisher Scientific, IL, USA).

4.4.3.4. Analytical methods.

Nitrite concentration was estimated after diazotization by adding the sulfanilamide/naphthylethylene diamine dyhydrochloride reagent (Nicholas and Nason, 1957). Protein concentration was estimated by using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) with a standard curve of varying bovine serum albumin concentrations.

4.4.3.5. Nitric oxide determination.

Cells of *E. meliloti* were incubated micro-oxically or anoxically in MMN, harvested and washed similarly as for NR or Nir activity assays. Nitric oxide (NO) was measured amperometrically with a 2-mm ISONOP electrode APOLO 4000® (World Precision Inst., Sarasota, FL, USA) in a 3 ml thermostatted and magnetically stirred reaction chamber (Zhang and Broderick, 2000). The membrane-covered electrode was situated at the bottom of the chamber above the stirrer and reactants were injected with a Hamilton syringe through the port in the glass stopper. For determination of NO production rates, the 3 ml cuvette was filled with 1.410 ml of 25 mM phosphate buffer (pH 7.4), 250 µl (0.1-0.2 mg protein) of cellular solution, 100 µl of an enzymatic mix

containing glucose oxidase (*Aspergillus niger*) (80 units/2ml) and catalase (bovine liver) (500 units/2 ml), 90 µl sodium succinate 1M and 100 µl of glucose 320mM. When oxygen was consumed and a steady base line was observed, 50 µl of $NaNO_2$ 1 M was added to the cuvette to begin the reaction. Each assay was run until NO was detected. For determination of NO consumption rates, the electrode chamber was filled with 1.655 ml of 25 mM phosphate buffer (pH 7.4), 5 µl (0.02-0.04 mg protein) of cellular solution, 100 µl of an enzymatic mix containing glucose oxidase (*Aspergillus niger*) (80 units/2ml) and catalase (bovine liver) (500 units/2 ml), 90 µl sodium succinate 1M and 100 µl of glucose 320 mM. Once a steady base line was observed, 50 µl of a saturated NO solution (1.91 mM at 20 ºC) was added to the cuvette to begin the reaction. Each assay was run until NO detection had dropped to zero, that is, when all NO was consumed.

4.4.3.6. Nitrous oxide determination.

Cells of *E. meliloti* were incubated micro-oxically or anoxically in MMN. After 18 or 36 hours incubation, gaseous alicuots of 500 µl were taken from the micro-oxically culture headspaces for N_2O determination. In anoxic cultures, headspace was created by transferring 10 ml of liquid culture into a 20 ml headspace vial (Supelco®). Gas-liquid phase equilibration was made keeping them during 2 h at 30 ºC. In order to stop cell growing, 200 µl of $HgCl_2$ 1mg · ml^{-1} was added to each headspace vial. N_2O production in liquid cultures was corrected with dissolved N_2O Bunsen solubility coefficient (47.2% at 30 ºC). Then, N_2O was measured with a gas chromatograph type HP 4890D equipped with an electron capture detector (ECD). The column was packed with Porapak Q 80/100 MESH (6ft) and the carrier gas was N_2 at a flow rate of 23 ml/min. The injector, column and detector temperatures were 125, 60 and 375 ºC, respectively. N_2O peaks were integrated using GC ChemStation Software (Agilent Technologies© 1990-2003). The samples were injected manually through a Hamilton® Gastight syringe. Concentrations of N_2O in each sample were calculated from standards of pure nitrous oxide (Air Liquid, France).

4.4.3.7. Quantitative Real-Time PCR analysis.

For immediate stabilization of bacterial RNA, the RNAprotect Bacteria Reagent (Qiagen USA, Valencia, CA) was added directly to cells incubated micro-oxically or anoxically during 12 hours in MM or MMN. Bacterial lysis was performed by resuspension and incubation of the cell pellet in 1 mg/mL lyzozyme from chicken egg white (Sigma) in Tris-EDTA buffer pH 8.0. Total RNA was isolated using the RNeasy Mini kit (Qiagen). Isolated RNA was submitted to DNase (Qiagen) treatment. RNA was quantified on a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and intactness was verified by visual inspection of rRNA banding in electrophoretically separated total RNA (Sambrook et al., 1989). Reverse transcription reaction was performed on 0.8 μg of total RNA per reaction of the First Strand cDNA Synthesis kit for RT-PCR (Roche), using random hexamers. The cDNA synthesis reaction mixture was diluted 50 times with distilled water before use in real-time PCR analysis.

Primers for the PCR reactions were designed using the PrimerExprmess v3.0 software (PE Applied Biosystems, Foster City, CA, USA), to have a melting temperature of about 57 ºC to 62 ºC and to give a PCR product of about 50 to 100 bp. Primer sequences were as follows: *napA* (Forward, 5´-CCGGCTATCGTGGCAAGA-3´; Reverse, 5´-CGGGAAGCTGTCGACATTG-3´); *nirK* (Forward, 5´-CCGCGCGACGCAAA-3´; Reverse, 5´-TCGAGCGTATCGGCATAGG-3´); *norC* (Forward, 5´-AGCTCACAGAGCAGGAACTGAAC-3´; Reverse, 5´-TGATGCGGCTCGTCCATT-3´); and *nosZ* (Forward, 5´-CGAGGATCTCACGCATGGAT-3´; Reverse, 5´-GCGGTGCAACCTCCATGT-3´). *sMC00128* was used as internal standard (Krol and Becker, 2004; Glenn et al., 2007) (Forward, 5´-ACGAGATCGAGATCGCCATT-3´; Reverse, 5´-CGAACGAGGTCTTCAGCATGA-3´).

Each PCR reaction contained 7.5 μl of SYBR Green PCR master mix (PE Applied Biosystems), 5 μl of cDNA and different final concentration of each primer depending on the studied gene. This concentration was 0.2 μM for *norC* and *sMC00128*, and 0.4 μM for *napA*, *nosZ* and *nirK*. Final volume of PCR reactions was 15 μl. Real-time PCR reactions were run in a 7300 Real Time PCR System (PE Applied Biosystems). The initial denaturing time was 10 min., followed by 40 PCR cycles consisting of 95 ºC 15 s and 60 ºC 60 s. A melting curve was run after PCR cycles. Relative quantification was

performed using the comparative C_T method for *nirK* gen, and the standard curve method for the other genes. Data were analyzed using the 7300 System Software (PE Applied Biosystems).

4.4.4. Results.

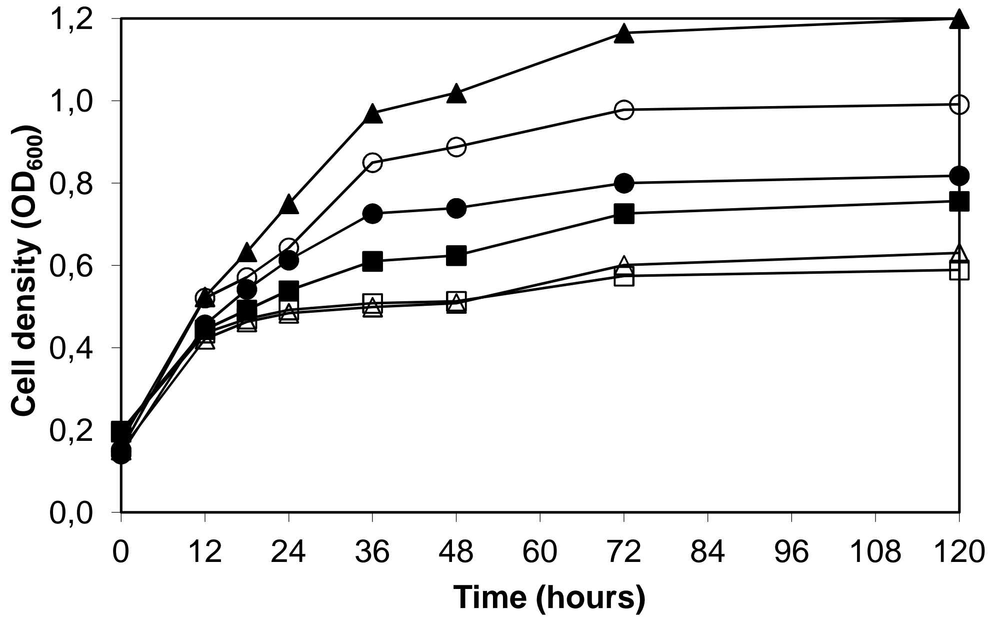
4.4.4.1. Nitrate-dependent growth under microoxic conditions.

