Doctoral Thesis

Global regulatory analysis in Bradyrhizobium japonicum from oxygen to iron control

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Global regulatory analysis in *Bradyrhizobium japonicum*:

from oxygen to iron control

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY
for the degree of
DOCTOR OF NATURAL SCIENCES

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Abstract

This thesis consists of two major parts reflecting the two different aspects of my Ph.D. work. Part I comprises two global approaches aimed at the identification of novel *Bradyrhizobium japonicum* genes that are controlled by the transcription activator NifA. Part II presents the functional and regulatory characterization of a heme uptake system in *B. japonicum*.

The redox-responsive NifA protein activates transcription of many genes required for symbiotic nitrogen fixation of *B. japonicum* living in soybean root nodules. Induction of NifA target genes depends on a specific sigma factor, RpoN, which enables RNA polymerase to recognize –24/–12-type promoters. New NifA-dependent genes were screened firstly by a competitive DNA-RNA hybridization approach focused on the 410-kb symbiotic region of the *B. japonicum* genome, and secondly, by a comparative proteome analysis of nifA mutant and wild-type cells.

The first screen resulted in the identification of three novel NifA-regulated genes, *nrgA*, *nrgB* and *nrgC*. Their NifA- and RpoN-dependent transcription was confirmed by transcript analysis. In addition, RpoN- and NifA-dependent expression of the *nrg* genes under anaerobic conditions was verified with translational lacZ fusions. While the deduced amino acid sequences of NrgA and NrgC display similarity to N-acetyltransferases and hydroxylases, respectively, no NrgB-homologous protein is present in the database. Individual mutant strains carrying null mutations in the *nrg* genes showed a wild-type phenotype with regard to anaerobic growth and symbiotic nitrogen fixation. Thus, the functions of NrgA, B and C remained enigmatic.

The comparative proteome analysis, which was performed in collaboration with the Laboratory for Protein Chemistry, ETH, Zürich, resulted in a list of 19 putatively NifA-regulated proteins. They were further characterized by MS-MS fingerprinting and, if applicable, by N-terminal amino acid sequencing. Using degenerated oligonucleotides deduced from the N terminus of one of these proteins, we identified the open reading frame orf110. The product of orf110 showed no similarity to any protein of known function. Studies with a translational orf110'-lacZ fusion indicated that orf110 expression only partially depends on NifA and RpoN and that the gene is maximally expressed under low-iron conditions.
In Part II, the genomic region adjacent to *orf110* was analyzed. Sequence analysis of a 16.7-kb region led to the discovery of a cluster of 9 genes that are involved in heme acquisition. Predicted products encoded in the *hmu* (heme uptake) gene cluster comprise: HmuR, an outer membrane heme receptor; HmuT, U and V, three proteins constituting an ABC transport system of the inner membrane; and a Ton system composed of TonB, ExbB, and ExbD. Mutant analysis proved that *hmuR*, *tonB*, *exbB* and *exbD* are essential for growth on heme as the sole iron source whereas *hmuTUV* are not absolutely required. Soybean plant infection tests indicated that the heme uptake system is dispensable for symbiosis and that the *hmu* genes are only weakly expressed in nodules.

In the past, the ability to use heme as iron source was described predominantly for pathogenic bacteria and opportunistic pathogens, which can use host hemoglobin as an iron source. We speculate that the heme uptake system enables *B. japonicum* to use leghemoglobin-derived heme during senescence of nodules.

Regulation of the divergently oriented *hmuTUV* and *hmuR* genes was studied with transcriptional *lacZ* fusions and transcript analyses. Maximal expression was observed under iron-depleted conditions. An A/T-rich imperfect inverted repeat (ICE = iron control element) located in the *hmuTUV-hmuR* promoter region is essential for iron control. We also analyzed the role of two iron-responsive regulatory proteins, Fur and Irr, which are known to be involved in iron and heme homeostasis in *B. japonicum*. It turned out that neither Fur nor Irr alone is absolutely required for iron-control of the *hmu* genes. We therefore postulate the existence of yet another iron regulatory system in *B. japonicum*, which might control expression of the *hmu* genes via ICE.
Kurzfassung


Expression von orf110 nur teilweise von NifA und RpoN abhängig ist und unter Eisenlimitierenden Bedingungen am höchsten ist.


In der Vergangenheit wurde die Fähigkeit, Häm als Eisenquelle zu nutzen, hauptsächlich für pathogene Bakterien und opportunistische Pathogene beschrieben. Diese können Hämaglobin des Wirtes als Eisenquelle nutzen. Wir vermuten, dass B. japonicum während der Seneszenz der Knöllchen mit dem Hmu-System Häm aus Leghämaglobin verwerten kann.

Part I

The NifA regulon
Chapter 1

Synopsis
1.1 Global analysis of bacterial genomes and proteomes

In the last decade a revolution in molecular biology occurred by the advent of whole genome sequencing. The development from single-gene to genome analysis was enabled mainly by improvements in DNA sequencing and innovations in computational biology, comprising new computer-based programs and the management of extensive databases. The nucleotide sequences of entire genomes are now available for a rapidly growing number of organisms, and predictions of the full complement of gene products can be made. About 50 microbial genome sequences are completed to date and more than 130 are in progress worldwide (http://www.tigr.org/tdb/mdb/mdbcomplete.html).

Research focused on systematic, large-scale study of genomes and their function is commonly referred to as ‘functional genomics’. The generation of single knockout mutants and subsequent analyses of phenotypes are first steps in order to explore functions of unknown genes. Traditional methods to investigate ‘functional genomes’ in large scales use the still powerful tool of transposable elements (Hamer et al., 2001). In addition to their application in random mutagenesis, transposable elements can be engineered to carry various reporter genes, epitope tags and/or regulatory elements and, therefore, can be used to study expression and regulation of the genome. Apart from understanding the biological function(s) of individual genes it is also important to study their regulation and their (potential) position within regulatory networks. Major questions are how cells react to changes in the environment and how they regulate differentially expressed genes. Ideally, studies of global responses and adaptation to different environments include the DNA, RNA and protein level. A simple diagram illustrates the regulatory mechanisms in microbial cells (Fig. 1.1). By analogy with the term genome, the proteome is defined as the complete set of proteins encoded by the genome. The term transcriptome comprises the entity of expressed RNAs and the term metabolome describes the total set of metabolites under certain conditions. In the following, selected techniques for profiling gene expression will be introduced. The aim is not to compile all known strategies of global screens, but rather to discuss advantages and disadvantages of transcriptome and proteome analysis.
Fig. 1.1 Regulatory mechanisms involved in cellular sensing, signal transduction and adaptation. Depicted is how a generic, intracellular or extracellular stimulus changes cell’s activities and functions. Each of these stages can be affected by feedback, as indicated by dashed arrows (modified from van Bogelen et al., 1999).

Differential display and microarrays
Differential display (DD) aims at the visualization of differences of transcriptomes in dependence of environmental conditions or between different cell types. The analysis of differential gene expression is based on the identification and quantification of mRNA profiles. This method, originally introduced by Liang and Pardee (1992), is based on a low-stringency reverse transcriptase (RT)-mediated PCR. The DDRT-PCR procedure consists of two major steps: (i) individual reverse transcription of RNA isolated from different cell populations with a set of anchored mRNA-specific primers to generate cDNA pools and (ii) PCR amplification of random partial sequences from the cDNA pool with the original anchored primer and an arbitrary upstream primer. The PCR products can be displayed on a gel, and differentially expressed DNA fragments can be directly sequenced or cloned. This method allows a rapid screening for genes that are expressed under specific conditions. The obvious advantages of this method are the
rapid performance with simple laboratory tools, the requirement of only very low amounts of RNA and the independence of genome sequence data. A critical and comprehensive evaluation of DD is given by Lievens et al. (2001) who used this method with the aim to identify genes involved in legume nodulation. Differential display has been used extensively for the analysis of eukaryotic genes but only to a limited extent for prokaryotic gene expression. This is explained by the difficulties in purification of bacterial mRNA from RNA pools, of which about 90-97% is rRNA (see below). However, recent modifications developed for specific bacterial species allowed the eliminations of rRNA during the RT-PCR step by using a collection of oligonucleotides instead of arbitrary primers (Fislage et al., 1997; Bhaya et al., 2000). For example, selected primers for the Enterobacteriaceae group display a statistically high probability to prime in the coding DNA (Fislage et al., 1997).

In comparison, the microarray technology allows monitoring of global gene expression in one single experiment (reviewed by Harrington et al., 2000; Lucchini et al., 2001). Microarrays are small glass slides to which gene-specific DNA fragments, amplified by PCR, or oligonucleotides are bound in an ordered manner. Thus, this technology is based on the availability of the respective genome sequence. Hybridization of microarrays is similar to the classical hybridization procedures. mRNA or cDNA is prepared from two different cultures and labeled in vitro with different fluorescent dyes prior to hybridization. A scanner is used to quantify the fluorescent hybridization signals. The expression profiles can be subjected to subsequent cluster analyses. Based on the observation that functionally related genes frequently show similar patterns of expression, clues may be obtained about the function of unknown genes that are co-regulated with known genes. This is nicely exemplified by microarray-based work with Caulobacter crescentus that resulted in the identification of novel cell cycle-dependent genes (Laub et al., 2000). Major disadvantages of microarray technology are the high costs of the technical equipment required for the production and application of microarrays and the dependence on genome sequence data.

The reliability of both DDRT-PCR and microarrays is mainly dependent on the quality of the original mRNA. Two critical steps that may strongly affect the results are the isolation procedure for the RNA and the generation of labeled probes or cDNA. In vivo, bacterial mRNA is much less stable than eukaryotic mRNA and its purification is more difficult because of the absence of polyadenylated 3' ends. This interferes with the
isolation of high-quality bacterial RNA and prevents the enrichment of mRNA from complex mixtures. Furthermore, the synthesis and labeling of cDNA is critical because the cDNA probe must represent the original population of mRNA species. Despite these difficulties, there are several reports where bacterial cDNA has been successfully synthesized and used for global expression studies. Examples include a microarray hybridization approach with RNA isolated from E. coli (Arfin et al., 2000) and a differential display technique applied to RNA from the symbiotic bacterium Sinorhizobium meliloti. For the latter approach, RNA was extracted from root nodules and directly used for RT PCR (Cabanes et al., 2000). In the PhD work presented here a variation of the aforementioned methods was used to study transcript patterns of a distinct genomic region. A suitable cosmid library, containing no rRNA genes was displayed on a macroarray used in a competitive DNA-RNA hybridization approach (Chapter 2). In this case high amounts of different RNAs were used in order to circumvent probably non-proportional cDNA amplification.

**Proteome analysis**

A global analysis of the whole proteome of a bacterial cell is possible by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (reviewed by Washburn and Yates, 2000; Fey and Larsen, 2001). 2D-PAGE separates proteins on the basis of both, their isoelectric point (pI) and molecular weight, and provides the highest resolution for the analysis of complex protein mixtures. Using 2D-PAGE, proteins can be analysed both qualitatively (post-translational modifications) and quantitatively (relative abundance). The identification of proteins separated by 2D-PAGE includes Edman peptide sequencing, immunoblotting and the use of matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and electrospray ionisation (ESI) (Celis et al., 2000 and references therein). The latter techniques, which rely on a comparison of peptide mass fingerprints, are fast and require only picomol amounts of proteins. Obviously, the performance of 2D-PAGE is time-consuming and laborious. Critical steps that can limit the application of the 2D-PAGE technique include the sample preparation and the sensitivity of protein detection. Because of their low solubility, membrane proteins are normally excluded from gel electrophoresis, and also very basic proteins have been proven difficult to resolve and often require separate gel
systems. Proteins spots are usually detected by Coomassie Blue or silver nitrate staining, yet, these procedures may be insufficiently sensitive to detect proteins of low abundance.

Like DDRT-PCR or microarray hybridization, 2D-PAGE can be used in comparative studies in order to compare different culture conditions or mutants. An example of a comparative proteome analysis is the study by Dainese-Hatt and co-workers (Dainese-Hatt et al., 1999), who compared the protein profiles of aerobically and anaerobically grown *Bradyrhizobium japonicum* cells (see Chapter 3 of this Ph.D. thesis). Furthermore, specific regulatory mutants were used to identify proteins that belong to distinct regulons.

### 1.2 Rhizobia and symbiotically relevant genes

**Rhizobia**

‘Rhizobia’ is the collective term for the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*, which all belong to the α-proteobacteria. Rhizobia can exist either free-living in the soil or as nitrogen-fixing symbionts in root nodules of specific leguminous plants (reviewed by Spaink et al., 1998). In compatible interactions, rhizobia invade root hair tips and penetrate into their host through infection threads. In parallel, root nodules develop from meristems induced below the cortex of infected roots. When infection threads reach subcortical nodule cells, rhizobia are released in the form of so-called symbiosomes (bacteria surrounded by a peribacteroid membrane) into the cytoplasm of plant cells where they eventually enlarge and differentiate into nitrogen-fixing bacteroids. Continuous exchange of chemical signals between the two symbionts coordinates expression of bacterial and plant genes required for the development of an effective symbiosis. Flavonoids released by legume roots are among the first signals exchanged in this molecular dialog (reviewed by Schultze and Kondorosi, 1998). By interaction with rhizobial regulators of the NodD family, flavonoids trigger the expression of nodulation genes. In turn, most nodulation genes participate in the synthesis and secretion of a family of lipochito-oligosaccharide molecules, the Nod factors, which are required for bacterial invasion and nodule development (reviewed by Broughton et al., 2000). In invading rhizobia,
low free-oxygen tensions coordinate the expression of genes involved in metabolic adaptation and nitrogen fixation. Low oxygen is the principal physiological signal that induces nitrogen fixation in rhizobia (see below). This means that nitrogen fixation is uncoupled from the bacterial nitrogen status. As a consequence, rhizobia provide their host legume with fixed nitrogen and thus allow plant growth on nitrogen-depleted soils. In return, the plant delivers carbon sources in the form of dicarboxylic acids to the bacteroids.

Although unique to prokaryotes, nitrogen fixation is not a feature exclusive for symbiotic bacteria. Besides symbiotic diazotrophs, different bacteria are known that exhibit nitrogenase activity under free-living conditions, as exemplified by *Klebsiella pneumoniae* and *Azotobacter vinelandii* (reviewed in Dixon, 1998). In contrast to rhizobia, the regulation of *nif* genes in free-living nitrogen-fixing bacteria is also affected by the cellular nitrogen status.

Symbiotic nitrogen fixation is not only restricted to rhizobia. Species of the actinomycete genus *Frankia* are able to fix nitrogen with a series of non-legumes (reviewed by Benson and Silvester, 1993), and very recently, nitrogen fixation was detected in a symbiotic Spirochaete, living in termite hindguts (Lilburn *et al.*, 2001). Thus, the term ‘symbiotic diazotroph’ comprises at least three different bacterial families.

### Genomic organization of symbiotic genes

Most of the bacterial genes involved in nodulation of legumes and nitrogen fixation are clustered within the rhizobial genomes. These symbiotic gene clusters are either carried on large symbiotic plasmids (pSym) or are located on the chromosome. Until recently, genomic studies of rhizobia were mainly focused on symbiotic regions. Most recently, however, the complete genome sequences of *Sinorhizobium meliloti* (6.5 Mb) and *Mesorhizobium loti* (7.6 Mb) have been established (Galibert *et al.*, 2001 and references therein for *S. meliloti*; Kaneko *et al.*, 2000 for *M. loti*).

*S. meliloti* and *Rhizobium* species NGR234 are representatives of rhizobia that contain symbiotic plasmids. In both species, the genomes are partitioned into three replicons, consisting of a chromosome of 3.5 Mb and 3.7 Mb, respectively, and two additional plasmids. In *S. meliloti*, both megaplasmids of 1.4 and 1.7 Mb, commonly named pSymA and pSymB, carry genes that are essential for symbiosis. The 536-kb symbiotic
plasmid pNGR234a of *Rhizobium* sp. NGR234 was the first rhizobial replicon to be sequenced (Freiberg et al., 1997). High-resolution transcriptional analysis revealed that many genes on pNGR234a are specifically induced during symbiosis (Perret et al., 1999). Yet, the chromosome and the 2-Mb plasmid pNGR234b of strain NGR234 also harbor symbiotic genes (Flores et al., 1998).

In contrast, *M. loti* and *B. japonicum* are two rhizobial species whose entire sets of symbiotic genes are located on the single chromosome. It was shown for *M. loti* strain ICMP3153 that the symbiotic genes are clustered in a 500-kb genetic element which is inserted within a phe-tRNA gene (Sullivan and Ronson, 1998). Most interestingly, this element is transmissible to other, non-symbiotic *Mesorhizobium* strains. On the basis of its similarity to pathogenicity islands of pathogenic bacteria (reviewed by (Lee, 1996) this region was termed ‘symbiotic island’. The genome sequence of *M. loti* strain MAFF303099 confirmed the presence of a similar ‘symbiotic island’ in the 7.6-Mb chromosome of this strain. It contains 30 genes related to nitrogen fixation and 24 genes involved in nodulation. Yet, even more genes with relation to these functions (46 and 39, respectively) were identified elsewhere on the chromosome (Kaneko et al., 2000).

Recently, the ‘symbiotic region’ of the *B. japonicum* 8.7 Mb chromosome was sequenced and analyzed (Göttfert et al., 2001; Chapter 2). The symbiotic region of 410 kb contains all *nif* genes and, except for *nodM*, all nodulation genes that were described previously in *B. japonicum*. Unlike the situation in *M. loti*, the borders of this symbiotic region are not well defined in *B. japonicum* and there is no evidence that it represents a mobile element.

Symbiotic plasmids and chromosomal symbiotic regions have two particular features in common. First, the G+C content is lower than that of the residual genome. Second, these regions are hot spots for repetitive sequences. More than 19% of the identified open reading frames in the *B. japonicum* symbiotic region, *M. loti* symbiotic island or symbiotic plasmid of pNGR234a are related to genes involved in transposition and recombination. It is attractive to consider that rhizobia received the genetic equipment for symbiosis from a common ancestral element, which has then co-evolved with the individual species.
1.3 Oxygen control is critical for symbiosis

The significance of oxygen in nitrogen fixation

Symbiotic nitrogen fixation requires a microaerobic environment. In fact, the oxygen tension in nodules corresponds to 5-30 nM free O$_2$, which is about 10$^4$-fold lower than that under normal aerobic conditions (Thumfort et al., 1994). On the one hand, the nitrogenase is inactivated by high oxygen concentrations. On the other hand, oxygen is required for respiration to synthesize sufficient ATP, which is needed for nitrogen fixation (Fig. 1.2). Rhizobia possess a high-affinity cbb$_3$-type terminal oxidase. The cbb$_3$-type oxidase of B. japonicum, for example, has a Km for O$_2$ of 7 nM that allows respiration in nodules (Preisig et al., 1996b). Mutant analyses have shown that the gene clusters fixNOQP, encoding the cbb$_3$-type oxidase, and fixGHIS, whose gene products are probably needed for the assembly of cbb$_3$-type oxidase, are essential for nitrogen fixation (Preisig et al., 1993; Preisig et al., 1996a). The FixNOQP oxidase and the nitrogenase are only synthesized under microaerobic conditions and the transcriptional activation of the respective genes is sensitive to oxygen (see below).

Microaerobiosis is brought about in nodules by an oxygen diffusion barrier in the cortex and by the host-derived leghemoglobin. A layer of tightly packed inner cortical cells forms an oxygen diffusion barrier that establishes microaerobic conditions in the central zone of the nodules. Leghemoglobin binds molecular oxygen. It makes up nearly 30% of the soluble protein in the cytosol of nodule cells and thus represents a kind of oxygen buffer. Moreover, the large amounts of cellular leghemoglobin store and supply sufficient oxygen to ensure constitutive respiration in bacteroids via FixNOQP.

\[
\text{N}_2 + 10 \text{H}^+ + 8 \text{e}^- + 16 \text{MgATP} \rightarrow 2 \text{NH}_4^+ + \text{H}_2 + 16 \text{MgADP} + 16 \text{P}_1
\]

**Fig. 1.2 Reaction of nitrogenase.** The amount of ATP required for the production of ammonia is theoretically 16 molecules. *In vivo*, this number can be higher, because the proportion of electrons allocated to proton reduction increases under conditions of limiting electron flux (Halbleib and Ludden, 2000).

**Oxygen-mediated control of symbiotic genes**

Upon the successful penetration of bacteria into the plant cells, the metabolic adaptation of bacteroids and expression of *nif* and *fix* genes is induced by low-oxygen tensions. Two different regulatory proteins, the sensor protein FixL and the transcription activator
NifA sense low-oxygen concentrations in bacteroids and mediate the control of numerous symbiotic genes (reviewed in Fischer, 1994; Kaminski et al., 1998). FixL as the sensor protein and cognate FixJ as the response regulator form a rhizobia-specific two-component regulatory system that regulates at least one other transcription activator, FixK. In general, FixLJ-FixK and NifA are found in all rhizobia. However, they are organized in complex regulatory networks that are species-specific. Particularly, regulation of nifA is distinct in different rhizobia (reviewed in Fischer, 1994; Fischer, 1996). In the following a short overview on the regulation cascades of B. japonicum, including FixLJ-FixK and NifA, is given as one example (Fig. 1.3).

The B. japonicum FixL is a hemoprotein whose heme iron alters the spin state depending on the oxygen condition (Gilles-Gonzalez et al., 1995). At low oxygen concentrations, FixL phosphorylates FixJ. Subsequently, FixJ-P032 activates transcription of the gene for the transcription regulator FixK2. Notably, two FixK homologues were identified in B. japonicum. FixK2 is controlled by FixLJ like in other rhizobia, and apart from regulating fixNOQP and fixGHIS, FixK2 is involved in the control of heme biosynthesis and nitrate respiration. Target genes for FixK1 are not known yet, however, its regulation is also dependent on FixK2 (Fig. 1.3). The regulatory proteins FixK2 and FixK1 are members of the Fnr-/Crp-family (reviewed by Fischer, 1994). Similarly to Fnr, FixK2 can operate as an activator or as a repressor depending on the target promoter. But in contrast to Fnr and FixK1, FixK2 lacks the characteristic cysteine motif and therefore is probably not directly involved in oxygen control.

The second oxygen sensor relevant for nitrogen fixation is the transcription activator NifA. The rhizobial NifA protein is redox sensitive probably due to a characteristic cysteine motif which may be involved in complexing redox-sensitive metal ions, for example iron. By contrast, this motif is absent in the NifA protein of free-living diazotrophs, such as K. pneumoniae or A. vinelandii, where redox control of NifA activity is mediated by interaction with an additional regulatory protein, NifL (reviewed by Dixon, 1998). Targets of NifA-dependent activation are the nif genes and several fix genes, which are essential for nitrogen fixation. The NifA protein binds to an upstream activator-binding site and initiates transcription in conjunction with RpoN-RNA polymerase. The transcription initiation catalyzed by NifA is energy-dependent and requires ATP. The RpoN-RNA polymerase recognizes −24/−12-type promoters.
(reviewed by Studholme and Buck, 2000; and references in Chapter 2.1) that are typical for all NifA-dependent genes.