To investigate the involvement of denitrification genes in the ability of *E. meliloti* to grow under micro-oxic conditions by using nitrate as electron acceptor, *E. meliloti napA*, *nirK*, *norC* and *nosZ* mutants (Table 6.1) were incubated micro-oxically in MM and MMN medium, and growth was determined by monitoring the optical density at 600 nm (OD_{600}) (Figs. 1A and 2). In contrast to *E. meliloti* 1021 wild-type (WT) strain, cells of the *napA* and *nirK* mutant strains showed a growth defect reaching a turbidity of about 0.72 and 0.80, respectively, compared with that of 1.16 determined in WT cells after 3 days incubation under micro-oxic conditions with nitrate (Fig. 6.1A). While WT and *napA* cells showed similar growth rates when the medium was not amended with nitrate (OD_{600} of 0.6), cells of the *nirK* mutant had increased growth rates (OD_{600} of about 1) under low oxygen conditions without nitrate (Fig. 6.1A). It might be possible that a mutation in the NirK enzyme increases electron flow from bc_1 complex to the cytochrome *c* oxidase resulting in an increased respiration capacity and consequently in better growth. In fact, cells of the *nirK* mutant had greater respiratory capacity than WT or *napA* cells after growth under micro-oxic conditions (data not shown).

As shown in Fig. 6.1B, nitrite was not observed in the *napA* growth medium supplemented with 10 mM nitrate. However, nitrite was accumulated in the culture medium of the WT and *nirK* mutant as a consequence of nitrate reduction (Fig. 6.1B). The *nirK* mutant accumulated around 8.26 mM nitrite in the growth medium after 120 h incubation indicating that almost all nitrate present in the culture medium was reduced to nitrite and nitrite was not further reduced. These observations suggest that in the *nirK* mutant Nir was not active *in vivo*. However, nitrite produced by the WT cells after 48 h incubation (5.14 mM) was consumed decreasing its concentration in the growth medium to zero after 120 h incubation (Fig. 6.1B). These results suggest that *E.*

meliloti nap and *nirK* genes are involved in *E. meliloti* nitrate-dependent growth under micro-oxic conditions. Under these conditions, nitrite derived from nitrate is produced by Nap and *nirK* is involved in nitrite reduction.

A)



B)

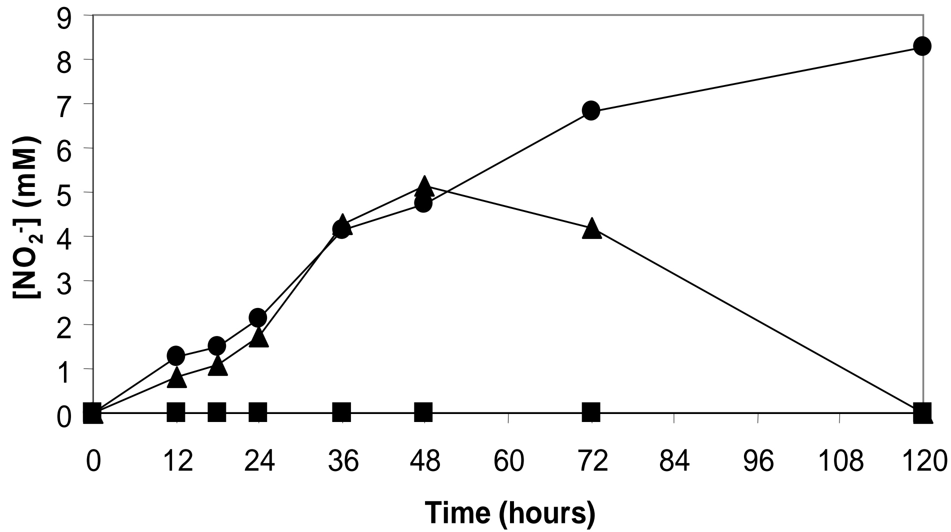


Figure 6.1. (A) Growth under micro-oxic (open symbols) or micro-oxic with nitrate (closed symbols) conditions and (B) extracellular nitrite concentration of wild-type *E. meliloti* 1021 (▲,Δ), and *napA* (■,□), and *nirK* (●, o) mutant strains. Shown are representative curves of three independent experiments run in triplicate.

As observed in Figure 6.2, a *norC* mutant strain had a significant growth defect when cells were incubated under micro-oxic conditions with nitrate reaching an OD_{600} of only 0.42 compared to that of 0.96 observed in wild-type cells under the same conditions. By contrast, cells of a *E. meliloti nosZ* mutant grew well when cultured micro-oxically with 10 mM KNO_3 reaching similar growth rates than WT cells (Fig. 6.2) suggesting that *nosZ* is not essential for growth under these conditions.

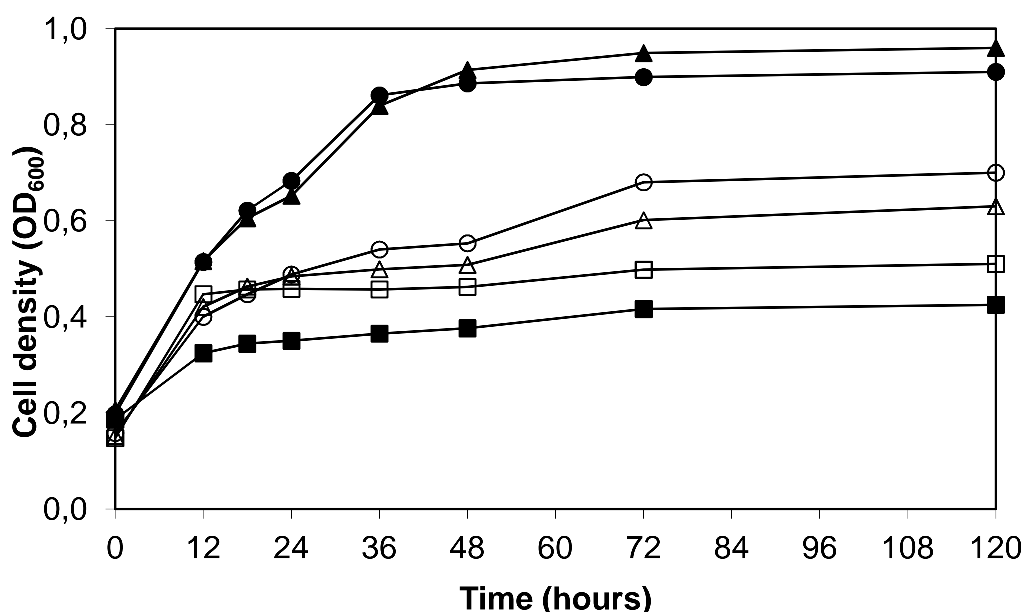


Figure 6.2. Growth under micro-oxic (open symbols) or micro-oxic with nitrate (closed symbols) conditions of wild-type *E. meliloti* 1021 (▲,Δ), and *norC* (■,□), and *nosZ* (●, o) mutant strains. Shown are representative curves of three independent experiments run in triplicate.

4.4.4.2. Activity of Nap, Nir, Nor and Nos enzymes under microoxic conditions.

Cells of the *napA* mutant incubated under micro-oxic conditions showed about 21-fold decrease of MV^{+}-NR activity compared to that observed in WT cells (Table 6.2). Similarly, levels of activity in *napA* cells incubated under low-oxygen concentration and in the presence of nitrate were about 11-fold lower than those detected in WT cells (Table 6.2). These results suggest that about 95% and 91% of the activity observed in

micro-oxic and micro-oxic with nitrate WT grown cells is due to the periplasmic nitrate reductase encoded by *napA*. MV^+-NR activity in WT cells was induced by low-oxygen conditions about 10 times compared to that detected in cells grown under fully oxic conditions (35.05 ± 1.80 nmol NO_2^- produced · mg of protein^{-1} · min^{-1}). However, cells of the *napA* mutant did not show an induction of MV+-NR in response to micro-oxic conditions since levels of activity under these conditions were very similar to those observed under oxic conditions (19.02 ± 1.31 nmol NO_2^- produced · mg of protein^{-1} · min^{-1}). Surprisingly, when nitrate was added to the growth medium of WT cells incubated under micro-oxic conditions, a slight decrease of MV+-NR activity (36 %) was observed in WT cells compared to that observed in the absence of nitrate (Table 6.2). These results indicate that in *E. meliloti*, low-oxygen conditions are required to induce Nap activity but this induction is not nitrate dependent. By contrast to MV^+-NR activity, the presence of nitrate in the micro-oxic incubation medium induced around 6-fold levels of MV^+-Nir activity in WT cells (Table 6.2). Under these conditions, levels of activity in cells of the *nirK* mutant were 10-fold lower than those detected in the parental strain (Table 6.2). These results suggest that the *E. meliloti nirK* gene is responsible for the Nir activity and it probably requires, besides oxygen limitation, the presence of nitrate or another derived nitrogen oxide for its maximal expression.

Table 6.2. MV^+-dependent nitrate reductase (MV^+-NR) and nitrite reductase (MV^+-Nir) activities of wild-type (WT) *E. meliloti* 1021, and *napA* and *nirK* mutant strains.

Growth conditions Strain	**Genotype**	**MV^+-NR[a]** (nmol NO_2^- produced· mg protein^{-1}·min^{-1})	**MV^+-NiR[a]** (nmol NO_2^- consumed·mg protein^{-1}·min^{-1})
Micro-oxic			
1021	WT	329.75 (14.76)	5.93 (1.03)
STM.3.02.F08	*napA*	15.56 (0.59)	ND
STM.1.13.B08	*nirK*	ND	2.68 (0.16)
Micro-oxic + nitrate			
1021	WT	210.93 (10.33)	32.57 (1,42)
STM.3.02.F08	*napA*	18.86 (3.79)	ND
STM.1.13.B08	*nirK*	ND	3.34 (0.26)

[a]MV$^+$-NR and MV$^+$-Nir activities were measured after 18 hours incubation under micro-oxic conditions in MM and MMN. Data are means with the standard error in parentheses from at least two different cultures, assayed in triplicate. ND = not determined.

We have also investigated the capacity of the *E. meliloti nirK* and *norC* mutants to produce nitric oxide. After 18 h incubation under micro-oxic conditions with nitrate, NO production rates were determined in an NO-electrode chamber after adding nitrite to the reaction mixture. As shown in Table 6.3A, a significant decrease in NO production was observed in the *nirK* mutant respect to that observed in the wild-type, suggesting that this mutant is not able to produce NO from nitrite reduction. By opposite, the *norC* mutant produced 4.6-fold more NO than the WT cells (Table 6.3A). The high levels of NO produced by the *norC* mutant are probably due to the defect in NO consumption activity. In fact, cells of the *norC* mutant incubated under micro-oxic conditions with nitrate showed a practically abolished NO consumption activity compared to that detected in WT cells (Table 6.3A). These results indicate that the respiratory CuNir encoded by *nirK* is the enzyme involved in NO production and the cNor encoded by *norC* is responsible of Nor activity in *E. meliloti* cells incubated under micro-oxic conditions with nitrate.

Table 3. (A) NO production and consumption activities and, (B) N_2O accumulation by wild-type (WT) *E. meliloti* 1021, and *nirK, norC* and *nosZ* mutant strains incubated under micro-oxic conditions with nitrate.

A)

Strain	**Genotype**	**NO production** [a] (nmol NO·mg protein^{-1}· min^{-1})	**NO consumption** [a] (nmol NO·mg protein^{-1}· min^{-1})
1021	WT	202.06 (14.79)	563.33 (21.81)
STM.1.13.B08	*nirK*	0.57 (0.19)	528.26 (20.86)
G1PELR32E8	*norC*	943.32 (4.52)	1.11 (0.01)

B)

Strain	**Genotype**	**N_2O accumulation** [b] (µmol N_2O·mg protein^{-1})	
		18 h	36 h
1021	WT	22.92 (2.01)	169.51 (16.01)
G1PELR32E8	*norC*	0.33 (0.11)	0.03 (0.01)
STM.5.07.B03	*nosZ*	185.78 (21.95)	353.11 (27.83)

[a]NO production and consumption activities were measured in cells harvested from cultures after 18 hours incubation, and [b]N_2O accumulation was measured in the headspace of the cultures after 18 and 36 hours incubation under micro-oxic conditions in MMN medium. Data are means with the standard error in parentheses from at least two different cultures, assayed in triplicate.

As previously reported (Torres et al., 2011), Table 6.3B shows that *E. meliloti* 1021 grown under micro-oxic conditions with nitrate is able to produce N_2O. Under these conditions, the *norC* mutant showed a clear defect in the capacity to produce N_2O after 18 or 36 h incubation compared to WT cells indicating that N_2O produced by the cells is due to the cNor activity (Table 6.3B). By contrast, cells of the *nosZ* mutant reached values of N_2O accumulation about 8-fold and 2-fold higher than those produced by the WT cells after 18 and 36 h incubation, respectively, under microoxic conditions with nitrate (Table 6.3B). These data suggest that *E. meliloti nosZ* mutant probably has a significant defect in the capacity to reduce N_2O and, therefore, this gas was accumulated in the headspace of the cultures. Although N_2 production was not determined in our experiments, the marked differences in N_2O production between the WT and the *nosZ* mutant suggests than N_2O is being consumed by NosZ enzyme in wild type cells and probably transformed to N_2.

4.4.4.3. Haem-*c* analysis.

Proteins from the membrane fraction of wild-type and *napC* and *norC* mutant strains were separated by SDS-PAGE and stained for covalently bound haem proteins in order to identify the *E. meliloti c*-type cytochromes corresponding to NapC and NorC. As previously reported by Torres *et al*. (Torres et al., 2013), four stained bands of 40, 33, 32, and 27 kDa were detected in *E. meliloti* 1021 cells grown under micro-oxic conditions (Fig. 6.3, lane 1). Although the identity of the 40 and 33 kDa proteins is at the moment unknown, the 32 kDa and 27 kDa *c*-type cytochrome have been identified as the *E. meliloti* FixP and FixO proteins, respectively, which are subunits of the cbb_3-type high-affinity cytochrome *c* oxidase encoded by the *fixNOQP* operon (Torres et al., 2013). The addition of nitrate to the growth medium revealed a haem-stainable band of approximately 16 kDa in membranes of wild-type cells (Fig. 6.3, lane 2). This protein was absent in the *norC* mutant incubated micro-oxically with nitrate (Fig. 6.3, lane 3), which identifies this *c*-type cytochrome as the NorC component of *E. meliloti* 1021

nitric oxide reductase. As shown in Fig. 6.3 (lane 4), membranes from a *napC* mutant presented a similar bands pattern than those from the wild type cells incubated under micro-oxic conditions with nitrate (Fig. 6.3, lanes 2 and 4). These results did not allow us to identify *E. meliloti* NapC protein which has a predicted size of 25 kDa. By contrast, in other rhizobia species such as *B. japonicum*, NapC has been detected by haem-staining analyses and identified as a protein of about 25 kDa (Delgado et al., 2003). As shown in Fig. 6.3 (lanes 1 and 2), NorC was detected in WT cells when nitrate was present in the medium. In addition to WT cells, NorC was also detected in membranes from the *napC* mutant incubated micro-oxically with nitrate (Fig. 6.3 lanes 2 and 4). However, densitrometric analysis of the 16 kDa band from *napC* cells indicated that it was about 3-fold lower than in those from WT cells. Since NapC is the electron donor for nitrate reduction *in vivo*, it might be possible that nitrate and a derived nitrogen oxide product of its reduction are both involved in the maximum expression of NorC under micro-oxic conditions.

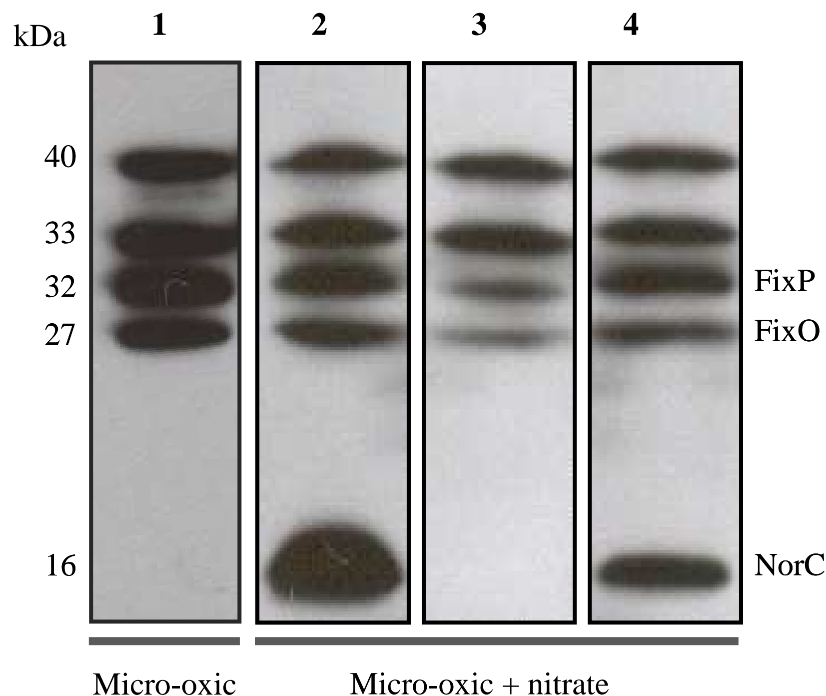


Figure 6.3. Haem-stained proteins of membranes prepared from wild-type *E. meliloti* 1021 (lane 1 and 2), and *norC* (lane 3) and *napC* (lane 4) mutant strains. Cells were incubated during 24 h under micro-oxic conditions in MMN (lane 2, 3 and 4) or MM (lane 1) medium. Each lane contains 25 µg membrane proteins. Haem-stained *c*-type cytochromes identified previously (FixP and FixO) and in this work (NorC) are specified at the right margin. Apparent masses of the proteins (kDa) are shown at the left margin.