In contrast to many *Rhizobium* species, *nifA* expression in *B. japonicum* does not depend on the regulatory proteins FixLJ. But instead, *nifA* is partially controlled by another two-component regulatory system, RegSR (Bauer *et al.*, 1998; Emmerich *et al.*, 1999). RegR activates *fixR*nifA expression under aerobic and microaerobic conditions. The signal of the RegSR system remains to be identified. Preliminary results indicated that it is also responding to low-oxygen conditions, yet, probably by indirect means (M. A. Scotti, unpublished data). In addition to the control by RegSR, NifA activates its own expression under microaerobic conditions.

In *B. japonicum*, two distinct regulatory cascades exist which sense oxygen at different levels (Fig. 1.3). The FixLJ-FixK2 cascade is connected to the RegSR-NifA cascade via *rpoN*, one of two *rpoN* genes (Fig. 1.3). With regard to the expression of symbiotic genes during nodule development, FixLJ-FixK2-regulated genes are probably expressed earlier than NifA-regulated genes. Atmospheric oxygen tensions of approximately 5% are already sufficiently low to induce FixLJ-dependent *fixK2* expression, yet they are too high for NifA-mediated activation and nitrogen fixation. In conclusion, the *cbb3*-type oxidase might be active before the nitrogenase is synthesized and therefore higher respiration rates further decreases the oxygen tension in bacteroids. Indeed, NifA-regulated genes, such as those encoding the nitrogenase, are significantly expressed only at oxygen concentrations of ≤ 0.5%, (M. A. Scotti, unpublished data).

1.4 Aims and structure of this thesis

The first part of this PhD thesis aimed at the identification of additional NifA-regulated genes in *B. japonicum*. The pleiotropic phenotype of a *nifA* mutant suggested the existence of yet undiscovered symbiotically relevant target genes of NifA. For this purpose, a competitive DNA-RNA hybridization approach was applied to the 410-kb symbiotic region of *B. japonicum*. Restriction fragments of a cosmid library representing the symbiotic region were displayed (blotted) on a nylon membrane ("macroarray") and hybridized with RNA isolated from wild-type and *nifA* mutant cells in a competitive manner. This strategy led to the identification of three new NifA-
dependent genes, which were further characterized at the functional and regulatory level (Chapter 2).

The second part of this work is based on a comparative proteome analysis of wild-type and nifA mutant cells which was performed in collaboration with Dainese-Hatt et al. (1999) (Chapter 3). This approach led to the discovery of a heme uptake (hmu) system in B. japonicum whose function and regulation is presented in Chapters 5 and 6 of this thesis. As an introduction to this subject, an overview of bacterial iron homeostasis is given in Chapter 4. The second part is completed by concluding remarks and perspectives described in Chapter 7.

Fig. 1.3 Regulation of nif and fix genes in B. japonicum. The two-component regulatory systems FixLJ and RegSR represent the top level of the two cascades that regulate microaerobically-induced genes. The sensor FixL, a hemoprotein, senses O2 directly and activates FixJ by phosphorylation under low oxygen tensions (< 5% O2). The precise nature of the signal for RegSR activation is not yet known. Oxygen tensions lower than 0.5% enable maximal activity of the redox-responsive NifA protein which is subject to autoregulation.

- $\oplus$ = transcriptional activation,
- $\ominus$ = transcriptional repression,
- $\square$ = unknown promoter type or -35/-10-type promoter,
- $\bullet$ = NifA- and RpoN-dependent -24/-12-type promoter.
Chapter 2

Three new NifA-regulated genes in the
*Bradyrhizobium japonicum* symbiotic gene region
discovered by competitive
DNA-RNA hybridization

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2.1 Summary

The so-called symbiotic region of the *Bradyrhizobium japonicum* chromosome (C. Kündig, H. Hennecke, and M. Göttfert, *J. Bacteriol.* 175:613-622, 1993) was screened for the presence of genes controlled by the nitrogen fixation regulatory protein NifA. Southern blots of restriction enzyme-digested cosmids that represent an ordered, overlapping library of the symbiotic region were competitively hybridized with *in vitro*-labeled RNA from anaerobically grown wild-type cells and an excess of RNA isolated either from anaerobically grown nifA and rpoN mutant cells, or from aerobically grown wild-type cells. In addition to the previously characterized *nif* and *fix* gene clusters, we identified three new NifA-regulated genes that were named *nrgA*, *nrgB* and *nrgC* (*nrg* stands for NifA regulated gene). The latter two probably form an operon, *nrgBC*. The proteins encoded by *nrgC* and *nrgA* exhibited amino acid sequence similarity to bacterial hydroxylases and N-acetyltransferases, respectively. The product of *nrgB* showed no significant similarity to any database entry. Primer extension experiments and expression studies with translational *lacZ* fusions revealed the presence of a functional −24/−12-type promoter upstream of *nrgA* and *nrgBC* and proved the NifA- and RpoN (σ54)-dependent transcription of the respective genes. Null mutations introduced into *nrgA* and *nrgBC* resulted in mutant strains that exhibited wild-type-like symbiotic properties, including nitrogen fixation, when tested on soybean, cowpea or mung bean host plants. Thus, the discovery of *nrgA* and *nrgBC* further emphasizes the previously suggested role of NifA as an activator of anaerobically induced genes other than the classical nitrogen fixation genes.
2.2 Introduction

Nitrogen-fixing rhizobia belonging to any of the four genera *Azorhizobium*, *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium* are able to establish an endosymbiotic interaction with specific leguminous host plants. The transition from the free-living to the symbiotic life style is initiated by the exchange of specific signal molecules between compatible symbiotic partners. Eventually this leads to the formation of root nodules (or in some instances stem nodules) hosting the bacterial partner as an intracellular microsymbiont (for reviews, see van Rhijn and Vanderleyden, 1995; Dénarié *et al.*, 1996). The induction of a number of symbiotic genes, including those specifying the nitrogen fixation apparatus is coordinated together with nodule development via the micro-oxic conditions prevailing in the central nodule tissue (Soupène *et al.*, 1995; Fischer, 1996). 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; Fischer, 1996; Kaminski *et al.*, 1998).

Two oxygen-responsive regulatory cascades are present in the soybean symbiont *Bradyrhizobium japonicum*, the FixLJ-FixK2 cascade and the RegSR-NifA cascade. In response to low-oxygen conditions, the FixJ protein of the FixLJ two-component regulatory pair becomes phosphorylated and activates expression of the subordinate regulatory gene *fixK2* which, in turn, controls a number of functions associated with microaerobic or anaerobic metabolism (Nellen-Anthamatten *et al.*, 1998). The environmental signal for the two-component system of the second cascade, RegSR (Bauer *et al.*, 1998), is not yet known; however, the activity of the transcriptional activator NifA, whose expression is partially controlled by RegR, is directly affected by the oxygen status (Fischer, 1996). Among the targets of NifA eight *nif* genes that are directly involved in nitrogen fixation and also the *fixRnifA* operon, which is subject to NifA-dependent autoregulation under low-oxygen conditions (Fischer, 1994; Bauer *et al.*, 1998). Furthermore, NifA controls expression of the *fixA* and *fixBCX* genes, which are essential for symbiotic nitrogen fixation.

NifA activates gene expression in concert with RNA polymerase containing the specialized σ factor, σ^{54}, which enables the core polymerase to recognize −24/−12-type promoters. Notably, two highly conserved genes encoding σ^{54} (*rpoN_1* and *rpoN_2*) are present in *B. japonicum* (Kullik *et al.*, 1991). Mutant analysis showed that their products
DNA-RNA hybridization can functionally replace each other with regard to their role in nitrogen fixation. NifA normally binds to upstream activator sequences (UAS) and interacts with the RNA polymerase holoenzyme via loop formation by the intervening DNA. DNA bending may be facilitated by the integration host factor (IHF) bound to a site located between the UAS and the core promoter region. Transcription is initiated by productive interaction of the holoenzyme with NifA, catalyzing open complex formation in an ATP-dependent reaction (see Dixon, 1998 and references therein).

The key role of *B. japonicum* NifA in symbiotic nitrogen fixation is documented by the pleiotropic phenotype of *nifA* mutants. Such mutants not only fail to fix nitrogen but also elicit numerous small nodules whose necrotic interior is reminiscent of a hypersensitive response characteristic for noncompatible host-pathogen interactions (Klement, 1982; Fischer *et al.*, 1986; Studer *et al.*, 1987). On the basis of this observation, we speculated that in the wild type, NifA may control as-yet-unknown bacterial genes involved in the suppression of a potential plant defense reaction and in the maintenance of a balanced host-symbiont interaction. In the search for such genes, we found two new NifA-dependent targets, namely, a chaperonin-encoding operon (*groESL*) (Fischer *et al.*, 1993) and a promoter (*ndp*) which is not closely associated with an obvious gene (Weidenhaupt *et al.*, 1993). Yet, neither of the two is essential for symbiosis.

In the present work, we have applied competitive RNA-DNA hybridization to explore the global regulatory scope of NifA. Our analysis was focused on a genomic region of approximately 400 kb of the 8 700-kb *B. japonicum* chromosome, as it turned out that many symbiotic genes are clustered in this region (the symbiotic region) (Kündig *et al.*, 1993). We speculated, therefore, that additional NifA targets might be located there. Moreover, this region was represented in an ordered cosmid library that was available in our laboratory and whose nucleotide sequence is currently being determined (Göttfert *et al.*, 2001). The NifA-dependent transcription pattern of the symbiotic region was analyzed by the competitive hybridization method (Perret *et al.*, 1994; Fellay *et al.*, 1995), which led to the identification of three novel NifA-regulated genes (termed *nrg*) of *B. japonicum*.
2.3 Results

Sections of the symbiotic region that are transcribed in a NifA-dependent manner

The competitive RNA-DNA hybridization technique described in Experimental procedures was used to probe the symbiotic region of *B. japonicum* for the presence of DNA segments that are transcribed under the control of NifA (Fig. 2.1). For comparison, we also performed analogous hybridization experiments with the *rpoN<sub>1/2</sub>* mutant N50-97 and with competing RNAs from aerobically and anaerobically grown wild-type cells to detect regions whose transcription is dependent on RpoN or induced by anaerobiosis, respectively. The hybridization patterns were highly similar in all three experiments (data not shown).

![Fig. 2.1 Transcription pattern of the *B. japonicum* symbiotic region determined by competitive DNA-RNA hybridization](image)

Radioactively labeled RNA from anaerobically grown wild-type cells was hybridized to membrane-bound, EcoRI-digested cosmid DNA in the presence of unlabeled competitor RNA isolated from anaerobically grown cells of nifA mutant A9 as described in Experimental procedures. Lane numbers refer to the respective cosmid number. Hybridizing EcoRI fragments carrying previously known NifA-dependent genes are marked with ellipses (class I genes; [Table 2.1]), those with newly identified NifA-regulated genes are marked with diamonds (class II), and those with other, potentially NifA-dependent *orfs* are marked with rectangles (class III). The hybridization signal of fragment B in lane 7 was only observed upon prolonged exposure times. Unmarked fragments were not further analyzed in this work. For explanation of letters see Table 2.1.
### Table 2.1 Genes and orfs located in NifA-dependently transcribed DNA regions identified by competitive hybridization as shown in Fig 2.1

<table>
<thead>
<tr>
<th>Location of hybridizing region</th>
<th>Hybridizing gene(s) or orf(s)</th>
<th>Reference(s) or comments</th>
</tr>
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<tr>
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\^a Numbers refer to the cosmid numbers indicated in Fig. 2.1.

\^b Letters refer to the specification of the respective fragment in Fig. 2.1.

\^c True NifA-dependent transcription of these orfs has not been demonstrated (see text).
The significance of the hybridization results was confirmed by the finding that many EcoRI fragments carrying previously characterized NifA- and RpoN-dependent nif and fix genes gave rise to strong hybridization signals. Examples include nif and fix genes of cluster I (Fischer, 1994) present on cosmids 6 and 7 (see Table 2.1). Similarly, the strong signal of the 11.5 kb-EcoRI fragment of cosmid 16 could be assigned to the NifA-dependent groESL3 operon by subsequent hybridization experiments with appropriate subclones of this cosmid (data not shown).

Based on (partial) sequence analyses and further hybridization with suitable subclones of selected EcoRI fragments, we divided NifA-dependent genes and open reading frames (orfs) into three classes (Table 2.1). Class I comprises 16 previously known NifA-dependent genes. Class II consists of four newly identified genes, nrgA, nrgB, nrgC and hemN1. A detailed study of nrgA and nrgBC is reported here. The analysis of the hemN1 gene will be presented elsewhere; briefly summarized, it turned out that B. japonicum possesses two hemN-like genes and that the hemN1 gene identified in this work apparently encodes a nonfunctional protein whose synthesis is only partially controlled by NifA and predominantly by FixK2. The alternative hemN gene, hemN2, whose sequence was previously deposited in the GenBank database (accession number AJ002517.1), is located outside of the symbiotic gene region. Class III (Table 2.1) includes three orfs (orf355-1, orf355-2, orf228) whose deduced products are highly homologous to the products of the previously reported B. japonicum orf355 (Ramseier and Göttfert, 1991) and orf228 (GenBank accession no. AB003134). orf228, which is located on the insertion element IS1632 of B. japonicum, is believed to encode a transposase. The potential NifA-dependent transcription and function(s) of class III orfs were not studied in greater detail (see also Discussion).

Identification of nrgA and nrgBC

Our work then focused on the analysis of the hybridizing 1.8-kb and 4.1-kb EcoRI fragments of cosmids 11 and 16, which were subcloned into pUC18, resulting in plasmids pRJ8227 and pRJ8611, respectively (Fig. 2.2). Sequence analysis of pRJ8227 revealed an orf, named nrgA, which specifies a predicted protein of 195 amino acids and a molecular mass of 21,466 Da. The hybridizing region on pRJ8611 was narrowed down to a 2.2-kb EcoRI-CIal fragment, whose nucleotide sequence revealed three partially overlapping orfs (orf110, nrgB, and nrgC). They encode putative proteins of
110 (orf110), 121 (nrgB), and 388 (nrgC) amino acids with molecular masses of 11,937, 14,004, and 41,013 Da, respectively. The nrgB and nrgC genes probably form an operon, since the two genes overlap by 20 codons and no obvious promoter was detected immediately upstream of nrgC. Moreover, nrgB and nrgC appeared to be coregulated as deduced from our studies with respective lacZ fusions (see below). While database searches revealed no entries with significant similarity to the products of orf110 and nrgB, the NrgA and NrgC proteins were found to be homologous to bacterial N-acetyltransferases and hydroxylases, respectively (see Discussion).

Fig. 2.2 Physical map of the EcoRI fragments carrying newly identified NifA-regulated genes. The orientation and location of nrgA on pRJ8227 (A) and of orf110 and nrgBC on pRJ8611 (B) are indicated. The structures of the nrgA and nrgBC mutations are shown along with the corresponding B. japonicum (Bj) strain numbers; hatched bars with horizontal arrows refer to inserted aphII (Km') cassettes and their orientation. Translational lacZ fusions to nrgA, orf110, nrgB and nrgC are indicated by horizontal bars fused to black arrows ('lacZ') and are specified by the number of the strain harboring the respective, chromosomally integrated fusion (Table 2.3). Relevant restrictions sites: B, BamHI; C, ClAI; E, EcoRI; EV, EcoRV; K, KpnI; N, NheI; S, Smal.
Transcriptional analysis of nrgA and nrgBC

By inspection of DNA regions upstream of both the nrgA and orfllO-nrgBC coding regions, we found DNA sequences that showed strong similarity to -24/-12-type promoters (nrgA: T287GGCAC-N5-TTGCA302 [Fig. 2.3A]; orfllO-nrgBC: T474GGCAC-N5-TTGCA489 [Fig. 2.3B]). Moreover, a putative binding site for the transcriptional activator protein NifA was present at an appropriate distance of approximately 100 bp upstream of the presumptive nrgBC promoter (T364GT-N10-ACA379 [Fig. 2.3B]). No consensus NifA binding motif was found upstream of nrgA. Interestingly, a perfect copy of this motif is present in the 5' coding region of nrgA (T530GT-N10-ACA545), yet its functional role at this unorthodox position is questionable.

The function of the putative promoters was confirmed by primer extension experiments using two different oligonucleotides for each of them (see Experimental procedures). Anaerobically grown cells of wild-type B. japonicum and of mutant strains A9 (nifA) and N50-97 (rpoNm) were used as sources for the isolation of template RNA. The results of these experiments are shown in Fig. 2.3. The 3' end of the dominant elongation product obtained with the primers for nrgA and wild-type RNA corresponded to C314 which is located at the appropriate distance of 11 nucleotides downstream of the predicted -24/-12 promoter of nrgA (Fig. 2.3A). The minor elongation product ending at C301 is not associated with an obvious promoter and may have resulted from premature termination of the reverse transcription reaction. Regardless of the primer used, no primer extension product was obtained with RNA isolated from the nifA mutant A9. The results of primer extension experiments with orfllO-nrgBC led to similar conclusions (Fig. 2.3B). The elongation products which were obtained with both primers and wild-type RNA, but not with RNA from the nifA or rpoNm mutant, indicated the existence of a NifA- and RpoN-dependent transcript starting at T497, i. e., at a correct distance from the predicted promoter. Its dependence on the oxygen-labile NifA protein was further documented by the absence of an elongation product in the experiment with primer 8611-2 and RNA from aerobically grown wild-type cells (Fig. 2.3B, right panel).
Fig. 2.3 Mapping of the transcription start sites of *B. japonicum* nrgA (A) and nrgBC (B) by primer extension. Total RNA was purified from anaerobically grown cells of wild-type *B. japonicum* (w) or mutant strain A9 (n) or N50-97 (r) and used for primer extension experiments with the primers indicated, as described in Experimental procedures. An additional control experiment was performed with RNA isolated from aerobically grown wild-type cells and primer 8611-2 (O2). The sequence ladders shown were generated with pRJ8227 (A) and pRJ8611 (B) plasmid DNA and the same primers used for the respective transcript mapping. The dominant transcription start sites for nrgA and nrgBC are marked by +1 and arrows. The nucleotide sequences of relevant DNA regions is shown at the bottom. A potential binding site for NifA (NifA-UAS), the −24/−12-type promoter core sequences, transcriptional start sites (+1) and putative translational start codons (ATG) are shown by white letters. Additional conserved nucleotides of −24/−12-type promoters are underlined. The numbering of nucleotide positions corresponds to that of the sequences deposited in the GenBank database.
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**nrgA**

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**nrgBC**

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**nrgB**

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DNA-RNA hybridization

**Analysis of nrgA, orf110, and nrgBC expression with lacZ fusions**

Translational lacZ fusions to nrgA, nrgB, nrgC, and orf110 were constructed as described in Experimental procedures (see also Fig. 2.2) in order to quantitate expression from the -24/-12-type promoters identified above and also to test whether the orfs and genes under investigation were translated. The fusions to nrgA, nrgB and nrgC were integrated into the chromosome of wild-type B. japonicum and mutants A9 (nifA) and N50-97 (rpoN\(_{1/2}\)). The orf110'-'lacZ fusion was introduced only into the wild type. Cells of all strains were grown under aerobic or anaerobic conditions, and β-galactosidase activity was determined (Table 2.2).

Regardless of the genetic background, no significant expression of any lacZ fusion was detected when cells were grown aerobically. By contrast, β-galactosidase activity derived from the fusions to nrgA, nrgB and nrgC was drastically induced in the wild-type background under anaerobic conditions (~190-, ~340-, and ~450-fold increases, respectively). No activities were detectable in the nifA or rpoN\(_{1/2}\) mutant strain grown under these conditions. No β-galactosidase activity was measurable also in strains harboring the orf110'-'lacZ fusion, from which we conclude that orf110 is not translated.

**Symbiotic phenotype of nrgA and nrgBC mutants**

The potential roles of nrgA and nrgBC in symbiotic nitrogen fixation were studied in infection tests with mutant strains 8236 (nrgA) and 8620 (nrgBC) (see Fig. 2.2) using soybean, cowpea and mung bean as the host plants. Nodulation and nitrogen fixation activity of 6 to 14 plants were evaluated 3 weeks after infection. The nitrogen fixation activities of both mutants did not differ significantly from that of the wild type on all three host plants tested (between 91 and 122% of wild-type Fix activity). The same result was found with regard to the size, the morphology, and the interior color of nodules, except for strain 8620, which elicited an increased number of nodules on cowpea (42 ± 12 and 21 ± 5 nodules for strain 8620 and the wild type, respectively). Thus, the products of nrgA and nrgBC are not essential for an effective B. japonicum-host plant symbiosis.

25
### Table 2.2 Expression of chromosomally integrated *B. japonicum* nrgA', nrgB', nrgC', and orf110'-lacZ fusions in cells grown under different oxygen conditions\(^a\)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>β-Galactosidase activity (Miller U)(^b)</th>
<th>Aerobic(^c)</th>
<th>Anaerobic(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8233</td>
<td>nrgA'-lacZ</td>
<td>1.0 ± 0.8</td>
<td>188 ± 64</td>
<td></td>
</tr>
<tr>
<td>A8233</td>
<td>nrgA'-lacZ nifA</td>
<td>0.4 ± 0.0</td>
<td>2.5 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>N8233</td>
<td>nrgA'-lacZ rpoN(_{1/2})</td>
<td>0.5 ± 0.2</td>
<td>1.5 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>8626</td>
<td>orf110'-lacZ</td>
<td>0.3 ± 0.0</td>
<td>0.4 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>8627</td>
<td>nrgB'-lacZ</td>
<td>0.5 ± 0.0</td>
<td>117 ± 64</td>
<td></td>
</tr>
<tr>
<td>A8627</td>
<td>nrgB'-lacZ nifA</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>N8627</td>
<td>nrgB'-lacZ rpoN(_{1/2})</td>
<td>0.3 ± 0.0</td>
<td>1.3 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>8628</td>
<td>nrgC'-lacZ</td>
<td>0.4 ± 0.0</td>
<td>161 ± 84</td>
<td></td>
</tr>
<tr>
<td>A8628</td>
<td>nrgC'-lacZ nifA</td>
<td>0.4 ± 0.0</td>
<td>1.2 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>N8628</td>
<td>nrgC'-lacZ rpoN(_{1/2})</td>
<td>0.3 ± 0.1</td>
<td>1.2 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The wild-type strain without an integrated lacZ fusion showed < 1.5 Miller units under all growth conditions.