4.4.4.4. Expression of *E. meliloti* denitrification genes under anoxic conditions.

In this work, we also investigated the capacity of *E. meliloti* to express the denitrification genes under anoxic conditions. By performing qRT-PCR analyses we have found that *napA, nirK, norC* and *nosZ* genes were induced about 4-, 48-, 84- and 32-fold by anoxia and nitrate relative to micro-oxic conditions without nitrate (Fig. 6.4). Except for the case of *nirK* expression that was induced 36-fold by micro-oxia and nitarte, it is worth noting that the presence of nitrate in micro-oxically grown cultures provoked an induction of only 1.46, 3.59 and 4.17-fold of *napA*, *norC* and *nosZ* expression compared to that observed in the absence of nitrate (Table 6.4). These results demonstrated that anoxia and the presence of nitrate are required for maximal expression of *E. meliloti napA*, *nirK*, *norC* and *nosZ* denitrification genes.

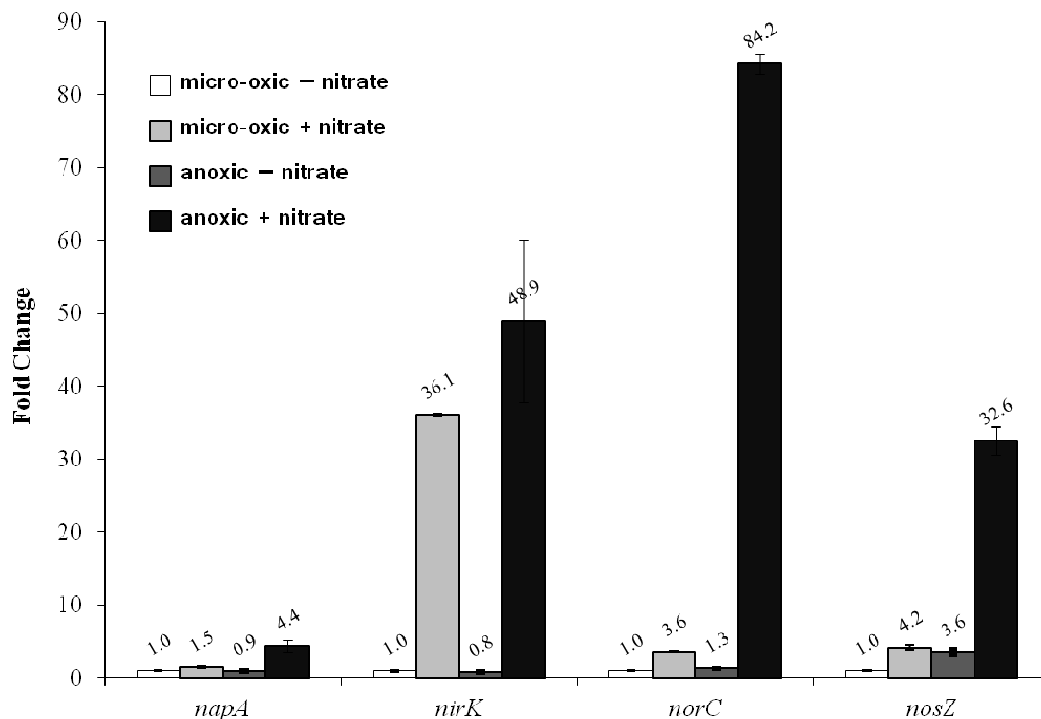


Figure 6.4. Expression of *E. meliloti* denitrification genes under micro-oxic and anoxic conditions. Transcription levels were quantified by qRT-PCR using total RNA samples as templates. RNA samples were prepared from *E. meliloti* 1021 cells incubated for 12 h under micro-oxic or anoxic conditions in MM and MMN medium. Data were analyzed by the standard curve method (*nirK* data were analyzed by the comparative C_T method) and the expression levels were normalized against the *E. meliloti smc00128* gene as an internal standard. The values are means of three independent experiments with standard deviations.

Haem-staining analyses in proteins from membranes of *E. meliloti* 1021 cells incubated under anoxic conditions showed a strong defect of FixP and FixO expression compared to micro-oxic conditions (Fig. 6.5, lanes 1 and 3). Only the proteins of about 40 and 33 kDa could be detected in anoxically-incubated cells. It has been reported that the 40 and 33 kDa proteins are also present in cells growing under oxic conditions (Torres et al., 2013). It might be possible that these proteins remain in the membranes from the cells that were grown aerobically previously to anoxic incubation. As observed in cells incubated under micro-oxic conditions with nitrate (Fig. 6.5, lane 2), the addition of nitrate to anoxically-incubated cells revealed the presence of the NorC protein. However, nitrate-dependent NorC expression was strongly reduced in anoxic conditions compared to micro-oxic conditions (Fig. 6.5, lanes 2 and 4). As observed for NorC, expression of FixP and FixO was also very weak in membranes from anoxically cells incubated in the presence of nitrate compared to that from micro-oxically grown cells (Fig. 6.5, lanes 2 and 4).

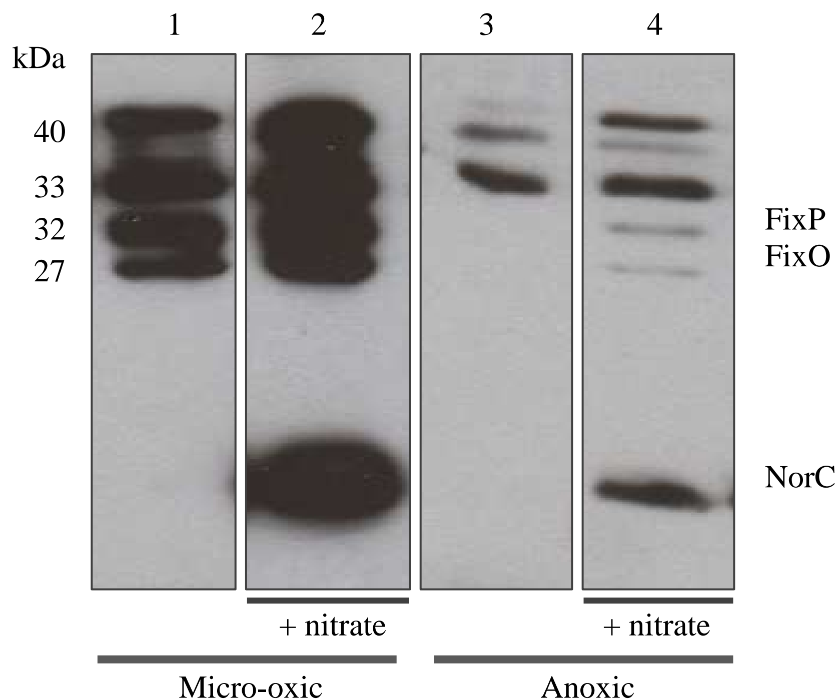


Figure 6.5. Haem-stained proteins of membranes prepared from wild-type *E. meliloti* 1021 incubated during 24 h under micro-oxic (lanes 1 and 2) or anoxic (lanes 3 and 4) conditions in MMN (lanes 2 and 4) or MM (lanes 1 and 3) medium. Each lane contains 25 µg membrane proteins (lanes 1 and 2) or 40 µg membrane proteins (lanes 3 and 4). Haem-stained *c*-type cytochromes identified previously (FixP and FixO) and in this work (NorC) are specified at the right margin. Apparent masses of the proteins (kDa) are shown at the left margin.

Finally, we have investigated the activity of the denitrification enzymes in cells incubated under anoxic conditions. As shown in Table 6.4A, MV^+-NR and Nir activities were detected in cells incubated under anoxia in the presence of nitrate. Although levels of MV^+-NR and MV^+-Nir activities in anoxically incubated WT cells were 3.7- and 3-fold lower than those observed under micro-oxic conditions (Table 6.2), these values were significantly higher than those detected in the *napA* and the *nirK* mutants. As observed in cells grown micro-oxically (Table 6.2), in anoxic cells, NR activity was not induced by nitrate, however Nir activity was induced about 3-fold when nitrate was added to the incubation medium (Table 6.4A). These results suggest that either Nap or NirK enzymes were active under anoxic conditions. However, the analysis of nitrite in the incubation medium revealed that the WT strain only accumulated 0.084 mM of nitrite after 48 h incubation (data not shown) under anoxic conditions with nitrate, while it accumulated about 5 mM nitrite under micro-oxic conditions with nitrate (Fig.1B). The *nirK* mutant was only able to accumulate 1.4 mM nitrite in anoxia and nitrate (data not shown), while it accumulated around 9 mM nitrite when was incubated under micro-oxic conditions with nitrate (Fig. 6.1B).