\(^b\) Numbers are mean ± standard errors of two (aerobic) or at least three (anaerobic; two independent experiments) cultures of individual strains, which were assayed in duplicate.

\(^c\) Aerobic cultures were grown for 3 days in PSY medium containing 100 μg spectinomycin per ml.

\(^d\) Anaerobic cultures were grown for 6 days in YEM medium containing 10 mM KN03 and 100 μg spectinomycin per ml.
2.4 Discussion

The DNA-RNA hybridization approach: advantages and drawbacks

In the present study, we have applied competitive DNA-RNA hybridization to screen the symbiotic region of the *B. japonicum* chromosome for sections that are transcribed under the control of the oxygen-responsive regulator NifA. An original version of this method was based on differential DNA-cDNA hybridization of an ordered *E. coli* cosmid library (Kohara *et al*., 1987), and it was applied to monitor alterations in the global transcription pattern in response to various external stimuli or regulatory mutations (Chuang *et al*., 1993). Subsequently, modifications were made to this technique, including competitive DNA subtraction hybridization and DNA-RNA hybridization, and were used by several authors for the detection of differentially expressed, often symbiotic, genes in different rhizobia (David *et al*., 1987; Bhagwat and Keister, 1992; Perret *et al*., 1994; Fellay *et al*., 1995; Girard *et al*., 1996; Perret *et al*., 1999). The success of our approach is documented by the identification of three new *B. japonicum* genes, *nrgA*, *nrgB* and *nrgC*, which are associated with a $\sigma^{54}$-dependent – 24/-12-type promoter that is activated by NifA. With the two newly identified promoters, a total of 11 transcriptionally mapped NifA–$\sigma^{54}$-dependent promoters in the symbiotic region of *B. japonicum* are now known (Kündig *et al*., 1993; Fischer *et al*., 1993; Weidenhaupt *et al*., 1993; Fischer, 1994). A comparable number of 16 (putative) NifA–$\sigma^{54}$-dependent promoters were found in a transcriptional survey of the 536-kb symbiotic plasmid of *Rhizobium* sp. NGR234 (Perret *et al*., 1999).

Critical prerequisites for our successful screening included the availability of (i) an ordered cosmid library representing the symbiotic region, (ii) regulatory nifA and rpoN₁/₂ mutants used as a source for the competing RNA, and (iii) the emerging DNA sequence information originating from a partial genomic sequencing project (Göttfert *et al*., 2001). Probably, we have not yet fully exploited the potential of the screening, as indicated by numerous hybridizing fragments that were not further characterized in this work. Additional candidate genes potentially controlled by NifA include, for example, *nijV*- and *nijM*-like genes whose products are involved in FeMo cofactor synthesis and activation of the nitrogenase Fe protein in free-living diazotrophs (Howard and Rees, 1994 and references therein). Intriguingly, no rhizobial homologues of these genes have been identified so far.
Surprisingly, we have identified as many as approximately 35 specifically hybridizing EcoRI fragments within the approximately 400 kb of genomic *B. japonicum* DNA represented by the 13 cosmids. However, this rather large number does not reflect truly disparate NifA-dependent loci, because we could show in at least two cases that the repetitive element RSRja was responsible for the observed hybridization signal (data not shown). Given the facts that the RSRja elements are extremely well conserved and that several of them are located in the symbiotic region (Kaluza *et al.*, 1985; Hahn, 1986), they have the potential to yield multiple hybridizing fragments. Hence, repetitive sequences which are transcribed in a NifA-dependent manner would render our approach less useful than initially anticipated.

All EcoRI fragments hybridizing in the experiment with competing RNA from the *nifA* mutant were also detected in the analogous experiment with competing RNA from the *rpoN*1/2 mutant (data not shown). This was to be expected in the light of the compulsory dependence on RpoN (σ54) of all NifA-activated promoters. The absence of a clear qualitative difference in the overall hybridization pattern in these two experiments indicated that the large majority of RpoN-dependent promoters in the symbiotic region are indeed activated by NifA and probably not by other activators working in concert with the σ54 RNA polymerase, such as NtrC. The only exception might be a gene present on the 10-kb EcoRI fragment of cosmid 13 that showed a strong differential hybridization with *rpoN* but not with *nifA* mutant RNA. Another notable aspect is that the hybridization pattern in the experiment with RNA from aerobically and anerobically grown wild-type cells resembled very much that observed in both of the other hybridization experiments. We interpret this to mean that in the DNA region investigated, transcriptional activation in response to anaerobiosis is predominantly brought about by NifA and not by another oxygen-controlled regulator. This finding was not necessarily predictable, because at least one additional oxygen-responsive regulation system exists in *B. japonicum*, i. e., the FixLJ-FixK2 cascade (Nellen-Anthamatten *et al.*, 1998). Interestingly, with *hemN*, a gene was identified that apparently belongs to both the FixLJ-FixK2 and the RegSR-NifA regulons. At least one additional member of the FixLJ-FixK2 regulon, namely *rpoN*1 (Kündig *et al.*, 1993), had been previously mapped to the symbiotic region, so in theory this gene ought to have been detected in our hybridization experiment. Reasons why this was not the case could be a weak expression of *rpoN*1, an interference with NifA- and RpoN-dependent genes
on the same EcoRI fragment, or a cross hybridization with the highly similar, constitutively synthesized rpoN2 mRNA.

**What is the role of the new Nrg proteins?**

The question regarding the potential function of the newly identified genes nrgA, nrgB, and nrgC was addressed by phenotypic analyses of appropriate mutants and by database homology searches. The results from the plant infection tests clearly indicated that, under the applied laboratory conditions, none of these genes is essential for nitrogen fixation in symbiosis with the three hosts tested. Notably, nodulation of cowpea by the nrgBC null mutant 8620 seems to be slightly disturbed as indicated by the elevated number of nodules. Principally, we cannot rule out that additional subtle effects of the nrg mutations might be detected under more competitive field conditions. It was shown recently that disruption of the Sinorhizobium meliloti phbC gene, encoding poly-ß-hydroxybutyrate synthase, resulted in a mutant that was outcompeted by the wild type in a mixed infection test, even though its ability to fix nitrogen was not affected (Willis and Walker, 1998). Alternatively, one could hypothesize that the function of the nrgA or nrgBC gene products in the respective mutants is replaced by potential homologues. However, Southern blot hybridization experiments performed under low-stringency conditions with suitable nrg probes were not indicative of this possibility (data not shown).

Searches with NrgA revealed that this predicted protein (195 amino acids) is similar to a number of N-acetyltransferases among which similarity to a puromycin N-acetyltransferase of Streptomyces anulatus was greatest (195 amino acids; 23% identity and 47% similarity [Fig. 2.4A]). Maximal similarity was found to a hypothetical protein of unknown function in Mycobacterium tuberculosis (201 amino acids; 33% identity and 53% similarity). Unfortunately, it was not possible to test the potential involvement of NrgA in puromycin resistance due to the intrinsic resistance of B. japonicum against this antibiotic (100 μg/ml). At any rate, it seems not compelling that NrgA is equivalent to a puromycin N-acetyltransferase, because the general similarity to several different N-acetyltransferases, particularly in the C-terminal half of the protein, allows for many possible substrates that could be envisaged for N acetylation by NrgA.

The search with NrgC (388 amino acids) identified hydroxylases from different bacteria as the homologous proteins (Fig. 2.4B). Examples include a putative hydroxylase of
Streptomyces violaceoruber (400 amino acids; 26% identity and 49% similarity), a hydroxylase of Rhodococcus erythropolis (393 amino acids; 24% identity and 49% similarity) and a phenol hydroxylase of Bacillus stearothermophilus (400 amino acids; 23% identity and 46% similarity). Some of these proteins were shown to have indole oxidation activity in a colorimetric plate test (Hart et al., 1990; Kim and Oriel, 1995). When we applied this test to microaerobically grown B. japonicum wild-type and nrgBC mutant cells, no indole oxidation activity was detectable in either strain. Thus, the function of NrgC and the substrate that might be hydroxylated by NrgC remain obscure. Interestingly, as in the case of NrgA, the most similar protein to NrgC was a hypothetical, functionally undefined protein from M. tuberculosis (376 amino acids; 34% identity and 56% similarity).

It is interesting that by and large, the products of both nrgA and nrgC display similarity to enzymes that modify potentially toxic compounds. It is known that during the early stages of the symbiotic rhizobium-legume interaction, the host plant induces the synthesis of low-molecular-weight, phenolic compounds (e.g., phytoalexins) which are synthesized in response to pathogenic interactions and have antimicrobial activity (Schmidt et al., 1992; for reviews, see Baron and Zambryski, 1995; Phillips and Kapulnik, 1995). Moreover, it was shown previously that glyceollin, the phytoalexin of soybean, is present at elevated levels in nodules elicited by a B. japonicum nifA mutant (Parniske et al., 1991). Therefore, it appears attractive to speculate that the NrgA and/or NrgC proteins contribute to overcoming the plant defense response. However, even if this were to be the case, the results from our plant infection tests imply that such a hypothetical function cannot be essential for the formation of a productive symbiotic interaction.

Fig. 2.4 Amino acid sequence alignments of the predicted B. japonicum NrgA and NrgC proteins to their best homologues. (A) Alignment of NrgA (BjNrgA) to M. tuberculosis hypothetical protein Rv0133 (MtRv01; accession no. CAB07039) and to S. anulatus puromycin N-acetyltransferase (SaPac; accession no. P13249). (B) Alignment of NrgC (BjNrgC) to M. tuberculosis hypothetical protein Rv3094c (MtRv30; accession no. CAB08386), a putative S. violaceoruber hydroxylase (SvGra; accession no. CAA09642), an R. erythropolis hydroxylase (ReBphC; accession no. BAA25602) and a B. stearothermophilus phenol hydroxylase (BsPheA; accession no. AAA85688). Identical amino acids are emphasized by white letters; those amino acids of the Nrg proteins that are identical in at least one homologue of NrgA and two homologues of NrgC are highlighted in gray. Numbering refers to amino acid positions in the NrgA (A) and NrgC (B) proteins. (see next page)
NifA, a global anaerobic rather than a nitrogen fixation-specific regulator in rhizobia

The identification of \textit{nrgA} and \textit{nrgBC} corroborates our earlier notion that NifA control is not restricted to genes directly concerned with nitrogen fixation (Fischer, 1994). Other examples include the \textit{groESL} operon and the \textit{glnII} gene of \textit{B. japonicum} (Martin \textit{et al.}, 1988; Fischer \textit{et al.}, 1993), rhizopine biosynthetic genes (\textit{mos}) of \textit{S. meliloti} (Murphy \textit{et al.}, 1988) and the tyrosinase (polyphenol oxidase) structural gene \textit{melA} of \textit{Rhizobium etli} (Hawkins and Johnston, 1988). Thus, NifA in rhizobia is a general regulator controlling microaerobically induced functions which may or may not be related to symbiosis. This implies that additional targets for NifA control might well be located outside of the symbiotic region of the \textit{B. japonicum} chromosome. Their identification could now be attempted by applying the method used in this study to a cosmid library representing the entire genome.

Acknowledgments

We are grateful to Rémy Fellay for helpful experimental advice. Franziska Biellmann, Roger Frei and Michael Spring are acknowledged for excellent technical assistance. This work was supported by a grant from the Swiss National Foundation for Scientific Research.

2.5 Experimental procedures

Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are listed in Table 2.3.
Table 2.3 Bacterial strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant phenotype or genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (Φ80lacZ∆M15) hsdR17 recA1 gyrA96 thi-1 relA1</td>
<td>Bethesda Research Laboratories, Inc., Gaithersburg, Md</td>
</tr>
<tr>
<td>S17-1</td>
<td>Sm’ Sp’ hsdR (RP4-2 kan::Tn7 tet::Mu, integrated in the chromosome)</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>B. japonicum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>110spc4</td>
<td>Sp’ wild type</td>
<td>Regensburger and Hennecke (1983)</td>
</tr>
<tr>
<td>A9</td>
<td>Sp’ Km’ nifA::aphII</td>
<td>Fischer et al. (1986)</td>
</tr>
<tr>
<td>N50-97</td>
<td>Sp’ Km’ Sm’ rpoN1::aphII rpoN2::Ω</td>
<td>Kullik et al. (1991)</td>
</tr>
<tr>
<td>8236</td>
<td>Sp’ Km’ nrgA::aphII (opposite orientation)</td>
<td>This work</td>
</tr>
<tr>
<td>8620</td>
<td>Sp’ Km’ nrgBC::aphII</td>
<td>This work</td>
</tr>
<tr>
<td>8233a</td>
<td>Sp’ Tc’ nrgA’-‘lacZ chromosomally integrated</td>
<td>This work</td>
</tr>
<tr>
<td>8626</td>
<td>Sp’ Tc’ orf110’-‘lacZ chromosomally integrated</td>
<td>This work</td>
</tr>
<tr>
<td>8627a</td>
<td>Sp’ Tc’ nrgB’-‘lacZ chromosomally integrated</td>
<td>This work</td>
</tr>
<tr>
<td>8628a</td>
<td>Sp’ Tc’ nrgC’-‘lacZ chromosomally integrated</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pUC18</td>
<td>Ap’</td>
<td>Norrander et al. (1983)</td>
</tr>
<tr>
<td>pSUP202</td>
<td>Ap’ Cm’ Tc’ oriT from RP4</td>
<td>Simon et al. (1983)</td>
</tr>
</tbody>
</table>
pSUP202pol4  Te\(^{r}\) (pSUP202) part of polylinker from pBluescript II KS+ between EcoRI and PstI

pSUP480  Te\(^{r}\), 'lacZ part from pNM480 in pSUP202pol4

pSUP481  Te\(^{r}\), 'lacZ part from pNM481 in pSUP202pol4

pBSL14/15  Ap\(^{r}\) Km\(^{r}\)

pRJ8227  Ap\(^{r}\) (pUC18) *B. japonicum* 1.8-kb EcoRI fragment of cosmid no. 11; *nrgA*

pRJ8610  Ap\(^{r}\) (pUC18) *B. japonicum* 11-kb EcoRI fragment from cosmid no. 16; *groESL*\(^{3}\)

pRJ8611  Ap\(^{r}\) (pUC18) *B. japonicum* 4.1-kb EcoRI fragment from cosmid no. 16; *nrgBC*

Cosmids

Lorist6  Km\(^{r}\), bacteriophage \(\lambda\)-replicon

Cosmids 6-18  Km\(^{r}\), ordered library of the symbiotic region in *B. japonicum*  
(Sau3A fragments of *B. japonicum* genomic DNA in Lorist6)

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The same *nrgA*\(^{r}\)-, *nrgB*\(^{r}\)- and *nrgC*\(^{l}\)-lacZ fusions were also integrated into the chromosome of *B. japonicum* A9 and N50-97. The resulting strains were given the same numbers preceded by A (A9 derivatives) or N (N50-97 derivatives); see Table 2.2.
Media and growth conditions
Luria-Bertani (LB) medium (Miller, 1972) was used for growth of *Escherichia coli* cells and, contained the following concentrations (micrograms per milliliter) of antibiotics for plasmid selection: ampicillin, 200; kanamycin, 30; tetracycline, 10. Peptone-salts-yeast extract (PSY) medium (Regensburger and Hennecke, 1983) supplemented with 0.1% L-arabinose was used for routine aerobic cultures of *B. japonicum*, whereas yeast extract-manitol (YEM) medium supplemented with 10 mM KNO₃ (Daniel and Appleby, 1972) was used for anaerobic *B. japonicum* cultures and corresponding aerobic control cultures. Anaerobic cultures were kept under argon in rubber-stoppered serum bottles. Concentrations (micrograms per milliliter) of antibiotics for use in *B. japonicum* cultures were as follows: spectinomycin, 100; kanamycin, 100; streptomycin, 50; tetracycline, 50 (solid media) or 25 (liquid media).

DNA work and sequence analysis
Recombinant DNA work and Southern blotting were performed according to standard protocols (Sambrook *et al.*, 1989). For homologous hybridizations, we used digoxigenin-labeled probes generated by PCR or by elongation of random hexanucleotides with the Klenow fragment of DNA polymerase (DIG DNA Labeling Kit, Roche Diagnostics, Rotkreuz, Switzerland). *B. japonicum* chromosomal DNA was isolated as described previously (Hahn and Hennecke, 1984). For computer-assisted analyses of DNA and protein sequences, we used the software package (version 8) of the Genetics Computer Group of the University of Wisconsin, Madison and the MAC program DNA-STRIDER version 1.2. Homology searches were performed by using the National Center for Biotechnology Information BLAST network server (http://www.ncbi.nlm.nih.gov/BLAST/).

RNA extraction and labeling
*B. japonicum* cells were grown anaerobically in 500 ml serum bottles filled with 400 ml YEM medium for at least 4 to 7 days to an optical density (600 nm) of 0.2 to 0.4. Spectinomycin was the only antibiotic used in these cultures. After the cultures were cooled on ice-water, cells were harvested by centrifugation and washed with 0.9% NaCl (w/v), and the cell pellets were immediately frozen in liquid nitrogen and stored at −80°C. For RNA isolation, 200 to 400 mg cells (wet weight) were resuspended in 10 ml
of cold 20 mM Na-acetate (pH 5.5) - 1 mM EDTA plus 0.5% (wt/vol) (final concentration) sodium dodecyl sulfate (SDS), followed by extraction with 10 ml of prewarmed (65°C) acidic phenol (pH 5.5). The phenol extraction was repeated with 10 ml phenol-CHCl₃-isoamylalcohol (49.5:49.5:1), and RNA was ethanol precipitated. The samples were treated with RQ1 RNase-free DNase (5 U) (Promega, Madison, WI) for 15 min at 37°C to remove potential contaminating DNA. After an additional phenol extraction, the RNA was ethanol precipitated and dissolved in diethyl pyrocarbonate-treated H₂O. RNA yields were approximately 100 µg per 100 mg (wet weight) of cells as determined by spectrophotometry. Radioactive end labeling of RNA was performed as described previously (Fellay et al., 1995). RNA (15-22 µg) was partially hydrolyzed by incubation in NaOH (125 mM final concentration) for 25 min on ice and labeled with T4 polynucleotide kinase (20 U) (MBI Fermentas, Vilnius, Lithuania) and [γ-³²P]ATP for 90 min at 37°C. Unincorporated label and low-molecular-weight RNA fragments were removed by gel filtration (Sephadex G-50; Amersham Pharmacia Biotech), and 5 x 10⁷ to 10 x 10⁷ cpm of labeled RNA was used for competitive hybridizations.

Competitive RNA-DNA hybridizations

Competitive hybridizations were performed as described previously (Perret et al., 1994; Fellay et al., 1995) with minor modifications. At least 1 µg of each of 13 cosmids (numbered from 6 to 18) representing the symbiotic region of the B. japonicum chromosome was digested with EcoRI. The resulting DNA fragments were separated on 1% agarose gels and transferred by Southern blotting to Hybond-N nylon membranes (Amersham Pharmacia Biotech). Prehybridizations were performed in 30 ml PHS solution (50 mM Tris-HCl [pH 7.4], 1 M NaCl, 1% SDS, 0.2% bovine serum albumin, 0.2% Ficoll 400, 0.2% polyvinylpyrollidone, 0.2% Na-pyrophosphate) at 65°C for 8 h with at least 130 to 200 µg of nonlabeled RNA isolated from anaerobically grown B. japonicum strains A9 or N50-97 or from aerobically grown wild-type cells. Subsequently, RNA isolated from anaerobically grown wild-type cells, end labeled as described above, was added to the prehybridization solution, and competitive hybridizations were performed at 65°C for 16 h. Membranes were washed three times for 30 min at 65°C in prewarmed 1x SSC (150 mM NaCl, 15 mM sodium citrate)-1% SDS (20 ml solution per wash step) and finally for 15 min at room temperature in 20 ml
of 0.2x SSC. Hybridizing bands were analyzed with a PhosphoImager (Molecular Dynamics) after exposure of the membranes for at least 24 h.

**Transcript mapping**
The transcriptional start sites of *nrgA* and *nrgBC* were mapped with primer extension experiments. Two 30-mers were used as primers for *nrgA* mapping (oligonucleotide 8227-4: 5'-GTTTGCATTCGCACATTTGATATCCGACTC<sub>515</sub>-3'; oligonucleotide 8227-5: 5'-CCAAATTTTCTGTCTACCTGTCAGAGTTAC<sub>466</sub>-3' [position numbers refer to those in the sequence deposited in the GenBank database]). A 28-mer (oligonucleotide 8611-1: 5'-CTCATACGTCGGACAAGCCGGGTCGAGC<sub>557</sub>-3') and a 25-mer (oligonucleotide 8611-2: 5'-GGGCATGCGATGTCATGTCTTCTCC<sub>597</sub>-3') were used for *nrgBC* mapping. RNA was isolated as described previously (Babst et al., 1996) from *B. japonicum* strains 110<sup>spc4</sup> (wild type), A9 (*nifA*), and N50-97 (*rpoN<sub>1/2</sub>*) grown anaerobically for 5 days in YEM medium containing 10 mM KNO<sub>3</sub> and, for a control, also from wild-type cells cultured aerobically for 3 days in the same medium. The latter cells had to be harvested by centrifugation at 14 000 x g because of the pronounced synthesis of extracellular slime. Approximately 5 μg of RNA and at least 100 000 cpm of radiolabeled primer (200 to 500 fmol) were used for each primer extension experiment, which was performed as described previously (Babst et al., 1996; Bauer et al., 1998). Extension products were purified by phenol extraction followed by ethanol precipitation before they were loaded on 6% denaturing polyacrylamide gels.

**Construction of *B. japonicum nrgA* and *nrgBC* mutant strains**
The *nrgA* gene was mutagenized by insertion of a 1.2-kb *KpnI* kanamycin resistance gene cassette (*aphII*) isolated from pBSL15, into its unique *KpnI* site (see Fig. 2.2A). The *nrgBC* genes were mutated by deleting a 0.56 kb *EcoRV*-*Nhel* fragment, which was replaced by a *Nhel*-*SmaI* *aphII* fragment from pBSL14 (see Fig. 2.2B). Appropriate DNA fragments containing the mutated *nrgA* or *nrgBC* genes were cloned into the vector pSUP202pol4 and mobilized into *B. japonicum* 110<sup>spc4</sup> as described previously (Hahn and Hennecke, 1984). Cointegrate-containing exconjugants (resulting from single crossover) were distinguished from true marker exchange mutants (resulting from double crossover) by the vector’s tetracycline resistance. The correct genomic structures of all mutant strains were confirmed by Southern blot analysis of genomic DNAs.
Construction of chromosomally integrated nrgA\(^{-}\), nrgB\(^{-}\), and nrgC\(^{-}\)-lacZ fusions

Translational lacZ fusions were constructed by making use of gene-internal restriction sites (see Fig. 2.2) and the mobilizable lacZ fusion vectors pSUP480 (for orf110 and nrgC) and pSUP481 (for nrgA and nrgB). The nrgA gene was fused at a BamHI site corresponding to Arg-70 in the predicted NrgA protein. The overlapping reading frames orf110 and nrgB were fused at their common EcoRV site corresponding to Asp-70 in the putative Orf110 protein and Ala-61 in the NrgB protein. The fusion to nrgC was constructed at a SmaI site corresponding to Pro-151 of the NrgC protein. The lacZ fusion constructs including appropriate portions of B. japonicum upstream DNA were conjugated into B. japonicum \(\text{ospc4} (\text{wild type})\), A9 (nifA), and N50-97 (\(\text{rpoN}_{1/2}\)). Those clones that contained the entire lacZ fusion plasmid integrated via single crossover at the homologous chromosomal position were selected by plating the exconjugants on tetracycline-containing plates. The genomic structures of all resulting strains were verified by Southern blot analysis.