Cells of *E. meliloti* 1021 incubated under anoxic conditions with nitrate also showed significant levels of NO consumption rates being only 1.7-fold lower than those observed in micro-oxically induced cells (Table 6.4B and Table 6.3). Anoxically incubated cells were also able to accumulate N_2O in the headspace of the medium after 18 and 36 h incucation (Table 6.4C). Interestingly, levels of N_2O accumulated per mg of protein by *nosZ* cells were 8-fold and 2-fold higher than those accumulated by the WT cells after 18 and 36 h incubation, respectively, under anoxic conditions with nitrate (Table 6.4C). These results strongly suggest that Nor and NosZ enzymes were active under anoxic conditions. However, as observed for nitrite production, the amount of N_2O accumulated in the headspace of WT cultures after 36 h incubation anoxically was only 142 µM, while it accumulated about 2.3 mM under micro-oxic conditions (data not shown). With respect to the *nosZ* mutant, it accumulated 547 µM N_2O under anoxic conditions and 4 mM under micro-oxic conditions (data not shown). These results suggest that the low levels of either nitrite or N_2O produced by *E. meliloti*

under anoxia are a consequence of the growth impairment under these conditions rather than a defect in the expression of denitrification genes.

Table 4. (A) MV^+-NR, and MV^+-Nir activities of wild-type (WT) *E. meliloti* 1021. (B) NO consumption activity of WT and *norC* mutant, and (C) N_2O accumulation by WT and *nosZ* mutant strain.

A)

Strain	Genotype	Growth conditions	MV^+-NR[a] (nmol NO_2^- produced· mg protein^{-1}·min^{-1})	MV^+-Nir[a] (nmol NO_2^- consumed·mg protein^{-1}·min^{-1})
1021	WT	Anoxic	87.19 (2.97)	3.62 (0.55)
1021	WT	Anoxic + nitrate	62.96 (5.70)	10.522 (1.465)

B)

Strain	Genotype	Growth conditions	NO consumptiona[b] (μmol NO·mg protein^{-1}· min^{-1})
1021	WT	Anoxic + nitrate	335.88 (32.12)
G1PELR32E8	*norC*	Anoxic + nitrate	2.84 (0.78)

C)

Strain	Genotype	Growth conditions	N_2O accumulation[c] (μmol N_2O·mg protein^{-1})	
			18 h	36 h
1021	WT	Anoxic + nitrate	15.57 (2.64)	78.53 (6.26)
STM.5.07.B03	*nosZ*	Anoxic + nitrate	124.84 (13.82)	156.26 (24.24)

[a]MV^+-NR and MV^+-Nir activities were measured in cells harvested from cultures after 18 hours incubation under anoxic conditions in MM and MMN minimum. [b]NO consumption activity was measured in cells harvested from cultures after 18 hours under anoxic conditions in MMN. [c]N_2O accumulation was measured in the headspace of the cultures after 18 and 36 hours incubation under anoxic conditions in MMN. Data are means with the standard error in parentheses from at least two different cultures, assayed in triplicate. ND = not determined.

4.4.5. Discussion.

E. meliloti has been considered a partial denitrifier due to its traditionally reported inability to use nitrate as electron acceptor for ATP generation and growth at low-oxygen tensions (Garcia-Plazaola, 1993; García-Plazaola, 1996). Recent results from our group have demonstrated that *E. meliloti* is able to use nitrate as respiratory substrate under micro-oxic conditions (Torres et al., 2011). However, up to date, the involvement of *E. meliloti* denitrification genes in nitrate respiration and denitrification has not been investigated. In this work, we have demonstrated that the periplasmic nitrate reductase (Nap) enzyme, encoded by *napA*, is the responsible for the MV^{+}-NR activity observed in cells grown under micro-oxic conditions with or without nitrate. The low levels of activity observed in a *napA* mutant incubated under micro-oxic conditions with nitrate explain the growth defect and the inability of this strain to produce nitrite under these conditions. Most of the best characterized denitrifying bacteria use the membrane-bound nitrate reductase (Nar) to catalyze the first step of denitrification. In contrast to Nar, which has a respiratory function, Nap systems have a range of physiological functions that include the disposal of reducing equivalents during aerobic growth on reduced carbon substrates or anaerobic nitrate respiration (Richardson et al., 2001; Gonzalez et al., 2006; Richardson et al., 2007; Kraft et al., 2011; Richardson, 2011). Our results support the proposed role of Nap on nitrate respiration. In fact, some rhizobial species such as *Pseudomonas* sp. G179 (actually *Rhizobium galegae*), and *Bradyrhizobium japonicum* can express *nap* genes under anaerobic conditions and disruption of these genes is lethal for growth under denitrifying conditions (Bedzyk et al., 1999; Delgado et al., 2003). Whereas deletion of *nosZ* did not have a significant effect on growth rates under micro-oxic conditions with nitrate, we were unable to detect any nitrate-dependent growth in the *nirK* or *norC* mutants under micro-oxic conditions, probably due to the toxicity of the intermediates nitrite and nitric oxide, respectively. In fact, either nitrite or NO were accumulated by *nirK* and *norC* mutants, respectively, incubated under micro-oxic conditions due to the strong inhibition of Nir and Nor activity observed in those mutants compared to WT levels. Similar phenotypes of *nirK* and *norC* mutants were reported in *B. japonicum* (Velasco et al., 2001; Mesa et al., 2002) and in *Rhizobium etli* (Gomez-Hernandez et al.,

2011). The increased levels of N_2O accumulated by the *nosZ* mutant relative to WT cells indicate that this gene is involved in nitrous oxide reduction in *E. meliloti*. Similar observations were found in a *B. japonicum nosZ* mutant (Velasco et al., 2004).

In this work we have shown that nitrate is not required to induce Nap activity either under micro-oxic or anoxic conditions. These results agree with those found recently in *Agrobacterium tumefaciens* where *nap* expression under low-oxygen conditions was not influenced by nitrate (Shapleigh, 2011). By contrast, the presence of nitrate was necessary to obtain maximal levels of Nir activity. Similarly, expression of NorC monitored by heam-staining in WT cells incubated under low-oxygen conditions required the presence of nitrate. The fact that NorC is also present in *napC* cells but at lower concentration than in WT cells suggest that nitrate and a nitrogen oxide derived from its reduction are required for maximal expression of NorC. This disparate nitrate dependence of denitrification genes expression was confirmed by qRT-PCR results where expression of *nirK*, *norC* and *nosZ* under low-oxygen conditions was highly induced by nitrate. However, a very weak induction of *nap* genes was observed after addition of nitrate to the cells incubated under micro-oxic or anoxic conditions. Supporting these observations, recent *E. meliloti* transcriptomic and qRT-PCR experiments have demonstrated that expression of *nirK* and *nor* is upregulated in the presence of NO, an intermediate in nitrate reduction; and that they are targets of the NnrR regulatory protein, a member of the FNR/CRP family, that is involved in the response to NO (Meilhoc et al., 2010). Similarly, in *R. sphaeroides* (Kwiatkowski and Shapleigh, 1996; Tosques et al., 1996), *P. denitrificans* (Van Spanning et al., 1999), *P. aeruginosa* (Arai et al., 1999) and *P. stutzeri* (Vollack and Zumft, 2001) NO has been proposed as the signal molecule for the coordinated activation of *nirK* and *nor* operon expression.

Previous work from our group has reported the inability of *E. meliloti* to grow under anoxic conditions with nitrate as respiratory substrate (Torres et al., 2011). In this work, we have demonstrated that *E. meliloti* denitrification genes are fully induced by anoxia and nitrate. Furthermore, denitrification enzymes are active after incubation of the cells under anoxic conditions plus nitrate since we were able to detect significant levels of MV^+-NR, MV^+-NiR, and Nor activities, as well as N_2O production

under these conditions. By contrary to the high expression of *norC* and Nor activity in response to anoxia and nitrate, levels of NorC as well as FixP and FixO components of the high affinity *cbb*$_3$ oxidase were very weak after incubation of the cells under anoxic conditions.