\(\beta\)-Galactosidase assays

\(\beta\)-Galactosidase activity assays were done as described previously (Fischer et al., 1993).

Plant infection test

The symbiotic phenotypes of the B. japonicum nrgA and nrgBC mutants were determined in infection tests using soybean [Glycine max (L.) Merr. cv. Williams], cowpea (Vigna unguiculata cv. Red Caloona) and mung bean (Vigna radiata) as host plants. The tests were performed as described previously (Hahn and Hennecke, 1984; Göttfert et al., 1990). Soybean seeds were kindly provided by P. M. Gresshoff (University of Queensland, Australia), whereas cowpea and mung bean seeds were kind gifts from W. D. Broughton (University of Geneva, Switzerland).

Nucleotide sequence accessions numbers

The nucleotide sequences of the B. japonicum nrgA and nrgBC genes have been deposited in the GenBank database under accession numbers AF190732 and AF190733, respectively.
Chapter 3

Search for new NifA-regulated genes
by proteome analysis
3.1 Introduction

The proteome of wild-type \textit{B. japonicum} and \textit{nifA} mutant A9, grown under anaerobic conditions, was analyzed by 2-dimensional polyacrylamide gel electrophoresis. Proteins were identified and characterized by N-terminal sequencing and MS-MS fingerprinting. This work was done in collaboration with Dainese Hatt \textit{et al.} (1999). Protein spots, which were present in the wild type but not or with at least 3-fold lower intensity in the \textit{nifA} mutant, were considered as putatively \textit{NifA}-regulated gene products. A selection of such proteins is depicted in Fig. 3.1 and Table 3.1. For some of the identified proteins, the \textit{NifA}-dependent synthesis under anaerobic conditions had already been shown previously (Fischer \textit{et al.}, 1993). For example, proteins 1 and 29 belong to this class; these proteins correspond to GroES\textsubscript{3} and GroEL\textsubscript{3}, the small and large subunit of the GroE\textsubscript{3} chaperonin. Spot 17.2 shows best homology to NifS, a putative \textit{NifA}-dependent cysteine desulfurase of \textit{Rhizobium} sp. NGR234.

In order to identify new \textit{NifA}-regulated genes, we intended to make use of the N-terminal amino acid sequence obtained from selected proteins. We focused on proteins with unknown function, comprising those that showed either no similarity to proteins in the database or to proteins with unknown function.

\textbf{Fig. 3.1} Comparison of the proteomes of \textit{B. japonicum} wild type (A) and \textit{nifA} mutant A9 (B). Two-dimensional gel electrophoresis was performed with total protein extracts. The cells used for protein preparation were grown anaerobically. Selected proteins are numbered and marked with white circles.
Table 3.1 N-terminal amino acid sequences of putatively NifA-controlled proteins, considered in this work (adapted from Dainese Hatt et al., 1999)

<table>
<thead>
<tr>
<th>Protein no.</th>
<th>N-terminal amino acid sequence</th>
<th>FASTA / TFASTA search</th>
<th>Identification by MS-MS fingerprinting</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MKFRPLHDRVVV</td>
<td>GroES\textsubscript{3}</td>
<td>GroES\textsubscript{3}</td>
</tr>
<tr>
<td>4</td>
<td>MFIAMNRFQVK</td>
<td>no match /homology</td>
<td>no match /homology</td>
</tr>
<tr>
<td>5.1</td>
<td>PNASEXE(G/W)REXRP</td>
<td>Orf\textsuperscript{a} NGR234</td>
<td>Orf\textsuperscript{a} NGR234</td>
</tr>
<tr>
<td>11</td>
<td>MDKMRIDKGEVL</td>
<td>no match /homology</td>
<td>no match /homology</td>
</tr>
<tr>
<td>17.2</td>
<td>N-terminally blocked</td>
<td>no match /homology</td>
<td>NifS in NGR234</td>
</tr>
<tr>
<td>29</td>
<td>SAKEVKFGVNRDR</td>
<td>GroEL\textsubscript{3}</td>
<td>GroEL\textsubscript{3}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Accession no. AE000073.em_ba, bases 3008-3214

3.2 Results

Choice of putative NifA-regulated proteins

The N terminus of three out of seven chosen proteins was blocked for Edman degradation. From the remaining four candidates, we have chosen proteins 4, 5.1 and 11, whose N termini seemed to be suitable for the deduction of degenerated oligonucleotides (Table 3.1). We also compared the respective N termini with the protein database deduced from the symbiotic gene region of *B. japonicum* (Göttfert et al., 2001). Indeed, the N terminus of protein 5.1 corresponds to the N terminus of Id249 (accession no. AAG60813), matching 10 out of 11 determined amino acids (Fig. 3.2). The calculated molecular mass of Id249 (14 283.73 Da) coincides with the size of protein 5.1, estimated from the two-dimensional protein gel.

\textbf{Fig. 3.2 Alignment of N-termini of the proteins 5.1 and Id249} (Göttfert et al., 2001; acc. no. AAG60813).
The sequence upstream of *id249* was inspected for putative RpoN binding sites, a prerequisite for NifA-dependent regulation (Fischer, 1994). About 200 bp upstream of the coding region for *id249*, we found a DNA sequence [GGGCAAGCGACAGCA] that showed some similarity to -24/-12-type consensus promoters [TGGCAC-N₅-TTGC²²; the highly conserved core region is underlined]. However, no NifA-binding site was found upstream of *id249*. Furthermore, *id249* is located on a 13 882-bp EcoRI fragment that gave a strong signal in the differential DNA-RNA hybridization performed to identify NifA-regulated genes (Chapter 2). In the context of this work, however, the analysis of *id249* was not further pursued.

**Search for the genes encoding proteins 4 and 11**

Two different mixtures of degenerated oligonucleotides deduced from the N terminus of protein 4 were synthesized and used in a Southern blot hybridization experiment with *B. japonicum* chromosomal DNA digested with various enzymes (Fig. 3.3). Both mixtures of degenerated oligonucleotides hybridized to distinct DNA fragments in each digest. A partial genomic library comprising *BamH*-*EcoRI* fragments of approximately 2 kb was constructed and used in a subsequent screen. This yielded plasmid pRJ8632, which showed strong hybridization to the oligonucleotide mixture 'spot4-forward' (Fig. 3.5).

A parallel approach using degenerated oligonucleotides deduced from the N terminus of protein 11 was less successful. Although we used several different hybridization temperatures (40-53°C) we could not detect distinct hybridizing DNA fragments with the oligonucleotide pool ‘spot11-1forward’ as a probe (Fig. 3.4). Using ‘spot11-1reverse’, we detected distinct bands, which, however, could not be confirmed with ‘spot11-1forward’. For this reason we did not continue the search for the gene encoding protein 11.
Proteome analysis

Fig. 3.3 Southern blot hybridization to identify the gene coding for protein 4. Radiolabeled, degenerated oligonucleotides deduced from the N terminus of protein 4 were used as a probe. Chromosomal *B. japonicum* DNA was digested with one or two of the following restriction enzymes: *XhoI* (X), *BamHI* (B), *EcoRI* (E) and *PstI* (P). The size (kb) of the detected DNA fragments is indicated at the left and right margin. Some DNA fragments of the size markers (lane M) cross-hybridized with the probe. The hybridization temperature was 45°C. The arrow indicates the 2-kb *EcoRI-BamHI* fragment which was subsequently cloned in pRJ8632.

Fig. 3.4 Southern blot hybridization to identify the gene coding for protein 11. Radiolabeled, degenerated oligonucleotides deduced from the N terminus of protein 11 were used as a probe. Chromosomal *B. japonicum* DNA was digested with the following restriction enzymes: *XhoI* (X), *BamHI* (B), *EcoRI* (E), *HindIII* (H) and *PstI* (P). The hybridization temperature was 40°C. Digested λ DNA that was used as a size marker (M) cross-hybridized with the “spot11-1forward” oligonucleotides.
Identification of orf110, the gene coding for protein 4

Upon sequence analysis of plasmid pRJ8632 we identified the 5'-end of an open reading frame orf110 that codes for a putative protein whose N terminus exactly corresponds to that of protein 4 (Fig. 3.5). Since the 3'-end of orf110 was not located on plasmid pRJ8632, we cloned the adjacent chromosomal region. Using probes derived from pRJ8631 (Fig. 3.6) and genomic plasmid libraries we obtained the overlapping plasmids pRJ8633 and pRJ8635, which were sequenced. Upstream of orf110, we identified an open reading frame, hmuR, which shows similarity to genes encoding TonB-dependent outer membrane heme receptors. Downstream of orf110, we identified four additional open reading frames: orfl68 and three open reading frames with similarity to a so-called Ton system comprising the genes exbBD and tonB (Fig. 3.6). Since the detailed analysis of these genes will be described in Chapter 5, the following is restricted to data related to orf110.

Fig. 3.5 The 5' end of orf110 present on pRJ8632. The nucleotides that coincide with the degenerated nucleotides are highlighted in black. The name of the oligonucleotide mixture is shown adjacent to the sequence. The underlined positions indicate 1:1 ambiguities of the following nucleotides: S = G or C  Y = C or T  R = A or G. At the bottom, the N terminus of the protein deduced from orf110 is shown. Bold letters refer to the experimentally determined N terminus of protein 4. The indicated nucleotide position number corresponds to the sequence deposited in the Genbank database (AJ311165) (Fig. 3.6).
Proteome analysis

Fig. 3.6 Genetic map of the *B. japonicum* locus encompassing *orf110* which codes for protein 4. Nucleotide positions correspond to the sequence deposited in the Genbank database (AJ311165). Relevant restriction sites are indicated as follows: BamHI (B), ClaI (C), EcoRI (E), HincII (H), NotI (N) and XhoI (X). The inserts of the initially constructed plasmid are depicted at the top along with the respective plasmid number. The insert of plasmid pRJ8635 extends the region depicted here and reaches the position 9698 upstream of *hmuR* (compare to Fig. 5.1). At the bottom, fragments used for construction of a deletion mutant and a translational *lacZ* fusion, are depicted with the respective restriction sites. The ‘Δ’ symbolizes the deletion that was replaced by an *aphII* cassette of 1.2-kb length; the orientation of the *aphII* gene is indicated by an arrow.

The role of NifA in *orf110* regulation

We did not identify a −24/−12-type consensus promoter upstream of *orf110* or upstream of the later identified *hmuR* gene. In order to test the suspected NifA dependency of *orf110* expression, we constructed a translational *orf110*-′*lacZ* fusion, which was integrated into the chromosome of *B. japonicum* wild type 110spc4, nifA mutant A9 and rpoN1/2 double mutant N50-97 (Fig. 3.6). The aerobic and anaerobic expression of *orf110*-′*lacZ* was examined in a β-galactosidase assay (Table 3.2).
Table 3.2 Expression of a chromosomally integrated orf110'-lacZ fusion in wild-type, nifA- and rpoN12-mutant cells grown under different oxygen conditions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>β-Galactosidase activity in Miller Units²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Aerobic</strong>²</td>
</tr>
<tr>
<td>8634</td>
<td>wild type</td>
<td>441.8 ± 37.2</td>
</tr>
<tr>
<td>A8634</td>
<td>nifA::aphII</td>
<td>380.3 ± 19.7</td>
</tr>
<tr>
<td>N8634</td>
<td>rpoN1::aphII, rpoN2::Ω</td>
<td>430.1 ± 4.7</td>
</tr>
</tbody>
</table>

² Shown are data of an individual experiment. Numbers are mean values ± standard errors from two cultures of individual strains which were measured in duplicate.
³ Aerobic cultures were grown for 3 days in PSY medium containing 100 µg of spectinomycin per ml.
⁴ Anaerobic cultures were grown for 6 days in YEM medium containing 10 mM KNO₃ and 100 µg of spectinomycin per ml.

Expression of orf110'-lacZ could be detected under anaerobic but, surprisingly, also under aerobic conditions at even high levels. Because different media were used for anaerobic and aerobic growth the absolute values cannot be compared directly. Aerobic expression levels of orf110'-lacZ in the mutant backgrounds are similar to that in the wild type. The aerobic expression of orf110 contradicts its presumed NifA dependency. Yet, nifA and rpoN mutations have a pronounced influence on orf110'-lacZ expression under anaerobic conditions by causing an approximately 5-fold decrease as compared with the wild type. Additional measurements with multiple parallel cultures confirmed that orf110'-lacZ expression is significantly decreased in the nifA mutant background (54 ± 12%) as compared to the wild type (100 ± 31%; see also Discussion).

Iron-dependent regulation of orf110

When orf110'-lacZ expression was measured in cells grown under different iron conditions, an effect of iron availability was observed (Table 3.3). Regardless of the oxygen conditions, the expression level of orf110'-lacZ increased under iron-limited conditions. The induction of orf110'-lacZ expression in iron-deplete (no Fe supplementation) as compared with iron-replete conditions (100 µM FeSO₄) was about 70-fold under aerobic conditions and more than 5-fold under anaerobic conditions. We tried to enhance iron limitation by adding 100 µM dipyridyl (DPD), a specific iron
Proteome analysis

chelator. However, this treatment did not further enhance the expression of orf110′-lacZ. Therefore, we concluded that normal PSY and YEM medium are already limiting for iron.

Table 3.3 Expression of a chromosomally integrated orf110′-lacZ fusion in B. japonicum strain 8634 grown under different oxygen and iron conditions

<table>
<thead>
<tr>
<th>Fe conditions</th>
<th>β-Galactosidase activity in Miller Units&lt;sup&gt;a&lt;/sup&gt; under different oxygen conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>100 μM DPD</td>
<td>421.4 ± 0.8</td>
</tr>
<tr>
<td>no Fe supplementation</td>
<td>465.5 ± 7.3</td>
</tr>
<tr>
<td>100 μM FeSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>6.7 ± 0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Shown are data of one single measurement; numbers are mean values ± standard errors from two cultures which were measured in duplicate.
<sup>b</sup> Aerobic cultures were grown for 3 days in PSY medium containing 100 μg of spectinomycin per ml.
<sup>c</sup> Anaerobic cultures were grown for 6 days in YEM medium containing 10 mM KN03 and 100 μg of spectinomycin per ml.
<sup>d</sup> na = not applicable. B. japonicum does not grow anaerobically in the presence of ≥ 20 μM DPD.

Phenotype of orf110 mutants in symbiosis

We tested strain 8634 (chromosomally integrated orf110′-lacZ fusion) and the deletion mutant 8648 (ΔhmuR-orf110; Fig. 3.6) in a soybean plant infection test as described in Experimental procedures. It is important to note here that both mutations could have a potential polar effect on downstream genes. Nodulation and nitrogen fixation activity of at least eight plants was evaluated three weeks after infection. The size, morphology, N2-fixing abilities and the interior color of nodules elicited by both mutant strains did not differ from those that were formed by the wild type. Thus, the product of the orf110 gene is not essential for an effective symbiosis of B. japonicum with its soybean host plant.
3.3 Discussion

What is the function of the orf110 gene product in B. japonicum?

Since Orf110 possesses no obvious signal peptide and was detected by two-dimensional gel electrophoresis, we assume that it is a soluble cytosolic protein. The soybean plant infection test did not point out an essential function of orf110 in symbiosis. A BLAST search with the deduced gene product of orf110 revealed four proteins in the database, which exhibit significant amino acid sequence similarity (Fig. 3.7). Unfortunately, these homologous proteins were found by genome analyses and none of them was investigated with respect to its function. Although the alignment displays strikingly conserved regions, no known functional motif could be identified. Four proteins resulting from this comparison with Orf110 originate from rhizobial genomes and they are more than 50% identical to each other. Very strikingly, the respective genes are clustered with those that have a putative function in heme acquisition. We therefore assume that orf110 and its homologues might be involved in rhizobial heme or iron acquisition (see also Chapter 5).

Fig. 3.7 Alignment of the Orf110 protein and its homologues. B. japonicum (Bj) Orf110 (accession AJ311165); M. loti (Mi) Mlr1159 (accession BAB48597); R. leguminosarum bv. viciae (Ri) OrfA (accession CAC34386); S. meliloti (Sm) Smc01518, http://sequence.toulouse.inra.fr/meliloti.html; D. radiodurans (Dr) DR0446 (accession B75517).
A potential effect of NifA on iron homeostasis

The amount of protein 4 (Orf110) was significantly decreased in the proteome of the nifA mutant as compared with the wild-type proteome. The expression studies with the orf110'-lacZ fusion confirmed that orf110 expression is decreased in a nifA mutant. These data indicate that the transcription regulator NifA has some, possibly indirect, effect on orf110. It is clear that NifA is not absolutely required for transcription of this gene, which is consistent with the lack of a canonical $\sigma^{54}$-type promoter and an upstream NifA-binding site and also with the aerobic expression of orf110. From proteome studies, it is known that nifA mutants are unable to synthesize more than 20 proteins that are normally made by B. japonicum wild-type cells under anaerobic conditions (Dainese-Hatt et al., 1999). Since several of these proteins contain iron (e.g. nitrogenase and ferredoxins), we speculate that in a nifA mutant the effective concentration of available iron might be higher than in wild-type cells. This may cause the repression of orf110 and presumably also of further iron-repressed genes. The high fluctuations of orf110'-lacZ expression that we observed in the nifA mutant are possibly caused by slightly variable intracellular iron concentrations that are close to a threshold level critical for the iron response.

3.4 Experimental procedures

Strains and plasmids

The bacterial strains and plasmids used in this part of the work are listed in Table 3.4.
Table 3.4 Bacterial strains, cloning vectors and recombinant plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or properties</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 gyrA96 thi-1 relA1</td>
<td>Bethesda Research Laboratories, Inc., Gaithersburg, Md</td>
</tr>
<tr>
<td></td>
<td>Sm' Sp' hsdR (RP4-2 kan::Tn7 tet::Mu, integrated in the chromosome)</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>B. japonicum</td>
<td>Sp' wild type</td>
<td>Regensburger and Hennecke (1983)</td>
</tr>
<tr>
<td>110spec4</td>
<td>Sp' Km' Sm' rpoN::aphII rpoN2::Ω</td>
<td>Kullik et al. (1991)</td>
</tr>
<tr>
<td>N50-97</td>
<td>Sp' Km' nifA::aphII</td>
<td>Fischer et al. (1986)</td>
</tr>
<tr>
<td>A9</td>
<td>Sp' Tc' orfl10'-lacZ chromosomally integrated in B. japonicum 110spec4</td>
<td>This work</td>
</tr>
<tr>
<td>8634^</td>
<td>Sp' Km' hmuR::aphIIΔorfl10 (aphII and hmuR oriented in opposite directions)</td>
<td>This work</td>
</tr>
<tr>
<td>8648</td>
<td>Ap' Km'</td>
<td>Alexeyev (1995)</td>
</tr>
<tr>
<td>Plasmids</td>
<td>Ap' Km'</td>
<td>This work</td>
</tr>
<tr>
<td>pBSL15</td>
<td>Ap' (pUC18) 1.2-kb XhoI-EcoRI fragment with 'hmuR, orfl10'</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ8631</td>
<td>Ap' (pUC18) 2-kb BamHI-EcoRI fragment with 'hmuR, orfl10'</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ8632</td>
<td>Ap' (pUC18) 3.8-kb EcoRI fragment with hmuT, hmuR, orfl10'</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ8633</td>
<td>Ap' (pUC18) 5-kb fragment with hmuR, orfl10, orfl67, exbBD, tonB</td>
<td>This work</td>
</tr>
<tr>
<td>pSUP202</td>
<td>Ap' (pUC18) 2.1-kb SmaI-EcoRI fragment with 'hmuR, orfl10'</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>pSUP202pol5</td>
<td>Ap' Te' pSUP202pol4 derivative with BglII linker inserted into SmaI site</td>
<td>This laboratory</td>
</tr>
<tr>
<td>pSUP480</td>
<td>Ap' Te' lacZ part from pNM480 in pSUP202pol4</td>
<td>This laboratory</td>
</tr>
<tr>
<td>pUC18</td>
<td>Ap'</td>
<td>Norrander et al. (1983)</td>
</tr>
</tbody>
</table>

^ The same orfl10'-lacZ fusion was also integrated into the chromosome of B. japonicum A9 and N50-97. The resulting strains were given the same number 8634 preceded by A (A9 derivate) or N (N50-97 derivative) (Table 3.2).
**Media and growth conditions**

Luria-Bertani (LB) medium (Miller, 1972) was used for growth of *E. coli* cells; it contained the following concentrations of antibiotics for plasmid selection (µg/ml): ampicillin, 200; kanamycin, 30; tetracycline, 10. Peptone-salts-yeast extract (PSY) medium (Regensburger and Hennecke, 1983) supplemented with 0.1% L-arabinose was used for routine aerobic cultures of *B. japonicum* whereas yeast extract-mannitol (YEM) medium supplemented with 10 mM KNO₃ (Daniel and Appleby, 1972) was used for anaerobic *B. japonicum* cultures. Anaerobic cultures were kept under argon in rubber-stoppered serum bottles. Concentrations of antibiotics for use in *B. japonicum* cultures were as follows (µg/ml): spectinomycin, 100; kanamycin, 100; streptomycin, 50; tetracycline, 50 (solid media) or 35 (liquid media). For growth under high-iron conditions, wild-type strain 110spc4 and its derivatives were cultivated in PSY medium supplemented with 100 µM FeSO₄, whereas normal PSY medium was supplemented with only 1.2 nM FeCl₃. For low-iron conditions, we used either PSY medium lacking the FeCl₃ supplement or containing 20 to 100 µM dipyridyl (DPD).

**DNA work and sequence analysis**

Recombinant DNA work and Southern blotting were performed according to standard protocols (Sambrook *et al.*, 1989). *B. japonicum* chromosomal DNA was isolated as described previously (Hahn and Hennecke, 1984). For computer-assisted analyses of DNA and protein sequences, the software package (version 10) of the UWGCG (Genetics Computer Group of the University of Wisconsin, Madison, WI) was used. Homology searches were performed by using the National Center for Biotechnology Information BLAST network server (http://www.ncbi.nlm.nih.gov/BLAST/).

**Labeling of oligonucleotides and DNA probes**

The degenerated oligonucleotides were radiolabeled with [γ-³²P]ATP with T4 polynucleotide kinase (MBI Fermentas). For colony hybridization, a 1.2-kb genomic *EcoRI-Xhol* fragment of pRJ8631 was used as a probe, which was radiolabeled by nick translation using [α-³²P]dATP. Prior to the hybridization procedure, the probe was denatured 5 min at 95°C and immediately chilled on ice water.
Chapter 3

Southern blot hybridization under low stringency conditions

A Southern blot containing different digestions of chromosomal DNA from *B. japonicum* (5 μg per lane) was pre-hybridized for at least 5 h at the indicated temperature (40-55°C) in 6x SSC hybridization buffer [6x SSC, 0.5% SDS, 20 mM Na-phosphate buffer pH 6.8, 5x Denhardt solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin) and 200 μg/ml sonicated salmon sperm DNA]. Upon 15 h hybridization with denatured oligonucleotides (approx. 3 - 4x 10⁷ cpm) the membrane was washed 3 times for 35 min with 6x SSC and 0.1% SDS at the same temperature as used for hybridization.

**Colony hybridization**

Colony hybridization was performed according to standard protocols, using the following hybridization buffer: 6x SSC, 0.4% SDS, 20 mM phosphate buffer, 5x Denhardt solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin). The nylon filters with blotted colonies were pre-hybridized for at least 4 h at 60°C, and hybridization with radiolabeled DNA probe (~10⁶ cpm) was done over night at 60°C. The membranes were washed for 20 min at 60°C with 6x SSC, 0.1% SDS. Finally, the washing step was repeated for 30 min at room temperature in the same buffer.

**Strategy for cloning the genes encoding protein 4 and 11**

Four different mixtures of radiolabeled, degenerated oligonucleotides deduced from the N-termini of protein 4 and 11 were used. As probe for the gene encoding protein 11 we used spot11-forward (5'-ATGGAYAARATGCGSATCGAYAA-3') and spot11-reverse (5'-TCICCCTTTCATGTCGATAGCATYTT-3'); for the definition of the underlined letters see legend Fig. 3.5. The degenerated oligonucleotides spot4-forward and spot4-reverse that were used in a screen for the gene coding for protein 4 are depicted in Fig. 3.5. The latter probes hybridized specifically to a genomic BamHI- EcoRI fragment of approximately 2 kb which was cloned in plasmid pRJ8632 by screening a partial genomic library based on pUC18. In an additional cloning experiment, using a partial genomic library of genomic XhoI-EcoRI fragments of about 1-1.5 kb, we isolated plasmid pRJ8631 (Fig. 3.6). Subsequently, the entire *B. japonicum* **orf110** region was cloned in plasmids pRJ8633 and pRJ8635 using the insert of...
pRJ8631 as an initial probe for hybridizing colonies representing appropriate plasmid libraries of *B. japonicum* chromosomal DNA.

**Construction of lacZ reporter fusions**

The translational orf110·lacZ fusion was constructed by making use of an orf110-internal HincII restriction site (corresponding to Val-59 in the putative Orf110 protein) and the mobilizable lacZ fusion vector pSUP480 (Fig. 3.6). The lacZ fusion construct comprising a 475-bp HincII fragment of genomic DNA was conjugated into *B. japonicum* 10spc4 (wild type), A9 (nifA) and N50-97 (rpoN1/2).

**Construction of a B. japonicum Δhmur-orf110 mutant strain**

The hmuR and orf110 genes were simultaneously mutated by deleting a 1.2-kb ClaI-NolI fragment which was replaced by a 1.2-kb Smal fragment (aphII) from pBSL15 (mutant strain 8648; Fig. 3.6). An appropriate DNA fragment containing the region to be mutated was cloned into pSUP202pol5 and mobilized into *B. japonicum* 10spc4 as described previously (Hahn and Hennecke, 1984). Co-integrate-containing transconjugants (resulting from single crossover) were distinguished from true marker exchange mutants (resulting from double crossover) by the vector’s tetracycline resistance. The correct genomic structures of all mutant strains were confirmed by Southern blot analysis of genomic DNA.

**β-Galactosidase assays**

β-Galactosidase activity assays were done as described previously (Fischer et al., 1993).

**Plant infection test**

The symbiotic phenotype of the *B. japonicum* wild type and mutant strains 8634 and 8648 was determined in infection tests using soybean (*Glycine max* (L.) Merr. cv. Williams) as host plant and nitrogenase activity was measured in an acetylene reduction assay (Hahn and Hennecke, 1984; Göttfert et al., 1990). Soybean seeds were kindly provided by P. M. Gresshoff (University of Queensland, Australia).
Part II

Heme uptake in

*Bradyrhizobium japonicum*
Chapter 4

Compendium about bacterial iron homeostasis

Incidentally, we identified a gene cluster (hmu) in B. japonicum whose predicted products show high similarity to heme uptake systems (Chapter 3). This was quite an unexpected finding because, to date, genes coding for an outer membrane heme receptor have been identified predominantly in pathogenic bacteria which use heme derived from their host as a major iron source. It was decided to study the heme uptake system of B. japonicum in more detail with respect to its function and iron-dependent regulation (Chapters 5 and 6). In this chapter 4, a compendium about bacterial iron homeostasis is presented with the goal to set the contents of chapters 5 and 6 in a context with bacterial iron metabolism.
4.1 The ‘irony of iron’

Iron is the second most abundant metal, (after aluminum), and the fourth most abundant element in earth’s crust (Crichton, 1991). In nature, iron is found mostly as a constituent of insoluble oxyhydroxide polymers (FeOOH). Free Fe$^{3+}$ in an aerobic aqueous environment is limited to an equilibrium concentration of approximately $10^{-9}$ M at pH 7 (Ratledge and Dover, 2000), a value far below that required to sustain optimal growth of plants and microbes. Because of the very low solubility of iron, bacteria evolved different mechanism to acquire this important trace element. Characteristic properties of bacteria with respect to iron acquisition are the presence of outer membrane receptors and high-affinity uptake systems for siderophores (“iron carrier”) or the use of alternative host-derived iron-sources like hemoglobin or transferrin. Siderophores are specific iron chelators of low molecular weight that are secreted to scavenge iron from the environment (reviewed by Crosa, 1989; Ratledge and Dover, 2000). Generally, bacteria are able to use not only endogenously synthesized siderophores but they also evolved additional receptors for exogenous types of iron chelates (reviewed by Wooldridge and Williams, 1993).

Iron is an essential trace element for most bacteria, because it is used in the catalytic centre of several important enzymes (see below) and is the preferred cofactor in numerous biological redox processes. The unique suitability of iron in a proteinaceous environment comes from the extreme variability of Fe$^{3+}$/Fe$^{2+}$ redox potential, which spans from $+1150$ mV to $-750$ mV (Pierre and Fontecave, 1999). The fine-tuning of the iron redox potential is dependent on the nature and the geometry of iron complexes with a maximum coordination number of 6 ligands. Different types of iron-containing proteins are known. Largely, they can be divided into two classes, hemoproteins and non-heme iron proteins, as for example Fe/S proteins. The most prominent group of Fe/S proteins are ferredoxins, in which at least two iron atoms are bound to sulphide (known as acid-labile sulphur). The Fe/S cluster is usually linked to the polypeptide chain by the thiol groups of cysteine residues. Ferredoxins play roles in electron transfer reactions related to various enzymatic processes (Crichton, 1991). Examples of Fe/S proteins are enzymes of the citrate cycle (aconitase and succinate dehydrogenase) or nitrogenase. In contrast to Fe/S proteins, the catalytic centre of di-iron enzymes consists of two iron atoms chelated by histidine and glutamate residues, like for example in...
methane monooxygenase or the R2 subunit of ribonucleotide reductase (Krebs and Huynh, 1999). The second class of iron proteins (hemoproteins) contains heme (Fe-protoporphyrin IX; Fig. 4.1) which represents the most abundant iron cofactor. Enzymes with a prosthetic heme group are involved in electron transfer coupled to the generation of cellular energy (cytochrome b and c), oxidative biotransformation (cytochrome P450) or hydrogen peroxide elimination (catalase and peroxidase).

![Fig. 4.1 The chemical structure of heme (Fe-protoporphyrin IX).](image)

Ironically, iron is not only essential but iron excess can be highly toxic under aerobic conditions. An iron overload can cause oxidative damage to DNA, lipids and proteins that might result in cellular death. This is due to the easy oxidation of Fe$^{2+}$, which is accompanied by the formation of hydroxyl radicals (Fenton reaction; Fig. 4.2). Whereas hydroxyl radicals can damage any biological molecule, superoxide is not extremely reactive by itself; for instance it cannot attack DNA. However, it is suggested that in vivo it releases and probably reduces iron from iron-containing molecules and thus favors the Fenton reaction by increasing the amount of free Fe$^{2+}$ (Pierre and Fontecave, 1999; Fig. 4.2).

Therefore, iron homeostasis is tightly controlled in bacteria and probably all other organisms. In order to prevent an iron overload, bacteria have evolved two strategies. The first one is the synthesis of bacterioferritins that are able to bind excess iron in a protein scaffold. These multimeric iron storage proteins are members of the ubiquitous ferritin family that have a characteristic hollow cavity to accommodate at least 2000 ferric ions (reviewed by Andrews, 1998). The second strategy consists in minimizing
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the iron amount being taken up from the environment by controlling the uptake pathways (reviewed by Braun, 1997a).

\[ \text{OH}^- + \text{OH}^- \xrightarrow{\text{Fe}^{3+}} \text{O}_2^- \]

**Fig. 4.2 Formation of reactive hydroxyl radicals in the Haber-Weiss cycle.** The Fenton reaction (left part) is the iron-salt-dependent decomposition of dihydrogen peroxide, generating the highly reactive hydroxyl radical, possibly via an oxoiron(IV) intermediate. Addition of a reducing agent such as superoxide leads to a cycle, which increases the damage to biological molecules (Pierre and Fontecave, 1999).

4.2 Iron-dependent regulation in bacteria

In many bacteria, control over iron uptake systems and biosynthesis of siderophores is exerted at the level of transcription by the ferric uptake regulatory protein, Fur that is best characterized in *E. coli* (reviewed by Touati, 2000; Hantke, 2001). Fur is active as a dimer, it binds Fe\(^{2+}\) as a cofactor at two distinct binding sites and represses the transcription of target genes by binding to a specific iron box in the promoter regions (Escolar *et al.*, 1999). When cytoplasmic iron levels are low, the ferrous iron is released from the Fur repressor protein, which then dissociates from the DNA. However, in addition to the repression of genes directly involved in iron transport, Fur also regulates three types of subsidiary regulatory proteins which belong either to two-component signal transduction systems, or to AraC-like regulators or sigma factors (Crosa, 1997; Vasil and Ochsner, 1999). The significance of Fur is not restricted to the regulation of iron acquisition systems. The Fur regulon comprises genes involved in metabolism (aconitase), iron storage (bacterioferritin), oxidative-stress response (catalase, superoxide dismutase) and virulence factors (Shiga-like toxins, hemolysins) (reviewed by Hantke, 2001). Proteins required under iron-replete conditions, like bacterioferritin, are positively regulated by Fur. However, the mechanism of this type of control is not yet fully understood.

Fur-mediated regulation does not only respond to iron but it is interwoven with the oxidative stress response. The regulatory systems SoxRS and OxyR regulate the
expression of fur in response to reactive oxygen species (ROS). Thus, Fur can be regarded as a global regulator, and it seems to be essential in some bacteria such as Pseudomonas aeruginosa, Neisseria meningitides and Vibrio anguillarum.

Another type of iron-dependent regulation involves the iron response regulator Irr that has been characterized in B. japonicum. Irr is suggested to coordinate the synthesis of heme with iron availability. Under iron-deplete conditions, it represses the heme biosynthesis gene hemB, and thus prevents the accumulation of toxic protoporphyrin IX. Furthermore, mutant analysis led to the suggestion that Irr might activate iron citrate uptake. Irr is a Fur homologue, but in contrast to Fur, it is conditionally unstable and detectable only under iron-limited conditions (Hamza et al., 1998; 1999). Transcription of irr is modestly regulated by B. japonicum Fur; however, the Irr protein concentration is mainly controlled at the post-translational level via heme-dependent degradation (Hamza et al., 2000).

In many Gram-positive bacteria, members of another protein family mediate iron regulation, exemplified by the diphtheria toxin regulator DtxR of Corynebacterium diphtheriae (reviewed by Hantke, 2001). Though DtxR-like proteins regulate a set of genes similar to those controlled by Fur in Gram-negative bacteria they share no sequence similarities with Fur, and the consensus DNA-binding sequence of DtxR differs from the Fur box.

Recently, bifunctional iron-regulatory proteins have been identified in E. coli and B. subtilis that are involved in post-transcriptional regulation (Alen and Sonenshein, 1999; Tang and Guest, 1999). By analogy with eukaryotic systems, the iron response proteins (IRPs) of these bacterial species are able to bind to mRNA at an iron responsive element (IRE). Depending on the location of the IRE, the IRPs either stabilize the transcripts or promote their rapid degradation. IRP of E. coli is a Fe/S-protein, which bears two iron-dependent functions. Under high iron conditions, the protein has aconitase activity involving a catalytic [4Fe-4S] cluster. By contrast, under low iron conditions, the aconitase becomes inactive due to the conversion of the [4Fe-4S] cluster to a [3Fe-4S] cluster, which converts the protein into an mRNA-binding protein. Only few examples of this posttranscriptional regulation mechanism have been elucidated to date. They include aconitase itself and ferritin, an iron storage protein (see below) (reviewed by Hantke, 2001).
4.3 High-affinity iron uptake

The “gated pore” for iron chelators

The outer membrane of Gram-negative bacteria is an important protective barrier acting as a molecular sieve for nutrient uptake. Porins, water-filled channel proteins, allow passive diffusion of different molecules smaller than 600 Da. More specific channels and receptor proteins mediate the specific transport of larger solutes, e.g. maltose, vitamin B₁₂ or fatty acids (Nikaido, 1996). A diversity of high affinity receptors has evolved in bacteria either to bind siderophores or to directly bind host iron proteins, such as transferrin or hemoglobin (reviewed by Ratledge and Dover, 2000). The active transport of siderophores, heme or vitamin B₁₂, which occurs against a concentration gradient, is dependent on the Ton system and proton motive force (pmf; see below). The receptors involved are classified as “TonB-dependent outer membrane proteins” which display unique structural features. Recently, the three-dimensional structure of two different siderophore receptor proteins from E. coli, FhuA and FepA, has been solved (Ferguson et al., 1998; Locher et al., 1998; Buchanan et al., 1999). The common structural organization comprises a huge C-terminal 22-stranded β-barrel that is gated by an N-terminal plug domain. The plug domain, also called ‘cork-domain’, is located inside the barrel and thus obstructs the channel interior (Fig. 4.3). A FhuA deletion variant that lacks the entire N-terminal plug domain was shown to insert into the outer membrane and form large pores that allow diffusion of large substances, like ferrichrome or maltotetraose (Braun et al., 1999). The structures of FepA and FhuA suggest a general mechanism for specific transport of metal chelates. Buchanan and colleagues (1999) presented the following model. The receptor may function like an air lock comprising two hatches. The first hatch is formed by the extracellular loops. Ligand recognition and binding leads to closure of the receptor at the extracellular side of the outer membrane. The second hatch is created by the plug domain. Upon binding of the ligand, a TonB-mediated signal opens the pore on the periplasmic side of the outer membrane and, simultaneously the gate at the extracellular side is closed. Either conformational rearrangement or a concerted movement of the N-terminal domain could accomplish the formation of this channel. It is unlikely that the entire plug domain is moved to gain access to the periplasm because this process would require large
activation energy. This system using two hatches prevents the flow of ions and small molecules through this gate in both directions.

![Fig. 4.3 A ribbon representation of the plug-barrel structure of the ferrichrome receptor FhuA (modified from Ferguson et al., 1998).](image)

(A) The FhuA ferrichrome-iron complex and a single LPS molecule noncovalently associated with its membrane-embedded surface; OM = outer membrane; PP = periplasm. Residues 621 to 723 of the barrel (blue) have been removed to allow a view of the cork domain (yellow). The ferrichrome-iron molecule is represented as a ball-and-stick model. The iron atom is indicated as a large sphere. (B) FhuA as viewed from the external environment along the barrel axis. The ferrichrome-iron molecule is represented as a ball-and-stick model.

**The Ton system**

The Ton system is a cytoplasmic membrane-localized complex that is present in many Gram-negative bacteria. The protein complex comprises TonB, ExbB and ExbD and transduces energy to high-affinity transporters located in the outer membrane (reviewed by Moeck and Coulton, 1998). The TonB protein is associated with both the inner and outer membrane (Letain and Postle, 1997). Its association with the cytoplasmic membrane requires an N-terminal signal anchor. TonB spans the periplasm and physically interacts with the TonB-dependent receptor protein at conserved regions (Fig. 4.4). Thus, TonB provides the structural link between the pmf of the inner membrane and high affinity transporters of siderophores, heme or vitamin B12 through
the outer membrane. ExbB spans the cytoplasmic membrane three times, and most of the protein is located in the cytoplasm. ExbD is arranged similar to TonB with the N terminus in the cytoplasmic membrane and the remainder of the polypeptide in the periplasm (reviewed by Braun, 1997a). Recently, cross-linking experiments led to the hypothesis that ExbB and ExbD form a 3:3 heterohexamer with a total of 12 transmembrane domains (Higgs et al., 1998). The molecular mechanism of how the Ton system transduces energy is not yet known. A major question is whether the ExbBD complex is involved autonomously in proton translocation or whether it interacts with an unidentified proton translocator.

Currently, the following model is favoured: TonB cycles between different conformations, one of which provides the energy for ligand transport through the receptor. Interconversion of the conformationally different states requires pmf and ExbB and ExbD to reset TonB in an energized state within the cytoplasmic membrane (Letain and Postle, 1997; Larsen et al., 1999). A previous model suggested that TonB rotates in the ExbBD-complex. Very recently, the structure of the C-terminal domain from *E. coli* TonB (residues 164 to 239) was solved and provided first evidence that this region dimerizes (Chang et al., 2001).

**Heme acquisition in bacteria**

Until recently, the ability to use heme as a source of iron was thought to be a characteristic property of a number of bacterial pathogens including species of the genera *Vibrio*, *Shigella*, *Escherichia*, *Pseudomonas*, *Haemophilus*, *Yersinia*, *Neisseria* and *Serratia* (reviewed by Lee, 1995; Wandersman and Stojiljkovic 2000; Genco 2001). In serum, heme is bound primarily to hemoglobin, hemopexin, albumin and lipoproteins. Hemoglobin is the most amenable heme source for pathogenic bacteria. A TonB-dependent outer membrane receptor can directly bind heme or hemoproteins. Upon binding of heme, it is taken up via the receptor and transported into the periplasm (Fig. 4.4). Once in the periplasm, heme is transferred to a specific periplasmic binding protein, passed on to a permease which transports heme through the inner membrane in an ATP-dependent process. The permease is a member of the ABC transporter family (reviewed by Köster, 2001). In the cytoplasm, heme might be directly incorporated into hemoproteins, or iron is removed from the porphyrin ring probably by the action of a
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heme oxygenase. Unfortunately, heme processing in the cytoplasm is currently only poorly understood.

Fig. 4.4 Simplified model of heme import in Gram-negative bacteria. Heme uptake through the outer membrane (OM) is mediated by a TonB-dependent outer membrane receptor (R). The receptor binds heme directly or interacts with a hemoprotein such as hemoglobin (Hb). The Ton system (orange) consists of TonB (T) and the heterohexamer ExbB (B) and ExbD (D). The Ton complex transduces proton motive force (pmf) to the receptor protein. A periplasmic binding protein (PBP) transfers heme to a heme permease (P) of an ABC transport system (blue) in the inner membrane (IM).

Heme uptake systems are similar to uptake systems used for siderophores. The overall structure of heme receptors is expected to be very similar to that of siderophore receptors (Fig. 4.3). In fact, protoporphyrin IX can be considered as a kind of iron-carrier = siderophore (Fig. 4.1). Two principle mechanisms for the exploitation of hemoproteins as iron source can be defined (reviewed by Wandersman and Stojiljkovic 2000; Genco and Dixon, 2001). The first includes receptor proteins that directly interact with heme or hemoproteins. This class is exemplified by HemR from Y. enterocolitica whose ability to bind heme or hemoglobin has been demonstrated biochemically (Bracken et al., 1999). Receptors of this type can either be highly adopted to one specific heme protein or be able to use a broad spectrum of hemoproteins. The second
mechanism depends on a secreted helper protein, termed hemophore, which binds extracellular heme and delivers it to the actual outer membrane receptor. The best-studied example is the HasR-HasA system in *S. marcescens* (Ghigo *et al.*, 1997). The hemophore HasA, a small protein of 19 kDa, is secreted by a specific ABC-exporter in a process that involves the carboxy-terminal secretion signal of HasA (Wandersman, 1998). Heme uptake by the receptor HasR is not absolutely dependent on HasA but the hemophore increases the uptake efficiency. HasA, however, is essential for heme acquisition from other heme sources such as hemopexin and myoglobin (Létoffé *et al.*, 1999). HasA binds one heme per molecule with very high affinity, and binding does not appear to alter the fish-like conformation of HasA (Fig. 4.5; Arnoux *et al.*, 1999). The crystal structure of the heme-containing HasA revealed that the heme moiety is highly exposed. Both heme-free and heme-loaded HasA bind to HasR, indicating direct protein-protein interactions between HasR and HasA (reviewed by Wandersman and Stojiljkovic, 2000). However, it is not clear how the HasR removes heme from the hemophore.

**Fig. 4.5 Backbone structure of HasA from *S. marcescens* (modified from Arnoux *et al.*, 1999).** Ribbon diagram with helices colored in red and strands in blue. The axial ligands of the heme (His-32 and Tyr-75) and the heme molecule are shown in ball-and-stick representation.

A special situation is represented by the neisserial hemoglobin-haptoglobin receptor HpuAB (Lewis *et al.*, 1997). It consists of two outer membrane proteins, a TonB-dependent receptor (HpuB) and a lipoprotein (HpuA). Both subunits of this bipartite receptor are required for heme acquisition. The function of the accessory lipoprotein is
not known. It may represent a docking site for hemoglobin, which enhances the efficiency of ligand binding to the receptor similar to the function of the aforementioned hemophore HasA. Yet another receptor variant is found in *Porphyromonas gingivalis*. It was suggested that the heme iron transporter lhtB of this bacterium has reverse chelatase activity that removes iron from extracellular heme prior to iron uptake. Unlike other heme receptors and most remarkably, lhtB confers *P. gingivalis* the ability to use c-type cytochromes as iron source, which contain the heme prosthetic group in covalently bound form (Dashper *et al.*, 2000).