One of the reasons of the limited growth of *E. meliloti* under anoxic conditions with nitrate might be the low protein levels. We suggest that cells would be trapped without energy after oxygen depletion and they are unable to produce proteins required to cope with oxygen-limiting conditions probably due to the lack of energy. Supporting this hypothesis, it has been reported in *Pseudomonas* sp.G59, that the formation of nitrate reductase and nitrous oxide reductase did not occur in aerobic or anaerobic conditions, but was observed in microaerobic incubation. These indicate that the dependence on microaerobiosis for the formation of these reductases was due to an inability to produce energy anaerobically until these anaerobic respiratory enzymes were formed (Aida et al., 1986). Recent studies have shown that the soil bacterium *Agrobacterium tumefaciens* appears to be unable to perform a balanced expression of denitrification if oxygen depletion happens too fast (Bergaust et al., 2008; Bergaust et al., 2010). Similarly, the soil bacterium *P. denitrificans* appears unable to switch effectively from oxic to anoxic respiration, leaving a large fraction of the population in anoxia without a chance to express the denitrification proteome (Bergaust et al., 2010). As suggested by Nadeem and coworkers (Nadeem et al., 2012), "microaerobic" denitrification is an essential trait for securing efficient transition to anaerobic denitrification. Similarly as we reported in *E. meliloti* (Torres et al., 2011), *Campylobacter jejuni* requires oxygen to support nitrate respiration (Sellars et al., 2002). The *C. jejuni* genome encodes a single class I-type ribonucleotide reductase (RNR) which requires oxygen to generate a tyrosyl radical for catalysis (Jordan and Reichard, 1998). Thus, an oxygen requirement for DNA synthesis can explain the lack of anaerobic growth in this bacterium. This is not the case for *E. meliloti* that contains a class II cobalamin (vitamin B_{12})-dependent RNR that is very common in bacteria from the *Rhizobiales* order (Jordan and Reichard, 1998; Lundin et al., 2009), http://rnrdb.molbio.su.se/). Recently, it was demonstrated the requirement of this class II RNR in symbiosis with alfalfa plants (Taga and Walker, 2010). Considering the

possibility that anaerobic growth in *E. meliloti* is cobalamin-dependent, culture medium was supplemented with vitamin $B_{12.}$ However, an increase in the optical density at 600 nm was not observed (data not shown). Since other rhizobia species such as *B. japonicum* containing class II RNR are able to grow under anaerobic conditions by using nitrate as respiratory substrate (Bedmar et al., 2005), we roled out a defect in DNA synthesis to explain the inability of *E. meliloti* to grow under anaerobic conditions. Considering that *B. japonicum* is a slow growth bacteria and *E. meliloti* is a fast growth bacteria, it might be possible that the transition from aerobic to anaerobic metabolism is different in these species. Supporting this suggestion, we have observed that *B. japonicum* cells are able to express FixO and FixP subunits of the cbb_3 oxidase under anoxic conditions (E. Bueno, personal communication). However, as we have shown in this work, *E. meliloti* does not express FixO and FixP proteins under anoxia. It might be possible that a lack of energy necessary for protein synthesis is contributing to the inability of *E. meliloti* to grow anoxically with nitrate. *B. japonicum* is a symbiont of soybean plants that are typical from tropical climates subjected to flooding stress. However, *E. meliloti* is symbiont of plants typical from temperate climates. Thus, it would exist the possibility that denitrification capacity by these two rhizobial species has evolved differently according to the different oxygen environments where they usually have lived.

5. GENERAL DISCUSSION

La función primordial de la respiración, es la obtención de la energía a través de la fosforilación oxidativa, proceso que consiste en la transferencia de electrones (e^-) desde sustratos carbonados reducidos hasta aceptores terminales de e^-. Debido a la diferencia de potencial redox entre el donador y aceptor, la energía libre, producida durante este proceso de transferencia electrónica, es usada para crear un gradiente electroquímico de protones a través de la membrana o fuerza protón motriz (Ap) que puede ser utilizada por la célula para multitud de procesos celulares, siendo el más significativo la síntesis de ATP a través de la F_1F_0 ATP-sintasa asociada a la membrana, aunque también destacan el movimiento flagelar o la adquisición de solutos. A diferencia del sistema respiratorio mitocondrial eucariótico, que consta de una única oxidasa terminal, todas las especies bacterianas aeróbicas se caracterizan por presentar cadenas respiratorias ramificadas con múltiples oxidasas terminales que presentan distinta afinidad por el O_2 como aceptor terminal de e^-. Esta particularidad permite a las bacterias adaptarse a medios con tensiones de oxígenos muy variables.

Este es el caso de los rizobios, que también poseen una cadena respiratoria ramificada con diferentes oxidasas terminales con distinta afinidad por O_2 (Delgado *et al.*, 1998), que los capacita para respirar en condiciones microóxicas (p.e. en el interior de los nódulos). Como ya hemos comentado anteriormente, *B. japonicum* posee un sofisticado circuito de regulación que responde a condiciones limitantes de oxígeno, los sistemas FixLJ-$FixK_2$ y NifA/RegSR. RegSR de *B. japonicum* pertenece a la familia de sistemas reguladores de dos componentes de respuesta a potencial redox descrita en bacterias. Estos reguladores, activan la expresión de diferentes regulones implicados en procesos que utilizan equivalentes de reducción como son fijación de nitrógeno, y fijación de CO_2, entre otros y además reprimen la expresión de genes implicados en

procesos que generan equivalentes de reducción como es el caso de la oxidación de hidrógeno entre otros (Elsen *et al*., 2004, Bauer and Wu, 2008; Bueno et al., 2012).

En cuanto al sistema RegSR de *B. japonicum*, se ha demostrado en varios artículos científicos el control de RegR sobre el gen regulador clave de la fijación de nitrógeno, *nifA* y de otros 250 genes, aproximadamente (Bauer et al., 1998; Lindemann et al., 2007). Los resultados obtenidos en esta Tesis Doctoral, han permitido ampliar el número de genes controlados por RegR hasta 1700, en células crecidas en condiciones desnitrificantes (anoxia y presencia de nitrato) en un medio mínimo con succinato como fuente de carbono, estableciendo la importancia de esta proteína reguladora en el crecimiento de *B. japonicum* en dichas condiciones. En concreto, los resultados obtenidos en los experimentos de microarrays y qRT-PCR han demostrado el control que ejerce RegR sobre genes estructurales de la desnitrificación como son *nor* y *nos*, así como de otros genes que intervienen en dicho proceso, como los responsables de la síntesis de los citocromos c_{550} (*cycA*) y c_2 (*cy2*). . Nos es la primera vez que un sistema regulador de este tipo se relaciona con el control de genes de la desnitrificación. Estudios previos demostraron el control de los sistemas PrrAB de *R. sphaeroides* y ActSR de *A. tumefaciens* sobre *nirK* (Laratta et al., 2012; Baek et al., 2008). Sorprendentemente, *nap* o *nirK* no han aparecido como dianas de RegR en nuestros estudios de transcriptómica, sugiriendo que en *B. japonicum* existe un control diferencial de los genes de la desnitrificación con respecto a su dependencia de RegR. Al igual que se ha observado para RegR, estudios previos de nuestro grupo de investigación demostraron que sólo los genes *nap* y *nirK* se encuentran bajo el control directo de $FixK_2$, pero no es el caso de los genes *nor* o *nos* (Bueno et al., enviado para su publicación). Cabe destacar de los resultados de transcriptómica, la identificación

como posibles dianas de RegR de una agrupación de genes (*blr2806-09*) implicados en la detoxificación de NO y asimilación de nitrato, los cuales están siendo estudiados en profundidad en nuestro grupo de investigación (Cabrera et al., 2011). Resulta interesante que entre los genes controlados por RegR también se encuentren genes reguladores tales como bll4130 que codifica la síntesis de un regulador transcripcional de la familia LysR ó bll3466, que codifica un homólogo de la proteína $FixK_2$.

El estudio comparativo de la expresión de los genes *nor* de *B. japonicum* en la mutante *regR* cultivada en diferentes concentraciones de oxígeno ha demostrado que la activación de *nor* dependiente de RegR requiere condiciones anóxicas y la presencia de nitrato o un óxido de nitrógeno derivado la reducción del mismo. En este sentido, en *B. subtilis* se ha descrito que la inducción anaeróbica por sistema redox de dos componentes ResDE de *nasDE* y *hmp*, que codifican una nitrito reductasa y una flavohemoglobina capaz de detoxificar NO, respectivamente, requiere la presencia de NO.