Heme acquisition is not a characteristic restricted to Gram-negative bacteria. A heme uptake system similar to heme ABC transporters was identified in the Gram-positive bacterium *Corynebacterium diphtheriae*. The surface-exposed HmuT protein which is homologous to periplasmic heme binding proteins, is anchored in the cell membrane via a lipid moiety and predicted to function as a heme receptor (Drazek *et al.*, 2000). Recently, another hemoglobin-binding protein was identified in the Gram-positive organism *Gardnerella vaginalis*. Whether its receptor shows homology to HmuT of *C. diphtheriae* has yet to be analysed (Jarosik, 2001).

### 4.4 Iron partitioning in nodules

**Iron demand in nodules**

Nodules of different legumes so far analyzed have probably the highest iron-concentration of any plant tissue (reviewed in Guerinot, 1993). Iron-containing proteins such as nitrogenase, leghemoglobin and cytochromes are essential for the proper functioning of the nitrogen-fixing symbiosis between legumes and their rhizobial partners. In addition to being essential, these iron-containing proteins are also abundant. Leghemoglobin represents 25-30% of the total soluble protein in an infected plant cell and nitrogenase can constitute 10–12% of the total bacterial protein (Verma and Long, 1983). Thus, synthesis of these proteins presumably places a high demand for iron on the host plant. Indeed, iron-deficiency has been reported to decrease nodule number and nodule mass in a number of legumes and it also can affect nodule structure. Conversely, nodulated root systems of soybean induce stronger iron deficiency responses than non-nodulated roots (Guerinot, 1993 and references therein).
Plant strategies of iron acquisition

Faced with a deficit of iron in the soil (see above), plants have evolved two principal strategies for making this essential nutrient more available (reviewed by Guerinot and Yi, 1994; Mori, 1999). All plants, except grasses, release protons into the soil to increase the solubility of Fe$^{3+}$ by lowering the pH, and they induce the synthesis of a ferric (Fe$^{3+}$) chelate reductase (FRO) to reduce iron to the more soluble ferrous (Fe$^{2+}$) form. In parallel, a Fe$^{2+}$ uptake system is also induced (Eide et al., 1996; Robinson et al., 1999). Collectively, this reaction is referred to as a strategy I response. Grasses, which include many of the world’s prominent food crops such as wheat, rice and maize, release low-molecular-weight compounds called phytosiderophores (mugineic acids) from their roots in response to iron deficiency. These phytosiderophores solubilize iron by complexing Fe$^{3+}$. Plants belonging to this group are able to take up Fe$^{3+}$-phytosiderophore complexes via specific receptors (Curie et al., 2001). This response is designated strategy II.

Rhizobial strategies to acquire iron

Rhizobia are able to use ferric and ferrous ions directly and are able to acquire Fe via different siderophores. Noya and co-workers (1997) have shown that rhizobia are also able to use leghemoglobin and heme as sole iron source (see Chapter 5). Utilization of iron in a complexed form is significant only under low iron availability because the specific uptake systems and receptors are expressed only under these conditions (reviewed by Guerinot, 1994). Conversely, uptake of Fe$^{2+}$ and Fe$^{3+}$ probably plays a role mainly under conditions of abundant iron. However, little is known about direct Fe ion transport by rhizobia. In the genome sequence of M. loti and S. meliloti, gene clusters were identified which specify proteins showing similarity to the SfuABC system of S. marcescens. These proteins that belong to the ABC transporter family are involved in high-affinity uptake of Fe$^{3+}$ through the inner membrane (Angerer et al., 1990). A high affinity Fe$^{2+}$ uptake system (FeoAB) is known in several Gram-negative bacteria (Kammler et al., 1993; Velayudhan et al., 2000). It is suggested that the feo system plays a role for the uptake of Fe$^{2+}$ under both low and high iron availability because it is expressed constitutively. Since Fe$^{2+}$ is readily soluble and can enter the periplasmic space by diffusion through porins, specialized outer membrane receptors are not necessary for its uptake. Recently, an extracellular iron reductase was identified.
in *E. coli* and *P. aeruginosa*, which reduces and releases Fe also from a variety of ferric chelators (Vartivarian and Cowart, 1999). Homologues of FeoAB were not detected in the genome of the sequenced rhizobial genomes.

Free-living rhizobia in the soil often encounter iron-limitation, which makes siderophores become important factors for survival under these competitive conditions. Many rhizobia benefit from the availability of exogenous siderophores that are produced by other soil microorganisms. The production of rhizobial-specific iron chelators, defined as rhizobactins, was investigated in some *Rhizobium* species. Apparently, there is no particular siderophore common to all rhizobia but rather strain-specific siderophores are produced (reviewed by Neilands, 1993). The first identified rhizobactin of *S. meliloti* DM4 displayed a novel siderophore structure. It is an ethylenediamine derivate that is related to the pyruvate-containing opines. *S. meliloti* 1021 produces rhizobactin 1021 that is a unique citrate-hydroxamate. Very recently the genes involved in the biosynthesis of rhizobactin 1021 have been identified (Lynch et al., 2001). While siderophores are produced by numerous rhizobia under free-living conditions, little is known about their synthesis during symbiosis. Probably, they are not relevant during symbiosis, as indicated by the Fix<sup>+</sup> phenotype of *R. leguminosarum* bv. *viciae* siderophore mutants (Stevens et al., 1999; Yeoman et al., 1999). Rhizobactin 1021 is also not absolutely required for symbiotic nitrogen fixation by *S. meliloti*; yet, it is suggested that it contributes to the symbiotic efficiency under low iron-conditions (Gill et al., 1991; Barton et al., 1996).

Apparently, *B. japonicum* strains do not produce high-affinity iron chelators except citrate which is synthesized and excreted in large amounts by *B. japonicum* strain 61A152 but not strain 110 (Guerinot et al., 1990). Yet, both strains are able to use iron citrate via a high affinity iron uptake system, which is not made when cells are grown under iron-replete conditions. In addition to citrate, *B. japonicum* can acquire iron via the uptake of exogenous siderophores, for example ferrichrome, a fungi-derived hydroxamate-type siderophore (Plessner et al., 1993). Three iron-regulated outer membrane proteins (OMPs) in the size range of several known receptors for Fe<sup>3+</sup> siderophores were identified in *B. japonicum* 110. One of them was characterized as the hydroxamate-type siderophore receptor FegA (LeVier and Guerinot, 1996). Very recently, it was shown by mutant analysis that FegA is responsible for the transport of ferrichrome. Surprisingly, the *fegA* mutation severely affected nodule structure and
nitrogen fixation (Prince et al., 2001). The Fix− phenotype of a fegA mutant may suggest the presence of a FegA-specific siderophore in symbiosomes. Because no siderophore production was ever observed in free-living B. japonicum cells (see above) one may speculate about a symbiosis-specific or a plant-derived siderophore.

**Iron transport across the peribacteroid membrane**

The peribacteroid membrane (PBM) is the interface between the host plant and the bacteroids. Its role in controlling the flux of metabolites between the two compartments is likely to be important in the regulation of nitrogen fixation. The PBM possesses properties of the plant cytoplasmic membrane from which it is derived but it also comprises PBM-specific proteins (reviewed by Verma, 1992). *In vitro* experiments with isolated symbiosomes and bacteroids from soybean nodules have shown that Fe\(^{3+}\)-chelates were transported at much higher rates across the PBM than the bacteroid membranes, suggesting the presence of an iron storage pool in the peribacteroid space (LeVier et al., 1996). Furthermore, a ferric-chelate reductase activity which reduces Fe\(^{3+}\) to Fe\(^{2+}\) was found to be associated with the PBM. In the PBM, this activity might play a role in the transport of iron into soybean symbiosomes. Moreau et al., (1998) have shown recently that symbiosomes and bacteroids isolated from soybean nodules are able to take up ferrous (Fe\(^{2+}\)) iron, and ferrous iron was taken up by symbiosomes more efficiently than the ferric form. This indicates that the iron transport from the plant host cell to the microsymbiont *in vivo* might occur mainly in the ferrous form. Ferrous iron in the peribacteroid space is likely to be stable due to the low oxygen concentration and the low pH in this compartment. To date, no data are available about the iron concentration in the peribacteroid space. The suggested high iron concentration in symbiosomes implies that high-affinity uptake systems are not required by bacteroids.

**Iron storage proteins in nodules**

Ferritin is an iron storage protein localized in chloroplasts and other plastids (reviewed by Theil, 1987). Ferritin can house up to 4500 atoms of iron (Fe\(^{3+}\)) in its central cavity. Iron stored in this manner is nontoxic and is readily available to the cell. The ability to sequester iron raises the question if ferritin contributes to a controlled iron partitioning in nodules. Ragland and Theil (1993) have investigated the ferritin content and iron concentrations in developing soybean nodules. Both ferritin mRNA and protein
concentrations increased early in nodulation, but later, ferritin protein in the cytosol of nodule cells declined as leghemoglobin accumulated and nitrogenase activity increased in bacteroids. It is suggested that there is an inverse correlation between the amounts of iron stored in ferritin and in nitrogenase or leghemoglobin. Recently, the physiological and biochemical alterations during senescence of stressed soybean root nodules have been investigated including the fate of ferritin (Matamoros et al., 1999). Upon nitrate-addition, large deposits of ferritin particles accumulated in plastids and amyloplasts of nodule cells simultaneously with a decline in nitrogenase activity. Generally, ferritin synthesis in plants is regulated by iron and induced by various adverse conditions including iron overload and oxidative stress. Ferritin protein accumulation in senescent nodules may be interpreted as a plant response to oxidative stress or as deprivation of iron from the microsymbionts which are not needed anymore. In this context it is worth mentioning that rhizobia themselves might be able to store iron. Although bacterial ferritins are widespread, they have not yet been characterized in rhizobia. Incidentally, the free-living nitrogen-fixing bacterium A. vinilandii was the first bacterium described to synthesize ferritin, referred to as bacterioferritin cytochrome b557.5 (Stiefel and Watt, 1979; Grossman et al., 1992). Two homologues of bacterioferritin have been annotated in the genome of M. loti (www.kazusa.or.jp/rhizobase: mll0004 and mlr5526) but nothing is known about their function in symbiosis.
Chapter 5

Discovery of a heme uptake system in the soil bacterium *Bradyrhizobium japonicum*

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5.1 Summary

In *Bradyrhizobium japonicum*, the nitrogen-fixing symbiont of soybeans, we have identified a heme uptake system, Hmu, which comprises a cluster of nine open reading frames. Predicted products of these genes include HmuR, a TonB-dependent heme receptor in the outer membrane, HmuT, a periplasmic heme-binding protein; and HmuUV, an ABC transporter in the inner membrane. Furthermore, we identified homologues of ExbBD and TonB that are required for energy transduction from the inner to the outer membrane. Mutant analysis and complementation tests indicated that HmuR and the ExbBD-TonB system but not the HmuTUV transporter, are essential for heme uptake or heme acquisition from hemeoglobin and leghemoglobin. The TonB system seems to be specific for heme uptake since it is dispensable for siderophore uptake. Therefore, we propose the existence of a second TonB homologue functioning in the uptake of Fe-chelates. When tested on soybean host plants, *hmuT-hmuR* and *exbD-tonB* mutants exhibited wild-type symbiotic properties. Thus, heme uptake is not essential for symbiotic nitrogen fixation but it may enable *B. japonicum* to have access to alternative iron sources in its non-symbiotic state. Transcript analysis and expression studies with lacZ fusions showed that expression of *hmuT* and *hmuR* is induced under low iron supply. The same was observed in *fur* and *irr* mutant backgrounds although maximal induction levels were decreased. We conclude either that both regulators, Fur and Irr, independently mediate transcriptional control by iron or that a yet unknown iron regulatory system activates gene expression under iron deprivation. An A/T-rich cis-acting element, located in the promoter region of the divergently transcribed *hmuTUV* and *hmuR* genes, is possibly required for this type of iron control.
5.2 Introduction

Iron is an essential element for almost all bacteria when it serves as a cofactor in key metabolic processes such as nucleotide biosynthesis, electron transfer and energy transduction. Despite its abundance in nature, iron supply poses severe problems for organisms growing under aerobic conditions. Owing to its poor solubility, free Fe$^{3+}$ is present only at very low concentrations of approximately $1.4 \times 10^{-9}$ M at pH 7 (Ratledge and Dover, 2000), which are far below the concentration needed to sustain growth of bacteria. In order to gain iron, bacteria have developed four principal strategies (Guerinot, 1994; Braun and Killmann, 1999): (i) chelation of free Fe$^{3+}$ ions by siderophores, (ii) the use of alternative iron sources, (iii) reduction of Fe$^{3+}$ and uptake of the more soluble Fe$^{2+}$ (Vartivarian and Cowart, 1999), and (iv) the minimization of iron dependence by evolving iron-independent enzymes, as in *Lactobacillus plantarum* and *Borrelia burgdorferi* (Archibald, 1983; Posey and Gherardini, 2000). Siderophores are ferric ion-specific ligands of low molecular weight and comprise a broad spectrum of different compounds (Höfte, 1993). Iron acquisition via siderophores requires genes for their biosynthesis, excretion and uptake. The latter step comprises the binding of the iron-loaded siderophore to a specific receptor in the outer membrane. Alternative iron sources include iron-containing proteins, such as hemoglobin, transferrin or lactoferrin. These proteins can be used as iron sources by numerous Gram-negative pathogens by means of specialized iron acquisition strategies (Ratledge and Dover, 2000). To gain iron from host hemoproteins, these pathogens synthesize high-affinity heme receptors and, in some cases, also extracellular heme-binding proteins (for reviews, see Lee, 1995; Genco and Dixon, 2001).

Receptor-mediated uptake of siderophores or heme includes translocation of the ligands into the periplasm by an energy-dependent process that requires a functional Ton system (for reviews, see Braun, 1995; 1997a). The TonB protein, that is anchored in the cytoplasmic membrane and associated with ExbB and ExbD, spans the periplasm and interacts with the ligand-loaded receptor. The TonB-ExbBD system transduces the energy of the proton-motive force of the cytoplasmic membrane into transport energy required by the receptor protein (Moeck and Coulton, 1998).

For bacteria, it is critical to maintain an adequate cellular iron homeostasis because excess iron may cause serious damage to biomolecules by reactive oxygen species.
formed in the Fenton reaction (Pierre and Fontecave, 1999; Touati, 2000). Cellular components required for iron utilization are controlled at the transcriptional level in either a negative or positive fashion (Crosa, 1997). High concentrations of iron lead directly to a shut-off of the expression of many genes involved in iron uptake. The Fur protein, which is present in Gram-negative and some Gram-positive bacteria, represses transcription of target genes when loaded with ferrous iron by binding to a DNA motif that consists of at least three AT₃/TAT repeats. These so-called Fur boxes, which are associated with individual iron-regulated genes, can vary in number and orientation (Escolar et al., 1999). In addition to derepression under iron-limiting conditions, some transport genes are expressed only when the corresponding iron chelator is available. One well-studied example of such a positive control mechanism is the Fec system of *Escherichia coli* (Braun, 1997b).

Nitrogen-fixing rhizobia, living as endosymbionts in root nodules of legume host plants, have a high demand for iron because a number of ‘symbiotic’ proteins contain iron or heme (Guerinot, 1993). For example, the nitrogenase complex that makes up more than 10% of the total cellular protein of nitrogen-fixing bacteroids contains 34 iron atoms per molecule (Eady et al., 1980). Similarly, several different types of cytochromes are synthesized at high levels in bacteroids (Hennecke, 1993). Iron metabolism has been studied in some detail in *Bradyrhizobium japonicum*, the symbiont of soybean. *B. japonicum* can acquire Fe³⁺ ions chelated to siderophores (Plessner et al., 1993) or as Fe³⁺-citrate (Guerinot et al., 1990). To date, two structurally related iron-regulatory proteins, Fur and Irr, have been identified in *B. japonicum* (Hamza et al., 1998; 1999). Both regulators contribute to iron-mediated regulation of heme biosynthesis although they act at different levels via distinct mechanisms. In addition, Fur is required for normal iron regulation of *irr* transcription (Hamza et al., 2000). Recently, it was reported that several rhizobial species, including *B. japonicum*, are able to grow on hemoglobin, or heme, as sole iron source (Noya et al., 1997), a property hitherto described exclusively for pathogenic bacteria (Genco and Dixon, 2001). This observation suggested the existence of a heme uptake systems in these rhizobia; however, this hypothesis has not yet been supported by genetic or biochemical data. In this work, we describe the discovery of the genetic determinants for a heme acquisition system in *B. japonicum* consisting of a TonB-dependent heme receptor and an ABC transporter. The newly identified genes were functionally characterized and their
transcriptional regulation was studied in wild-type, fur and irr mutant cells grown under different iron conditions. In a parallel study, Wexler et al. (2001) report on a similar heme uptake system that they had discovered independently in *Rhizobium leguminosarum* biovar *viciae*.

5.3 Results

**Identification of the genetic determinants for a heme acquisition system**  
The DNA region located downstream of the recently described *hemN*₂ gene, which codes for an anaerobic coproporphyrinogen III dehydrogenase (Fischer et al., 2001), was cloned in the overlapping plasmids pRJ8658 and pRJ8638 and analyzed using sequencing (Fig. 5.1). In the sequenced region of 16 607 bp, a total of 14 open reading frames was identified (Table 5.1). Most interestingly, we identified a cluster of seven genes (*hmuTUV*, *hmuR*, *exbBD*, and *tonB*) whose predicted products displayed significant similarity to individual components of heme uptake systems described in numerous pathogenic bacteria or opportunistic pathogens. Specifically, the proteins encoded by *B. japonicum* *hmuTUV* are most similar to an ABC transporter of *Yersinia enterocolitica* (Stojiljkovic and Hantke, 1994), and the product of *hmuR* resembles the TonB-dependent heme receptor HasR, which is located in the outer membrane of *Serratia marcescens* (Ghigo et al., 1997). The most similar homologues of the *B. japonicum* proteins encoded by *exbBD* and *tonB*, are ExbBD and TonB of *Pseudomonas putida* (Bitter et al., 1993). The region downstream of *tonB* codes for the 3’ end of an open reading frame of which predicted product is similar to a putative periplasmic protein in *Campylobacter jejuni* (64% identity in 69 compared amino acids; accession no. C81344). Between *hemN*₂ and *hmuTUV* (Fig. 5.1), we identified five additional open reading frames. The deduced protein sequences of three of them (*orf236*, *orf319*, *orf206*) show significant similarity to different transcriptional regulators (Table 5.1).
Fig. 5.1 Physical map of the *B. japonicum* locus spanning from hemN to tonB. An overview of the entire region is shown in the upper part while the genes encoding the heme uptake system described in this study, are depicted as a blow-up in the lower part of the figure. Vertical numbers indicate the position of the start codons and stop codons of open reading frames and recognition sites of the following restriction enzymes: BamHI (B), BglII (Bg), Clal (C), EcoRI (E), NcoI (Nc), NruI (Nr), NotI (Nt), PstI (P), SacI (S). Only relevant restriction sites for BglII, NcoI and NruI are indicated. Nucleotide positions correspond to the sequence deposited in the GenBank database (16,607 bp; accession no. AJ311165). Open reading frames encoding proteins of unknown functions or which were not further studied in this work are specified by the numbers of codons (in italics). The inserts of the initially constructed plasmids are depicted at the top along with the respective plasmid number. The inserts of plasmids used for complementation experiments are shown above the enlarged *hmu* gene cluster. Horizontal arrows in opposite directions indicate the transcriptional start sites upstream of *hmuT* and *hmuR*. Open arrows refer to the location and orientation of the kanamycin resistance gene cassette (*aphII*), which was used for construction of the mutants deleted for the specified fragments (Δ). The structures of the transcriptional fusions of *hmuT* and *hmuR*, which were integrated into the chromosome, are depicted at the bottom. Numbers refer to the designation of the mutant strains indicated.
<table>
<thead>
<tr>
<th>B. japonicum ORF or gene</th>
<th>No. of amino acids</th>
<th>Homologous protein</th>
<th>Homologous organism</th>
<th>% Identity / Similarity</th>
<th>Accession no.</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>orf236</td>
<td>236</td>
<td>NnrR</td>
<td>R. sphaeroides</td>
<td>49 / 60</td>
<td>AF126490</td>
<td>Fnr-type regulator of denitrification genes</td>
</tr>
<tr>
<td>orf403</td>
<td>403</td>
<td>NnrS</td>
<td>R. sphaeroides</td>
<td>51 / 58</td>
<td>AF016258</td>
<td>Not known</td>
</tr>
<tr>
<td>orf319</td>
<td>319</td>
<td>Rv 3736</td>
<td>M. tuberculosis</td>
<td>29 / 40</td>
<td>CAA18058</td>
<td>Transcriptional regulator of the AraC family</td>
</tr>
<tr>
<td>orf206</td>
<td>206</td>
<td>FixJ</td>
<td>B. japonicum</td>
<td>32 / 45</td>
<td>P23221</td>
<td>Response regulator</td>
</tr>
<tr>
<td>orf560</td>
<td>560</td>
<td>ZK1055.7</td>
<td>C. elegans</td>
<td>25 / 33</td>
<td>T33320</td>
<td>Not known</td>
</tr>
<tr>
<td>hmuV</td>
<td>268</td>
<td>HemV</td>
<td>Y. pestis</td>
<td>45 / 53</td>
<td>Q56993</td>
<td>ATPase unit of ABC transporter</td>
</tr>
<tr>
<td>hmuU</td>
<td>363</td>
<td>DRB0015</td>
<td>D. radiodurans</td>
<td>46 / 54</td>
<td>AAF12576</td>
<td>Heme permease</td>
</tr>
<tr>
<td>hmuT</td>
<td>317</td>
<td>HemT</td>
<td>Y. enterocolitica</td>
<td>31 / 44</td>
<td>X77867</td>
<td>Periplasmic heme binding protein</td>
</tr>
<tr>
<td>hmuR</td>
<td>782</td>
<td>HasR</td>
<td>S. marcescens</td>
<td>30 / 53</td>
<td>CAA70172</td>
<td>TonB-dependent outer membrane heme receptor</td>
</tr>
<tr>
<td>orf110</td>
<td>110</td>
<td>OrfA</td>
<td>R. leguminosarum bv. viciae</td>
<td>52 / 61</td>
<td>CAC34386</td>
<td>Not known</td>
</tr>
<tr>
<td>orf167</td>
<td>167</td>
<td>OrfX</td>
<td>Y. pestis</td>
<td>44 / 54</td>
<td>T12066</td>
<td>Not known</td>
</tr>
<tr>
<td>exbB</td>
<td>258</td>
<td>ExbB</td>
<td>P. putida</td>
<td>56 / 72</td>
<td>Q05605</td>
<td>Energy transduction</td>
</tr>
<tr>
<td>exbD</td>
<td>148</td>
<td>ExbD</td>
<td>P. putida</td>
<td>64 / 76</td>
<td>Q05606</td>
<td>Energy transduction</td>
</tr>
<tr>
<td>tonB</td>
<td>276</td>
<td>TonB</td>
<td>P. putida</td>
<td>30 / 51</td>
<td>Q05613</td>
<td>Energy transduction</td>
</tr>
</tbody>
</table>
The heme uptake locus is required for iron acquisition from heme and hemoglobin

Deletion mutations were constructed in order to study the function of the genes encoding the putative heme acquisition system (Fig. 5.1). The corresponding *B. japonicum* mutants showed normal growth when cultivated in PSY-rich medium under aerobic conditions. Thus, *hmuT*, *hmuR*, *exbD*, and *tonB* are not essential under these conditions. Next, we tested the ability of wild-type and mutant cells to acquire iron from different sources. Wild-type cells clearly grew around filter disks that had been soaked in solutions of heme, hemoglobin or leghemoglobin and deposited on iron-limited PSY agar plates (Fig. 5.2; Table 5.2; for details, see Experimental procedures). By contrast, the mutants completely failed to grow with these Fe sources, but they all showed normal growth when iron sulphate, iron citrate or ferrichrome were provided as iron sources in the plate assays. Iron citrate and ferrichrome are known to be taken up by a TonB-dependent process in many Gram-negative bacteria (Braun, 1995). In the same series of experiments, we observed that none of the tested *B. japonicum* strains (including the wild type) was able to acquire iron from rhodotorulic acid, a fungal chelator, or from horse heart cytochrome c, a hemoprotein with a covalently bound heme (data not shown).

Mutant strains 8640 and 8654 were used for complementation tests with plasmids comprising one of the following: the entire gene cluster from *hmuV* to *tonB* (pRJ8695), the region spanning from *hmuR* to *tonB* (pRJ8694) or only *hmuR*, *orf110*, *orf167* (pRJ8696) (Fig. 5.1; Table 5.2). Growth of mutant 8640 with hemin or hemoglobin as sole iron source was restored by plasmid pRJ8695 and also by pRJ8694, even though the latter does not contain the entire *hmuTUV* genes. Likewise, strain 8654 regained the ability to utilize heme when it contained plasmid pRJ8694. No complementation was detected with plasmid pRJ8696, indicating that the mutation in 8640 was probably polar.

Symbiotic phenotypes of heme uptake mutants

The potential role of the heme acquisition genes in symbiotic nitrogen fixation was studied in a soybean plant infection test with the *B. japonicum* mutant strains 8640, 8646, 8654, 8655. Nodulation and nitrogen fixation activity of at least eight plants were evaluated three weeks after infection. The size, morphology, N2-fixing abilities and the interior color of nodules elicited by all mutant strains did not differ from those that were
formed by the wild type. Similarly, nitrogen fixation activity of all mutants did not significantly differ from the wild type. Thus, the products of the heme uptake genes are not essential for an effective symbiosis of *B. japonicum* with its soybean host plant.

**Fig. 5.2 Growth stimulation by heme, hemoglobin or FeSO₄ as sole iron sources.** *B. japonicum* wild type and mutant 8640 (ΔhmuT-hmuR) were pregrown in normal PSY medium. 10⁶ cells were then embedded into a top layer of soft agar poured on PSY plates containing the iron chelator EDDHA (25 μM). The photographs show the growth zones around filter disks soaked with solutions of hemoglobin (Hb; 250 μM), hemin (Hm; 1 mM) and FeSO₄ (Fe; 10 mM) or as control with H₂O (−) after 6 d incubation of the plates at 30°C. Similar results were obtained with mutants 8646, 8654 and 8655 (data not shown).
Table 5.2 Growth of *B. japonicum* wild-type and mutant strains on different Fe sources

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmid^a</th>
<th>FeSO₄ (10 mM)</th>
<th>Hemin (1 mM)</th>
<th>Hb (250 μM)</th>
<th>LegHb (500 μM)</th>
<th>FeCitrate (10 mM)</th>
<th>Ferrichrome (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>110spc4</td>
<td>Wild type</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GEM4</td>
<td>fur::Ω</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8640</td>
<td>ΔhmuT-hmuR</td>
<td>pPP375</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>—</td>
<td>nd</td>
</tr>
<tr>
<td>8646</td>
<td>ΔhmuT-hmuR</td>
<td>pRJ8694</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8654</td>
<td>ΔexbD-tonB</td>
<td>pPP375</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8655</td>
<td>ΔexbD-tonB</td>
<td>pRJ8694</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DES 122</td>
<td>Wild type</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>LODTM5</td>
<td></td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>nd</td>
</tr>
<tr>
<td>8666</td>
<td>hmuT-lacZ, hmuR^+</td>
<td>pRJ8694</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8683^d</td>
<td>P* hmuT-lacZ, P* hmuR</td>
<td>pPP375</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8664</td>
<td>hmuR-lacZ, hmuT^+</td>
<td>pRJ8694</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8687^d</td>
<td>P* hmuR-lacZ, P* hmuT</td>
<td>pRJ8694</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

^a The indicated plasmids were tested for their ability to restore growth of mutants 8640 and 8654 with hemin or hemoglobin as sole iron source.

^b +, Growth as detected by the appearance of distinct growth zones around the filter disks; (+), weaker cell densities as those formed by wild-type cells; —, no growth.

^c nd, not done.

^d For definition of P*, see Fig. 5.5.
Expression of \textit{hmuT} and \textit{hmuR} is subject to iron regulation

To study regulation, we inserted \textit{hmuT-lacZ} and \textit{hmuR-lacZ} transcriptional fusions into the chromosome of suitable strains (Fig. 5.1) and examined their expression under high- and low-iron conditions (Fig. 5.3). Maximal expression of these fusions was observed under iron-limiting conditions. The addition of 100 $\mu$M Fe$\text{SO}_4$ to the culture medium resulted in a 42- and 26-fold repression of \textit{hmuT} and \textit{hmuR} expression, respectively (Fig. 5.3A). Iron-dependent repression of \textit{hmuT-} and \textit{hmuR-lacZ} expression was also detected in the respective \textit{fur} mutant derivatives, indicating that the Fur protein is not directly involved in iron regulation of these genes. However, in the \textit{fur} mutant, maximal expression levels were decreased to 58\% (\textit{hmuT}) and 67\% (\textit{hmuR}), compared with the levels observed in the wild-type background. To study the potential involvement of Irr, a second iron regulatory protein known in \textit{B. japonicum}, the \textit{hmuR-lacZ} fusion was integrated into the chromosome of the \textit{irr} mutant LODTm5 and its isogenic parental strain USDA DES122. As was found in the background of strain 110\emph{spc}4, \textit{hmuR} expression was also repressed in USDA DES122 under iron-replete conditions. In the \textit{irr} mutant, repression was maintained but the maximal expression level under iron-depleted conditions was lowered to 52\% of the value observed in the corresponding wild type (Fig. 5.3B).

Transcriptional analysis of the \textit{hmuT-hmuR} divergon

The transcriptional start sites of \textit{hmuT} and \textit{hmuR}, which we predicted to be located in the 466-bp intergenic region between these oppositely oriented genes, were mapped precisely by primer extension experiments. Using two different primers for both genes, we localized the start of the \textit{hmuT} transcript at position C$\text{10\,645}$, 240 bp upstream of the putative \textit{hmuT} start codon (Fig. 5.4A), and that of the \textit{hmuR} transcript at position A$\text{10\,759}$, located 113 bp upstream of the \textit{hmuR} coding region (Fig. 5.4B). Both transcripts were much more abundant in cells grown under iron-limiting conditions, which is in good agreement with the results of the expression studies using \textit{lacZ} fusions. Moreover, in \textit{fur} and \textit{irr} mutant cells, the transcripts were similarly regulated as in the wild type. No \textit{hmuT} transcript was detected in the RNA isolated from the negative control strain 8640 because the DNA region corresponding to the priming site of primer \textit{hmuT-rev1} had been deleted in this strain. The transcription start sites of both \textit{hmuT} and \textit{hmuR} are preceded by putative promoter elements that exhibit significant similarity to those of –
35/-10-type promoters (underlined nucleotides in Fig. 5.4C). Inspection of the region between both promoters revealed a striking A/T-rich imperfect inverted repeat of 21 bp length (emphasized in white letters in Fig. 5.4C) of which the axis of dyad symmetry is located 52 and 62 nucleotides upstream of the \textit{hmuT} and \textit{hmuR} transcription start site, respectively.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5_3}
\caption{Expression of chromosomally integrated \textit{hmuR-lacZ} (8664) and \textit{hmuT-lacZ} (8666) fusions in \textit{B. japonicum} cells grown under different iron conditions. Aerobic cultures, that had been inoculated to an optical density at 600 nm of 0.02, were grown for 3 d in PSY medium supplemented with no additional iron (panel A; grey bars), 0.5 \textmu M (panel B; grey bars), 50 \textmu M (panel B; black bars) or 100 \textmu M FeSO\textsubscript{4} (panel A; black bars). \textbeta-Galactosidase activities of at least two cultures were determined in duplicate. Data from one out of three independent experiments are shown, which yielded comparable results. Low-iron cultures of the strains shown in panel B were supplemented with 0.5 \textmu M FeSO\textsubscript{4} because the irr mutant \textit{is unable} to grow in PSY medium lacking additional iron.}
\end{figure}
Fig. 5.4 Mapping of the transcription start sites of *B. japonicum* hmuT (A) and hmuR (B) by primer extension. RNA used for the extension reactions with the primers specified at the top was purified from cells of the indicated strains which were grown aerobically in PSY medium supplemented with different amounts of FeSO₄. The transcription start sites of both hmuT and hmuR (marked with arrow heads in panels A and B) were confirmed with a second primer for each gene (data not shown). The sequence ladders were generated with plasmid pRJ8638 and the same primers used for the respective transcript mapping. Note that the G lane is missing in panel B. The nucleotide sequence of the promoter region of the divergently oriented hmuT and hmuR genes in *B. japonicum* 110spc4 is shown in panel C. The transcription start sites for hmuT and hmuR are marked (+1) and the 5' end of the RNA transcripts of hmuT and hmuR are indicated by horizontal arrows. Conserved nucleotides of putative −35/−10-type promoters are underlined. White letters emphasizes an A/T-rich, imperfect inverted repeat located between the putative promoters, and the distance of its axis of dyad symmetry relative to the transcription start sites of hmuT and hmuR is indicated. The numbering of nucleotide positions (right margin) corresponds to that of the sequence deposited in the GenBank database (see Fig. 5.1).
Chapter 5

**Functional analysis of the A/T-rich cis element in the promoter region of hmuT and hmuR**

To study the potential regulatory function of the A/T-rich motif upstream of *hmuT* and *hmuR*, a 2-bp insertion was introduced between the imperfect inverted repeat (see Experimental procedures). The mutation was placed upstream of the *hmuR-lacZ* and the *hmuT-lacZ* fusion and chromosomally integrated. The relevant chromosomal structure of the resulting strains 8683 (P*, hmuT-lacZ) and 8687 (P*, hmuR-lacZ) is depicted in Fig. 5.5. Expression from the mutated promoters was measured in cells grown under low- and high-iron conditions (Table 5.3). The insertion of 2 bp resulted in a drastic reduction of both *hmuT-lacZ* (strain 8683) and of *hmuR-lacZ* expression (strain 8687) under low-iron conditions, and it also further diminished the expression levels in cells grown under high-iron conditions. Interestingly, *hmuR* expression from the mutated promoter was still weakly induced under low-iron conditions whereas that of *hmuT* was equally low under both iron conditions.

Because normal regulation of the divergently transcribed genes *hmuT* and *hmuR* was dependent on the same A/T-rich cis element, we rationalized that the 2-bp insertion in mutant 8683 not only affected *hmuT-lacZ* expression but at the same time also the expression of *hmuR*. Likewise, the mutation in strain 8687 would affect *hmuT* in addition to having consequences on *hmuR-lacZ* expression (Fig. 5.5). Therefore, these mutants were used to study the effects of reduced *hmuT* or *hmuR* promoter activity on the ability to utilize hemin or hemoglobin as iron source. Appropriate reference strains were the *lacZ* reporter derivatives 8666 and 8664, which contain the wild-type promoter upstream of *hmuT* and *hmuR*. As listed in Table 5.2, both reference strains (8666, 8664) as well as strain 8687, containing the mutated promoter upstream of *hmuT*, were able to grow in the plate assay. By contrast, the reduced activity of the *hmuR* promoter in mutant 8683 prevented growth of this strain. Thus, the ABC transporter (HmuTUV) is dispensable for heme utilization, whereas the heme receptor (HmuR) and/or the gene products encoded downstream of *hmuR* are essential for this trait.
Fig. 5.5 Structure of the chromosomally integrated hmuT- and hmuR-lacZ fusion in strains 8683 and 8687, respectively, containing a 2-bp insertion (open arrow) in the A/T-rich imperfect inverted repeat (solid box). For comparison, the wild-type situation is shown on top. The nucleotide sequence of the mutated element is depicted at the bottom with the nucleotides of the imperfect inverted repeat in white letters and the two newly introduced nucleotides denoted by lower case letters shaded in grey. The NruI site resulting from this insertion is marked. Note that wild-type copies of hmuT and hmuR are present in strains 8683 and 8687, respectively, in addition to the lacZ fusions to the respective mutant promoters.

Table 5.3. Expression of chromosomally integrated hmuT- and hmuR-lacZ fusions from mutated promoters (P*) carrying a 2-bp insertion in the A/T-rich cis element (see Fig. 5.5)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>β-Galactosidase activity (Miller Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50 µM FeSO₄ 0.5 µM FeSO₄</td>
</tr>
<tr>
<td>8666</td>
<td>hmuT-lacZ</td>
<td>26.4 ± 3.6 262.3 ± 32.5</td>
</tr>
<tr>
<td>8683</td>
<td>P* hmuT-lacZ</td>
<td>6.0 ± 0.2 6.5 ± 0.5</td>
</tr>
<tr>
<td>8664</td>
<td>hmuR-lacZ</td>
<td>50.6 ± 1.0 564.2 ± 105.7</td>
</tr>
<tr>
<td>8687</td>
<td>P* hmuR-lacZ</td>
<td>8.8 ± 0.3 22.1 ± 0.5</td>
</tr>
</tbody>
</table>

Two cultures of each strain were grown for 2.5 d at 30°C in PSY medium supplemented with the indicated concentrations of FeSO₄ and assayed in duplicate. Data are shown from a representative experiment, which was repeated once.
5.4 Discussion

While it had been reported previously that some rhizobia are able to grow on hemoglobin or heme as sole iron source (Noya et al., 1997), nothing was known of the determinants underlying this property. The results presented here show that acquisition of iron from heme by the facultative symbiotic soil bacterium *B. japonicum* is brought about by an uptake system that is highly similar to that used by pathogenic bacteria for capturing heme from their hosts.

Recent data from different laboratories indicates that *B. japonicum* is not exceptional among rhizobia with regard to the presence of a heme uptake system. Homologues of HmuR and HmuTUV are encoded in the genomes of *Sinorhizobium meliloti* (http://sequence.toulouse.inra.fr/meliloti.html) and *Mesorhizobium loti* (http://www.kazusa.or.jp/rhizobase/), and (Wexler et al., 2001) have identified a similar system in *R. leguminosarum* bv. *viciae*. The organization of the respective genes, including those for the ExbBD-TonB system, varies among these species. Interestingly, homologues of the functionally undefined *B. japonicum* orf110 are found associated with *hmu* genes only in rhizobial species but not in other bacteria.

**HmuR**

The predicted *B. japonicum* HmuR protein displays convincing similarity to outer membrane TonB-dependent receptors. These proteins have in common a unique plug-and-barrel organization as deduced from the structural analysis of different siderophore receptors (Koebnik et al., 2000, and references therein). The large β-barrel is gated by a flexible, N-terminal loop, the so-called plug domain. Our notion that HmuR is a receptor for heme is supported by the presence of characteristic domains and motifs, shared by TonB-dependent heme receptors (Fig. 5.6). For example, three typical motifs which presumably are involved in the interaction with TonB (TonB boxes I, II and III; Bitter et al., 1991; Kühn et al., 1996) are also found in the *B. japonicum* HmuR protein. The TonB box III is part of a conserved region of about 80 amino acids not far from the N terminus. This domain includes a conserved histidine residue which is probably located in the putative plug domain and essential for heme uptake in the HemR protein of *Y. enterocolitica* (Bracken et al., 1999). A second functional histidine is present within the region defined by the so-called FRAP/NPNL motif (Bracken et al., 1999;
This region is preceded by a short, absolutely invariant RYDX483 sequence (numbering refers to *B. japonicum* HmuR) whose function is not known. One may speculate that the tyrosine residues of this motif, and nine additional tyrosines conserved in HmuR-like proteins, possibly form a hydrophobic environment required for binding of the hydrophobic heme molecule or stabilizing HmuR in the outer membrane (see Fig. 5.6).

**Fig. 5.6** Amino acid sequence comparison of the *B. japonicum* (Bj) HmuR protein with homologues from other bacteria. The full-length HmuR protein is shown schematically as a rectangle. Conserved regions are indicated by black boxes with the corresponding amino acid sequence comparisons shown above or below the boxes (for further details, see text). Vertical lines marked by amino acid position numbers denote highly conserved tyrosine residues. Residues conserved in all proteins are highlighted on a solid background and represented by uppercase letters in the consensus line; residues conserved in at least six proteins are emphasized by white letters on a grey background, those conserved in five proteins by black letters on a light grey background. The proteins (accession no.) included are as follows: *Mesorhizobium loti* (Ml) HmuR (mlr115 in http://www.kazusa.or.jp/rhizobase/), *Sinorhizobium meliloti* (Rm) HmuR (SMc04205 in http://sequence.toulouse.inra.fr/meliloti.html), *Serratia marcescens* (Sm) HasR (CAA70172), *Pseudomonas aeruginosa* (Pa) HasR (AAG06796), *Haemophilus influenzae* (Hi) HxC (P45357), *Shigella dysenteriae* (Sd) ShuA (AAC27309) and *Yersinia enterocolitica* (Ye) HemR (CA48250). In the latter sequence, the histidines marked by asterisks had been shown to be essential for HemR function (Bracken *et al.*, 1999).
Heme uptake

HmuTUV
Based on sequence comparisons, the predicted products of the *hmuTUV* genes form a heme transport system with HmuV being the ATP-hydrolyzing subunit, HmuU the integral membrane component and HmuT the periplasmic binding protein that receives heme from HmuR and delivers it to the HmuUV transporter in the cytoplasmic membrane. The *hmuTUV* genes are most likely cotranscribed because they appear to be translationally coupled. Characteristic structural features support the predicted functions of HmuU and HmuV. A typical ATP-binding site consisting of Walker A (GPNGAGKST_{45}) and B motifs (LLLLD_{67}) is present in HmuV. HmuU is very hydrophobic, and computer analyses predict at least six membrane-spanning domains. Our data suggest that the *hmuTUV* gene products are not essential for iron acquisition because the *hmuT-hmuR* double mutant 8640 was complemented by plasmid pRJ8694, which provides *hmuR* and the distal genes but not *hmuTUV*. Our assumption is further corroborated by the phenotype of mutant 8687. This strain still is able to grow on heme although *hmuTUV* expression is impaired by the 2-bp insertion in the A/T-rich regulatory element. That the putative heme transporter is not essential for heme utilization has been described previously for *V. cholerae* (Occhino et al., 1998). This might be explained by the presence of an alternative ABC transporter that is able to transport heme, or at least the heme iron, through the inner membrane.

ExbBD-TonB
Downstream of *hmuR* and separated by two open reading frames of unknown function (*orf110, orf167*), we have identified the genes for the ExbBD-TonB system that is essential for heme uptake. The *exbBD-tonB* genes are likely to be organized in an operon with *hmuR, orf110* and *orf167* as indicated by the complementation experiments. Plasmid pRJ8696 is not able to correct the iron acquisition defect of mutant 8640, which is probably due to polarity of the deletion present in strain 8640. The overall similarity of *B. japonicum* TonB to homologues from other bacteria is rather low, yet this protein displays the central proline-rich region and numerous conserved amino acids in the C terminus, which are characteristic for TonB proteins (Braun, 1995). Furthermore, a conserved SXXXH motif is present in its N terminus (SQPEH_{45}), which had been shown to be critical for activity of *E. coli* TonB (Larsen and Postle, 2001). Interestingly, the *exbD-tonB* deletion mutants 8654 and 8655 are still able to use
the fungal siderophore ferrichrome or Fe-citrate as iron source. Because all iron-
complex receptors studied so far depend on TonB, we postulate that *B. japonicum*
possesses a second copy of *tonB* and probably also of *exbBD*. In fact, Southern blot
hybridizations with an *exbBD*-specific probe supported this assumption (data not
shown). The ability to express a TonB system that is specific for heme uptake is not
uncommon among bacteria. *P. aeruginosa* and *Vibrio cholerae*, for example, synthesize
two versions of TonB, one of which is dedicated to heme uptake (Occhino et al., 1998;
Zhao and Poole, 2000; Seliger et al., 2001).

**Transcriptional control of the heme uptake system**

Expression of *hmuT* and *hmuR* is repressed under high iron conditions. Neither the
ferric uptake regulator, Fur (Hamza et al., 1999), nor the iron response regulator, Irr
(Hamza et al., 1998), are solely responsible because this type of control was maintained
in the respective null mutants. Unfortunately, we were unable to construct a *fur-irr*
double mutant that made it impossible to evaluate whether these two regulators can
functionally replace each other or whether a third, as yet unknown regulatory system is
involved in iron control of the *B. japonicum hmu* system. However, *hmu* repression by
Irr alone seems improbable because this regulator is down-regulated at both the
transcriptional and post-transcriptional level under iron-replete conditions (Qi et al.,
1999; Hamza et al., 2000). Its predominant role is the coordination of heme biosynthesis
with iron availability.