Ejemplos de sistemas de dos componentes que han sido ampliamente estudiados son RegBA de *R. capsulatus* y ArcBA de *E. coli*. Estos sistemas son capaces de percibir los cambios en el estado estado redox a través de las quinonas presentes en la membrana o a través de una cisteína presente en la proteína sensora RegB o ArcB y en consecuencia, responder a estos cambios. Resulta interesante especular si ocurre lo mismo con la proteína sensora RegS de *B. japonicum*. Sin embargo y sorprendentemente, el control de RegR sobre la expresión de los genes *nor* en *B. japonicum* es independientemente de la proteína sensora RegS. Es posible que en esta bacteria exista una interacción/comunicación cruzada entre RegSR y otro/s sistema/s

de dos componentes, por lo que la la actividad de RegR podría estar modulada por otra proteína sensora. Tampoco podemos excluir la posibilidad de que RegR en su forma desfosforilada pueda actuar como un regulador transcripcional. De hecho, este fenómeno se ha observado en *Rhodobacter*, donde RegA y PrrA son capaces de unirse al ADN y activar la transcripción tanto en su forma fosforilada como desfosforilada (Ranson-Olson et al., 2006).

Otra proteína clave en la supervivencia de bacterias aerobias facultativas es la oxidasa cbb_3 de alta afinidad por el O_2, la cual las capacita para crecer en ambientes limitantes de O_2 (p.e. en vida libre en la rizosfera o en el interior del nódulo). En *B. japonicum,* esta oxidasa ha sido caracterizada en profundidad y se ha demostrado su implicación en la fijación simbiótica de nitrógeno (Preisig et al., 1993; 1996). Sin embargo, el conocimiento sobre la oxidasa cbb_3, de *E. meliloti* es bastante limitado. *E. meliloti*, a diferencia de *B. japonicum* o *A. caulonidans,* tiene 3 copias de los genes *fixNOQP*, responsables de la síntesis de la oxidasa cbb_3, situadas en el megaplásmido pSymA (http://genome.kazusa.or.jp/rhizobase/). Las copias 1 y 2 son muy parecidas entre sí, diferenciándose de ellas la copia 3 a la que se le ha atribuido un papel en el metabolismo del fósforo (Krol and Becker 2004). En cuanto a la copia 1, está localizada en una región génica que contiene el conjunto completo de los genes reguladores necesarios para la respiración microaeróbica y la fijación de nitrógeno (*fixLJ, fixK, fixT, fixM*), así como genes implicados en el ensamblaje de la oxidasa (*fixGHIS*). El contexto génetico en el que se encuentra esta copia, permite proponerla como la candidata potencial para ser la copia funcional de *E. meliloti* para crecer en condiciones limitantes de oxígeno, tanto en vida libre como en simbiosis. De hecho, el análisis de las proteínas con grupos hemo *c* en las membranas de una mutante *fixN*1 de *E.*

meliloti, nos ha permitido identificar dos proteínas de 32 y 27 KDa como los citocromos FixP y FixO de la oxidasa *cbb*$_3$, respectivamente. Además, la mutante *fixN*1 mostró un defecto en su capacidad de respirar y crecer en condiciones microóxicas, sugiriendo que la copia 1 de los genes *fixNOQO* es la copia funcional responsable del manteniento de la respiración en dichas condiciones. Con anterioridad, Bobik y colaboradores (2006) demostraron que *fixNOQP1* está controlada por FixJ tanto en vida libre como en simbiosis, mientras que la copia 2 solo se detecta en bacteroides. Todos estos datos considerados en conjunto, nos permiten proponer que las tres copias de *fixNOQP* pueden tener funciones diferentes en *E. meliloti*. En consonancia con nuestras observaciones, en *M. loti* que posee dos copias de los genes *fixNOQP*, se estableció que una copia era inducida principalmente en vida libre mientras que ambas se inducían en simbiosis (Uchiumi et al., 2004). En cuanto a la implicación de la copia 1 de los genes *fixNOQP* de *E. meliloti* en simbiosis, hemos demostrado en este trabajo que plantas de alfalfa inoculadas con la cepa deficiente en la copia 1 del gen *fixN* mostraron un defecto en biomasa, contenido de nitrógeno, así como longitud de tallo y raíz a las tres semanas tras la inoculación. Sin embargo, cuando las plantas crecieron por un periodo de 8 semanas se observó una recuperación de las mismas hasta alcanzar valores similares de los parámetros fisiológicos analizados que las plantas inoculadas con la cepa parental. Es posible que en periodos más largos de crecimiento ocurra una inducción de la segunda copia de *fixNOQP*, para compensar la mutación de la copia 1. Resultados similares se han descrito previamente por Trzebiatowski y colaboradores (2001) y Schlüler y colaboradores (1997), donde ambas copias de la *cbb*$_3$ fueron necesarias para la óptima fijación de nitrógeno. Sin embargo, en nuestros experimentos no pudimos detectar la proteína FixO sintetizada por la copia 2 de

fixNQOP, en membranas de bacteroides aislados de nódulos de plantas inoculadas con la cepa mutante *fxN1*, crecidas durante un periodo de 8 semanas. De hecho, estudios de transcriptómica realizados por Bobik et al., (2006) demostraron que los niveles de expresión en bacteroides de *fixNOQP2* fueron considerablemente más bajos que los de *fixNOQ1*. También es posible que otras oxidasas tales como la de alta afinidad de tipo *bd* o la quinol oxidasa *cyo*, las cuales se inducen en condiciones microóxicas en vida libre (Bobik et al., 2006) sean las responsables de la recuperación de las plantas de alfalfa inoculadas con la cepa mutante *fixN*1 tras 8 semanas de cultivo. En esta línea, existen otros casos en la bibliografía donde ambas copias tienen funciones no reiterativas, como ocurre con las dos copias de *R. etli*, donde la mutación en el gen *fixN* localizado en el plásmido d, pero no la mutación en *fixN* del plásmido f, afecta severamente la fijación de nitrógeno (Granados-Baeza et al., 2007).

Los estudios relacionados con la caracterización de la desnitrificación en *E. meliloti* eran muy escasos hasta el inicio de esta Tesis. Ello se debía principalmente al hecho que *E. meliloti* era incapaz de crecer a expensas de nitrato como aceptor de electrones en condiciones anóxicas, a pesar de poseer el conjunto completo de los genes de la desnitrificación. En el megaplásmido pSymA de *E. meliloti* están presentes todos los genes responsables de la ruta completa de la desnitrificación (Barnett et al., 2001; Torres et al., 2011). En concreto, *napEFDABC, nirK, norECBQD* y *nosRZDFYLX*, los cuales son responsables de la síntesis de las enzimas nitrato reductasa periplásmica, nitrito reductasa tipo Cu, óxido nítrico reductasa y óxido nitroso reductasa, respectivamente. Tras estudiar la capacidad de *E. meliloti* crecer y desnitrificar en diferentes concentraciones de oxígeno, concluimos que este rizobio no es capaz de crecer anaeróbicamente a expensas del nitrato o del nitrito como aceptores final de

electrones, pero si lo hace en condiciones microóxicas (Torres et al., 2011). Una vez demostrada la capacidad de *E. meliloti* de utilizar nitrato o nitrito como sustratos respiratorios en condiciones microóxicas, hemos llevado a cabo en este trabajo el análisis funcional de los genes *nap*, *nirK*, *nor* y *nos* de *E. meliloti* mediante la caracterización fenotípica de cepas mutadas en los mismos. De esta manera, hemos demostrado que una mutante *napA* mostró un defecto en su capacidad de crecer así como de producir nitrito cuando se incubó en condiciones microóxicas en presencia de nitrato. Al contrario que en una cepa mutada en *nosZ*, la cual creció de forma similar a la cepa parental, las cepas mutantes *nirK* o *norC* mostraron un defecto en su capacidad de crecer con nitrato como aceptor de electrones, probablemente debido a la toxicidad del nitrito y óxido nítrico, productos que se acumularon en el medio de cultivo así como en la atmosfera gaseosa de las mutantes *nirK* y *norC*, respectivamente. Igualmente, se había demostrado previamente en *B. japonicum* (Velasco et al., 2001; Mesa et al., 2002) y en *R. etli* (Gomez- Hernandez et al., 2011) similares fenotipos para las mutantes *nirK* y *norC* de estos rizobios. En esta Tesis también hemos demostrado la capacidad de una mutante *nosZ* de *E. meliloti* de acumular N_2O, lo que demuestra la implicación del gen *nosZ* en la reducción del óxido nitroso en *E. meliloti*. Velasco et al. (2004) observaron un fenotipo similar en la mutante *nosZ* de *B. japonicum*.

Los valores de actividad nitrato reductasa fueron muy bajos en la cepa mutante *napA*, lo que indica la implicación de este gen no sólo en el crecimiento a expensas de nitrato sino también en la expresión de la enzima Nap. Además, también hemos sido capaces de demostrar que en *E. meliloti* la actividad Nap es independiente de la presencia de nitrato en el medio, siendo la baja concentración de oxígeno el único

requerimiento para inducir la expresión de Nap. Resultados similares se han observado recientemente en *A. tumefaciens* (Shapleigh, 2011). Al contrario que ocurre con Nap, el nitrato si fue necesario para obtener los máximos niveles de actividad Nir, así como para la expresión de la proteína NorC. Esta diferente respuesta a nitrato de los genes *napA*, *nirK* y *nor* también se demostró mediante experimentos de qRT-PCR. Estos resultados indican que el nitrato o un producto de la reducción del mismo son necesarios para la máxima inducción de los genes *nirK* y *nor* en *E. meliloti*. En concordancia con estas observaciones, se ha demostrado recientemente que tanto *nirK* como los genes *nor* están positivamente controlados por NO a través de la proteína reguladora NnrR en *E. meliloti* (Meilhoc et al., 2010). De hecho, numerosos estudios han propuesto al NO como una molécula señal clave para la activación de la expresión de los genes *nir* y *nor* en bacterias desnitrificantes (Kwiatkowski and Shapleigh, 1996; Tosques et al., 1996; Van Spanning et al., 1999; Arai et al., 1999; Vollack and Zumft, 2001).

A pesar de la incapacidad de *E. meliloti* de crecer anaeróbicamente con nitrato como sustrato respiratorio, hemos podido demostrar que los genes de la desnitrificación se expresan en estas condiciones, alcanzando niveles superiores a los obtenidos en condiciones microóxicas . En estos experimentos se demostró que células incubadas anaeróbicamente en presencia de nitrato expresan actividad MV^+-NR, MV^+-NiR y Nor, así como son capaces de producir N_2O. Sin embargo, el análisis de citocromos *c* en células incubadas en condiciones anóxicas, indicaron que los niveles tanto de NorC como de FixP y FixO, componentes de la oxidasa terminal cbb_3, eran muy bajos en relación con los observados en condiciones de microoxia. Probablemente, la incapacidad de *E. meliloti* para crecer anaeróbicamente a expensas

del nitrato se deba a una limitación en la síntesis de proteínas en estas condiciones, y no a la expresión de los genes de la desnitrificación. Es posible que las células de *E. meliloti* cuando se encuentran en condiciones limitantes de oxígeno sean incapaces de obtener la energía necesaria para sintetizar toda la maquinaria enzimática requerida para el crecimiento celular a expensas de nitrato como aceptor final de electrones. De hecho, mientras que *B. japonicum* expresa la oxidasa cbb_3 en condiciones anóxicas, los niveles de esta oxidasa en *E. meliloti* incubada en las mismas condiciones son muy bajos y probablemente no sean los suficientes para obtener la energía que las células necesitan para sintetizar las proteínas desnitrificantes. En este sentido, se ha propuesto recientemente que en algunas bacterias desnitrificantes las condiciones microóxicas son necesarias para conseguir una transición efectiva hasta la desnitrificación anaeróbica (Nadeem et al., 2012). La diferencia observada entre *B. japonicum* y *E. meliloti* en cuanto a su capacidad de sintetizar las proteínas necesarias para crecer en condiciones anóxicas con nitrato puede deberse a que *B. japonicum* es un rizobio de lento crecimiento y *E. meliloti* es de rápido. Por otro lado, es importante tener en cuenta que mientras *B. japonicum* es un simbionte de la soja, una leguminosa típica de climas tropicales frecuentemente sometidos a encharcamiento y anoxia, *E. meliloti* es simbionte de plantas de alfalfa que se cultivan en climas templados. Por ello, es posible que la desnitrificación sea un proceso que haya evolucionado de forma distinta en ambos rizobios dependiendo de los diferentes ambientes de oxígeno en los se encuentran cada uno de ellos.

6. CONCLUSIONS

1. Transcriptome analyses of a *B. japonicum regR* mutant grown under anoxic conditions with nitrate as respiratory substrate revealed as RegR targets several denitrification genes (*nor*, *nos*, *cycA*, *cy2*), as well as genes involved in nitric oxide detoxification (*blr2806-09*), and regulatory genes (*bll3466*, *bll4130*).
2. DNA binding experiments showed a direct control of RegR at promoter regions of *norC*, *nosR*, the *fixK*-type *bll3466*, and the LysR-type *bll4130* genes.
3. Two transcriptional start sites located at about 35 (P1) and 22 (P2) bp from the putative translational start codon of NorC were identified in the *norC* promoter region. Whereas P2 is the principal start site and is modulated by RegR, P1 is the previously identified start site, whose expression depends on $FixK_2$.
4. Anoxia and nitrate are the signals involved in the RegR-dependent induction of *nor* genes, and this control is independent of the sensor protein RegS.
5. The copy 1 of the *fixNOQP* operon is involved in *E. meliloti* respiration and growth under microoxic conditions as well as in the expression of the FixOand FixP components of the cbb_3 oxidase.
6. The copy 1 of the *fixNOQP* is important for nitrogen fixation during the early steps of symbiosis. *E. meliloti* is able to grow under microoxic conditions using nitrate or nitrite as respiratory substrates..
7. *E. meliloti napA*, *nirK*, *norC, and nosZ* genes are involved in the ability of the cells to grow under microoxic conditions using nitrate as final electron

acceptor. as well as in the expression of the denitrification enzymes under microoxic conditions.

8. *E. meliloti napA, nirK*, *norC* and *nosZ* are expressed not only under microoxic, but also under anoxic conditions with nitrate. Furthermore, anoxically-incubated cells also expressed MV^{+}-NR, MV^{+}-Nir, Nor and Nos activities. Thus, the inability of *E. meliloti* to grow under anoxic conditions with nitrate is not due to a defect on the expression of denitrification genes.

9. Three membrane-bound *c*-type cytochromes of 32, 27, and 16 kDa have been identified as the *E. meliloti* FixO, FixP and NorC components of the cbb_3 oxidase and nitric oxide reductase.

1. El análisis del transcriptoma de una mutante *regR* de *B. japonicum* crecida encondiciones anóxicas con nitrato como sustrato respiratorio, permitió la identificación de nuevos genes controlados por la proteína RegR. Entre ellos se encuentran genes de la desnitrificación (*nor*, *nos*, *cycA*, *cy2*), genes implicados en la detoxificación de NO (*blr2806-09*) y genes reguladores (*bll3466*, *bll4130*).
2. La proteína RegR purificada interaccionó con la región promotora de Los genes *norC*, *nosR*, *bll3466* (codifica una regulador de tipo FixK) y *bll4130* (codifica un regulador de tipo LysR), lo cual sugiere un control directo de RegR sobre estos genes.
3. En la región promotora de *norC* se han identificadodos sitios de inicio de la transcripción situados a 35 pb (P1) y 22 pb (P2) pb del codón inicio de la traducción anotado de NorC. Mientras que P2 es el sitio de inicio principal y está modulado por RegR, P1 corresponde con el lugar de inicio de la transcripción identificado anteriormente cuya expresión depende de $FixK_2$.
4. Tanto anoxia como nitrato son las señales necesarias para la la inducción de los genes *nor* de forma por la proteína RegR. Este control no depende de la proteína sensora RegS.
5. La copia 1 del operon *fixNOQP* está implicada en la respiración y crecimiento de *E. meliloti* en condiciones microoxicas, así como en la expresión de los componentes FixO y FixP de la oxidasa terminal *cbb*$_3$.
6. La copia 1 de *fixNOQP* es importante para la fijación de nitrógeno durante los primeros estadíos de simbiosis.*E. meliloti* es capaz de crecer usando nitrato o nitrito como sustratos respiratorios en condiciones microóxicas.

7. Los genes *napA, nirK, norC* y *nosZ* de *E. meliloti* están implicados en la capacidad de *E. meliloti* para crecer en condiciones microóxicas con nitrato como aceptor final de electrones así como en la expresión de las enzimas de la desnitrificación en dichas condiciones.

8. Los genes *napA, nirK, norC* y *nosZ* de *E. meliloti* se expresan no solo en microaerobiosis sino también en anaerobiosis con nitrato. Además, células incubadas anoxicamente también expresaron las actividades MV^+-NR, MV^+-Nir, Nor y Nos. Por lo tanto, la incapacidad de *E. meliloti* de crecer en condiciones anaeróbicas con nitrato no es debido a un defecto en la expresión de los genes de la desnitrificación.

9. FixO, FixP and NorC de *E. meliloti* son tres citocromos *c* de membarana de 32, 27, and 16 kDa que forman parte de la oxidasa terminal cbb_3 (FixP, FixO) y de la óxido nítrico reductasa (NorC).

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