Maximal induction of *hmuT* and *hmuR* expression was decreased by 30-40% in the *fur*
mutant background, and the absence of a functional *irr* gene had a similar effect on
*hmuR* expression. However, *hmu* expression levels in both mutant backgrounds were
sufficiently high to sustain growth with heme as the sole iron source. Thus, a strict
dependence of *hmu* induction on either Fur or Irr can be excluded. The slightly reduced
*hmu* expression in the *fur* and *irr* backgrounds might be explained by an indirect effect
possibly via altered iron conditions in the mutant strains or by a partial involvement of
these regulators in positive control of *hmu* expression. In fact, it was suggested
previously that Irr has both positive and negative regulatory functions in *B. japonicum*
(Hamza et al., 1998). Similarly, in addition to its classical negative regulatory role, Fur
has been implicated in positive control in *E. coli* although this is probably the result of
an indirect effect too (Escolar et al., 1999; Dubrac and Touati, 2000). After all, positive
control seems to be quite common for siderophore uptake genes in various bacteria, and this includes a number of different mechanisms (for review, see Crosa, 1997). We have shown that the spacing of the half sites of an A/T-rich motif located between hmuT and hmuR is critical for induction of these genes under iron deprivation. The center of this cis element, which is rather dissimilar from the consensus Fur box, is located 62 and 52 nucleotides upstream of the transcriptional start of hmuT and hmuR, respectively, suggesting that it is involved in a positive control mechanism. We speculate that it represents the binding site for an additional (hypothetical) transcriptional regulator engaged in iron homeostasis in B. japonicum. At this point, one may extend these speculations on the potential involvement in iron regulation of orf319 and/or orf206 that are located downstream of the hmuTUV genes and the products of which show similarity to transcriptional activators.

What is the role of the heme uptake system in rhizobia?
The soybean plant infection tests performed with the B. japonicum hmu mutants indicated that the heme uptake system identified here is not important for symbiotic nitrogen fixation in soybean nodules under the experimental conditions applied in our assay. In this context, two aspects may be considered. First, it is rather questionable whether heme is available to bacteroids during the functional period of the symbiosis. Although leghemoglobin makes up 30% of the total soluble protein in infected plant cells (Verma and Long, 1983), bacteroids have no access to this iron pool because the peribacteroid membrane, separating them from the cytoplasm of the host cell, surrounds them. However, one may envisage that a heme uptake system is advantageous to non-terminally differentiated bacteroids when senescing nodules lose their integrity and leghemoglobin is released from its compartment. Second, the iron status of bacteroids is not well defined and surprisingly little is known about the bacterial iron acquisition strategies in planta. Probably, Fe(II) and Fe(III) are amenable to bacteroids because both forms of iron are transported across the peribacteroid membrane (Moreau et al., 1995; 1998). Finally, siderophores which are synthesized by numerous rhizobia under free-living conditions are probably not essential in planta as indicated by the Fix+ phenotype of R. leguminosarum bv. viciae siderophore mutants (Stevens et al., 1999; Yeoman et al., 1999).
5.5 Experimental procedures

Strains and plasmids
The bacterial strains and plasmids used in this work are listed in Table 5.4.

Media and growth conditions
Luria-Bertani (LB) medium (Miller, 1972) was used for growth of E. coli cells; it contained the following concentrations of antibiotics for plasmid selection (µg ml⁻¹): ampicillin, 200; kanamycin, 30 and tetracycline, 10. Peptone-salts-yeast extract (PSY) medium (Regensburger and Hennecke, 1983) supplemented with 0.1% L-arabinose was used for routine aerobic cultures of B. japonicum. It contains 1.2 µM FeCl₃.
Concentrations of antibiotics for use in B. japonicum cultures were as follows (µg ml⁻¹): spectinomycin, 100; kanamycin, 100; streptomycin, 50 and tetracycline, 50 (solid media) or 35 (liquid media). For growth under low-iron conditions, PSY medium containing no or 0.5 µM FeSO₄ was used. PSY cultures under high-iron conditions contained 50 or 100 µM FeSO₄. For growth experiments with different iron sources B. japonicum wild-type and mutant strains were grown for 3 d at 30°C in normal PSY medium. The cultures were diluted to approximately 10⁷ cells ml⁻¹, and 100 µl of the diluted cells were mixed with 5 ml PSY soft agar (0.8%, 40°C) and poured onto PSY plates (1.5% agar), both containing 25 µM of the iron chelator EDDHA (ethylenediaminedi[o-hydroxyphenylacetic] acid; Sigma-Aldrich). Sterile filter disks (6 mm diameter) were placed onto the plates and 6 µl of the following solutions were deposited on them: 1 mM bovine hemin in 0.1 N NaOH, 50% ethanol; 250 µM bovine hemoglobin in H₂O; 10 mg/ml (~550 µM) leghemoglobin from soybean in H₂O, 1 mM mitochondrial horse heart cytochrome c; 1 mM ferrichrome in H₂O, 1 mM rhodotorulic acid in H₂O, 10 mM FeSO₄, or 10 mM ferric citrate. These compounds were all purchased from Sigma-Aldrich, except for leghemoglobin, which was a gift from C. A. Appleby (Moruya, Australia).
Table 5.4 Bacterial strains, cloning vectors and recombinant plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or properties</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
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<tr>
<td>DH5α</td>
<td><em>supE44 ΔlacU169 (Φ80lacZΔM15) hsdR7 recA1 gyrA96 thi-1 relA1</em></td>
<td>Bethesda Research Laboratories, Inc., Gaithersburg, Md</td>
</tr>
<tr>
<td>S17-1</td>
<td>*Sm&lt;sup&gt;i&lt;/sup&gt; *Sp&lt;sup&gt;i&lt;/sup&gt; <em>hsdR</em> (RP4-2 <em>kan::Tn7 tet::Mu</em>, integrated in the chromosome)</td>
<td>Simon <em>et al.</em> (1983)</td>
</tr>
<tr>
<td><strong>B. japonicum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>110&lt;sup&gt;spc4&lt;/sup&gt;</td>
<td><em>Sp&lt;sup&gt;i&lt;/sup&gt; wild type</em></td>
<td>Regensburger and Hennecke (1983)</td>
</tr>
<tr>
<td>USDA DES122</td>
<td><em>wild type</em></td>
<td>O'Brian <em>et al.</em> (1987)</td>
</tr>
<tr>
<td>GEM4</td>
<td>*Sp&lt;sup&gt;i&lt;/sup&gt; *fur:*Ω parental strain USDA 1110</td>
<td>Hamza <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>LODTM5</td>
<td>*Km&lt;sup&gt;i&lt;/sup&gt; *Sm&lt;sup&gt;i&lt;/sup&gt; *irr:<em>Tn5 in strain LO (LO = spontaneously nalidixic acid-resistant derivative of USDA DES122)</em></td>
<td>Hamza <em>et al.</em> (1998)</td>
</tr>
<tr>
<td>8640</td>
<td>*Sp&lt;sup&gt;i&lt;/sup&gt; <em>Km&lt;sup&gt;i&lt;/sup&gt; <em>hmuT::aphII::hmuR</em> (aphII and hmuT oriented in the same direction)</em></td>
<td>This work</td>
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<td>8646</td>
<td>*Sp&lt;sup&gt;i&lt;/sup&gt; <em>Km&lt;sup&gt;i&lt;/sup&gt; <em>hmuT::aphII::hmuR</em> (aphII and hmuT oriented in opposite directions)</em></td>
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<td>*Sp&lt;sup&gt;i&lt;/sup&gt; <em>Km&lt;sup&gt;i&lt;/sup&gt; <em>exbD::aphII::tonB</em> (aphII and tonB oriented in opposite directions)</em></td>
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<tr>
<td>8655</td>
<td>*Sp&lt;sup&gt;i&lt;/sup&gt; <em>Km&lt;sup&gt;i&lt;/sup&gt; <em>exbD::aphII::tonB</em> (aphII and tonB oriented the same direction)</em></td>
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<td>8664&lt;sup&gt;a&lt;/sup&gt;</td>
<td>*Sp&lt;sup&gt;i&lt;/sup&gt; *Tc&lt;sup&gt;i&lt;/sup&gt; <em>hmuR-lacZ chromosomally integrated in <em>B. japonicum</em> 110&lt;sup&gt;spc4&lt;/sup&gt;</em></td>
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<tr>
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<td>*Sp&lt;sup&gt;i&lt;/sup&gt; *Tc&lt;sup&gt;i&lt;/sup&gt; <em>hmuT-lacZ chromosomally integrated in <em>B. japonicum</em> 110&lt;sup&gt;spc4&lt;/sup&gt;</em></td>
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<td>8683</td>
<td>*Sp&lt;sup&gt;i&lt;/sup&gt; *Tc&lt;sup&gt;i&lt;/sup&gt; <em>hmuT-lacZ chromosomally integrated in <em>B. japonicum</em> 110&lt;sup&gt;spc4&lt;/sup&gt; with 2-bp insertion in A/T-rich promoter element</em></td>
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<td>8687</td>
<td>*Sp&lt;sup&gt;i&lt;/sup&gt; *Tc&lt;sup&gt;i&lt;/sup&gt; <em>hmuR-lacZ chromosomally integrated in <em>B. japonicum</em> 110&lt;sup&gt;spc4&lt;/sup&gt; with 2-bp insertion in A/T-rich promoter element</em></td>
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<td><strong>Plasmids</strong></td>
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<td>pBBR1MCS-2</td>
<td><em>Km&lt;sup&gt;i&lt;/sup&gt;</em></td>
<td>Kovach <em>et al.</em> (1995)</td>
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<td>pBSL15/86</td>
<td>*Ap&lt;sup&gt;i&lt;/sup&gt; <em>Km&lt;sup&gt;i&lt;/sup&gt;</em></td>
<td>Alexeyev (1995)</td>
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<td>pME3535</td>
<td><em>Tet&lt;sup&gt;i&lt;/sup&gt;</em></td>
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<td>pRK290S</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt; pRK290 derivative with SacI linker inserted in EcoRI site</td>
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<td>pPP375</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt; pRK290 derivative</td>
<td>P. Putnoky (Biological Research Center, Szeged, Hungary)</td>
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<td>pSUP202</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt; oriT from RP4</td>
<td>Simon et al. (1983)</td>
</tr>
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<td>pSUP202pol4</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt; (pSUP202) part of polylinker from Bluescript II KS+ between EcoRI and PstI</td>
<td>Fischer et al. (1993)</td>
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<td>pSUP202pol5</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt; pSUP202pol4 derivative with BglII linker inserted into SmaI site</td>
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<td>pSUP480</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt; lacZ part from pNM480 in pSUP202pol4</td>
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<td>pUC18</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Norrander et al. (1983)</td>
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<td>pME8656</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; pBBR1MCS-2 derivative (pRJ5616) containing the B. japonicum rrn terminator cloned upstream of a polylinker and a 5.5-kb EcoRI-Sacl fragment with the lacZ gene from pME3535</td>
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<td>pRJ8638</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pUC18) 6.9-kb EcoRI-BamHI fragment with hmuT, hmuR, orf110, orf167, exbBD and tonB</td>
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<td>pRJ8658</td>
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<td>pRJ8663</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; (pME8656), 720-bp EcoRI-Sacl insert of pRJ8659, resulting in a transcriptional hmuT-lacZ fusion</td>
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<td>pRJ8668</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pUC18) 2.9-kb BglII fragment of pRJ8658 inserted into pRJ8638</td>
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<td>pRJ8681</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pUC18), derivative of pRJ8659, filled-in Clal site, resulting in a 2-bp insertion and a new NruI site</td>
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<td>pRJ8695</td>
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<td>Tc&lt;sup&gt;+&lt;/sup&gt; (pRK290S), 4.06-kb SacI fragment of pRJ8638</td>
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</tbody>
</table>

<a>a</a> The same hmuR-lacZ fusion was also integrated into the chromosome of the B. japonicum mutant strains GEM4 (fur) and LODTm5 (irr). The resulting strains were given the same number preceded by 'F' (fur mutant) or 'I' (irr mutant).

bThe hmuT-lacZ fusion was also integrated into the chromosome of the fur mutant strain GEM4. The resulting strain was given the same number preceded by 'F'.

DNA work and sequence analysis
Recombinant DNA work and Southern blotting were performed according to standard protocols (Sambrook et al., 1989). For homologous hybridization, we used digoxigenin-labeled probes generated by PCR or using elongation of random hexanucleotides with the Klenow fragment of DNA polymerase (DIG DNA Labeling Kit, Roche Diagnostics, Rotkreuz, Switzerland). B. japonicum chromosomal DNA was isolated as described (Hahn and Hennecke, 1984). For computer-assisted analyses of DNA and protein sequences, we used the software package (version 10) of the UWGCG (Genetics Computer Group of the University of Wisconsin, Madison, WI). Homology searches were performed by using the National Center for Biotechnology Information BLAST network server (http://www.ncbi.nlm.nih.gov/BLAST/).

Transcript mapping
The transcriptional start sites of hmuT and hmuR were mapped with primer extension experiments. Two 28-mers were used as primers for mapping of hmuR (hmuR-rev1, 5'-T10.888TAGCCCGCGTCAGCCATATCGCCAGCCAG-3'; hmuR-rev2, 5'-G10.927AAACCACCGACGCACCCAAAATCAAGG-3'). A 28-mer (hmuT-rev1, 5'-A10.364CCAGTCAGCAGGATCTGTGTGAGGGTG-3') and a 30-mer oligonucleotide (hmuT-rev2, 5'-G10.389TGCGGCAAATGTCATTGCGTACTTC-3') were used for hmuT mapping. Position numbers correspond to those of the sequence deposited in the GenBank database (accession no. AJ311165). RNA isolation and primer extension experiments were performed as described previously (Babst et al., 1996; Bauer et al., 1998).

Construction of B. japonicum ΔhmuT-hmuR and ΔexbD-tonB mutant strains
The hmuT and hmuR genes were simultaneously mutated by deleting a 2.55-kb NruI fragment that was replaced by a 1.2-kb SmaI fragment (aphII) from pBSL86 (mutant strains 8640 and 8646; Fig. 5.1). Similarly, the exbD and tonB genes were mutated by deleting a 960-bp NcoI fragment (mutants 8654 and 8655; Fig. 5.1). In this case, too, the deleted DNA region was replaced by a 1.2-kb SmaI fragment from pBSL15 (aphII). Appropriate DNA fragments containing the region to be mutated were cloned into derivatives of pSUP202 and mobilized into B. japonicum 110spc4 as described.
previously (Hahn and Hennecke, 1984). The correct genomic structures of all mutant strains were confirmed by Southern blot analysis of genomic DNA.

**Mutagenesis of A/T-rich cis element in the promoter region of hmuT and hmuR**

The *Clal* site located at position 10,696 in the center of the A/T-rich motif was used for the following mutagenesis procedure. Plasmid pRJ8659 was digested with *Clal*, the protruding ends were filled in with the Klenow fragment of DNA polymerase I and the plasmid was recircularized by ligation. In the resulting plasmid pRJ8681, the 2-bp insertion generated a new *NruI* site (see Fig. 5.5), which was verified. The mutated promoters of *hmuT* and *hmuR* containing the altered A/T-rich motif were designated P*.

**Construction of lacZ reporter fusions**

The transcriptional *lacZ* fusions to *hmuT* and *hmuR* were constructed by PCR amplification of the *hmuT-hmuR* intergenic region (spanning nucleotides 10,358 to 11,079) using plasmid pRJ8638 and the following primers: 8635-1F (G\_10,358ATGGTACCAGTCAGCAGGATCTGTGTGAG; a new *KpnI* site [underlined] was introduced by the nucleotide exchange marked in italics) and 8635-3R (C\_11,079ACGGGGAATTCAGCAGGCGTTGCCTGC; *EcoRI*). The 722-bp PCR product was digested with *KpnI* and *EcoRI* and subcloned into pUC18 resulting in pRJ8659; the insert was checked by sequencing. Subsequently, the *KpnI-EcoRI* fragment was excised from pRJ8659 and cloned into the identically digested vector pME8656. The resulting plasmid pRJ8661 carries a transcriptional *hmuR-lacZ* fusion. For construction of the transcriptional *hmuT-lacZ* fusion on pRJ8663, pRJ8659 was digested with *EcoRI* and *SmaI*, and the resulting 725-bp fragment cloned into *EcoRI-SmaI*-linearized pME8656. Translation of *hmuT* and *hmuR* of which the 5' ends were present on the PCR-amplified fragment initially used for this cloning strategy, is terminated in both *lacZ* fusions constructs due to the presence of in-frame stop codons located upstream of the translational start of the *lacZ* reporter gene. Appropriate DNA-fragments of pRJ8661 and pRJ8663 containing the *hmuR-lacZ* and *hmuT-lacZ* fusion, respectively, were then cloned into pSUP202pol4 and mobilized into *B. japonicum* wild type 110spc4 and GEM4. In addition, the *hmuR-lacZ* fusion was inserted into *B. japonicum* strains USDA DES122 (wild type) and LODTm5 (irr). Those clones, that contained the entire *lacZ* fusion plasmid integrated via single crossover at the homologous chromosomal position, were selected by plating the transconjugants on
tetracycline-containing plates. The genomic structures of all resulting strains were verified by PCR analysis. For construction of the P* hmuT-lacZ and P* hmuR-lacZ fusions, a similar strategy based on plasmid pRJ8681 was applied. In the final mutant strains, the presence of the mutation upstream of the hmuT-lacZ fusion (strain 8683) and the hmuR-lacZ fusion (strain 8687) was verified by PCR amplification of appropriate genomic DNA fragments followed by restriction analysis with NruI. Conversely, the wild-type promoter upstream of hmuR and hmuT in strains 8683 and 8687, respectively, was confirmed by ClaI digestion of suitable PCR-amplified promoter fragments (see Fig. 5.5).

β-Galactosidase assays

β-Galactosidase activity assays were done as described previously (Fischer et al., 1993).

Plant infection test

The symbiotic phenotypes of the B. japonicum wild type and mutant strains 8640, 8646, 8648, 8654 and 8655 was determined in infection tests using soybean (Glycine max (L.) Merr. cv. Williams) as host plant and nitrogenase activity was measured in an acetylene reduction assay (Hahn and Hennecke, 1984; Göttfert et al., 1990). Soybean seeds were kindly provided by P. M. Gresshoff (University of Queensland, Australia).

Acknowledgements

We thank Mark R. O'Brian (School of Medicine and Biomedical Sciences, Buffalo, New York, USA) for kindly providing the B. japonicum mutant strains GEM4 and LODTM5 and Andreas Nocker (ETH Zürich) for providing plasmid pRJ5616. We also thank A. Johnston (University of East Anglia, Norwich, UK) for sharing unpublished results and valuable comments on the manuscript. This work was supported by a grant from the Swiss National Foundation for Scientific Research.
Chapter 6

Supplementary experiments to elucidate the function and regulation of the heme uptake system
6.1 Expression of the *hmu* genes under symbiotic conditions and in dependence on heme availability

6.1.1 Introduction
The plant infection test indicated that the *hmu* gene cluster is not essential for symbiosis (Chapter 5). Because we did not know whether the heme uptake system is expressed in bacteroids, we attempted to measure expression levels of *hmuT* and *hmuR* in planta with the help of the available lacZ fusion constructs. Furthermore, we addressed the question whether an *hmu* mutant is able to compete with the wild type in a plant infection test.
Finally, we also were interested to learn whether heme affects *hmu* expression levels similarly as iron.

6.1.2 Results

Expression of *hmuT-lacZ* and *hmuR-lacZ* in bacteroids
A β-galactosidase assay was performed with re-isolated bacteroids (Table 6.1.1). Unlike in wild-type bacteroids lacking a reporter fusion we detected some β-galactosidase activity in bacteroids containing a *hmuT*- or *hmuR-lacZ* fusion; however, the activity levels were rather low in comparison with values observed previously with a *fixA*-′-*lacZ* and a *fixB*-′-*lacZ* fusion (Gubler and Hennecke, 1988). The latter reached approximately 150- to 400-fold higher expression levels, respectively (Gubler and Hennecke, 1988). When the measurements were repeated with bacteroids from plants grown for 28 d or 32 d, similar results were obtained (data not shown). In parallel, we also tested expression in nodules of plants that were treated with 3-(3,4-dichloro-phenyl)-1,1-dimethyl-urea (DCMU), an inhibitor that blocks photosynthesis (Takahashi and Watanabe, 1993). DCMU treatment is known to induce senescence in soybean (J. Müller, personal communication). Again, *hmu* expression levels did not differ significantly from that in untreated plants (data not shown). While nodule morphology was unaltered, DCMU treatment induced a color change of the leaves, which turned slightly yellowish and brownish.
Though β-galactosidase activities expressed as Miller Units cannot be directly compared in bacteroids and free-living *B. japonicum* cells, the low values detected in bacteroids, which are in the same range as that observed in iron-supplemented PSY medium (see Chapter 5), suggested that the *hmuT* and *hmuR* genes were not or only weakly induced under these conditions.

### Table 6.1.1 Expression of *hmuT-lacZ* and *hmuR-lacZ* in bacteroids isolated from soybean nodules 24 d after infection

<table>
<thead>
<tr>
<th>Strain used for soybean infection</th>
<th>Genotype</th>
<th>β-Galactosidase activity in Miller Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>110<em>spc</em>4 wild type</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>8664 <em>hmuR-lacZ</em></td>
<td>10.0 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>8666 <em>hmuT-lacZ</em></td>
<td>12.7 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>110<em>spc</em>4-46 <em>fixA'-lacZ</em></td>
<td>1 600*</td>
<td></td>
</tr>
<tr>
<td>110<em>spc</em>4-47 <em>fixB'-lacZ</em></td>
<td>4 801*</td>
<td></td>
</tr>
</tbody>
</table>

* For comparison, the values for strain 110*spc*4-46 and 110*spc*4-47 are shown that were published by Gubler and Hennecke (1988).

We tried to create iron-limited conditions in the plant assay by using different nutrient solutions and pH conditions. For this purpose, we had to switch to another plant cultivation system that allowed growth of soybean plants for longer than 3 weeks (Table 6.1.2; for details see Experimental procedures). As visible signs for iron limitation in the plants, we expected a change in the color of leaves (Fe-chlorosis) and reduced shoot development (Marschner, 1995). Yet, none of these alterations was observed even after prolonged plant growth of 6 weeks. We measured nitrogen fixation activity of at least 3 plants of each plant group. Nitrogen fixation activities did not differ significantly between the different plant groups (data not shown). However, the number of nodules within plants of group VI (pH 8) was 2-fold lower as compared with the plants grown with a nutrient solution of pH 6.2. The reduced nodule number was accompanied by an increase in nodule size that probably led to comparable nitrogen fixation activities.

We also examined the expression of an *hmuR-lacZ* fusion in bacteroids that were isolated from plants infected with strain 8664 (Table 6.1.2). No induction of *hmuR-lacZ* expression was observed under any plant growth regime, and the β-galactosidase activities were similarly low as in plants grown under routine conditions (Table 6.1.1).
Table 6.1.2 β-Galactosidase activity of bacteroids isolated from 25-d old soybean plants infected with B. japonicum 8664 (hmur-lacZ)

<table>
<thead>
<tr>
<th>Growth conditions of soybean plants</th>
<th>β-Galactosidase activity in Miller Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.2, Fe 107 µM</td>
<td>8.5 ± 1.1</td>
</tr>
<tr>
<td>pH 6.2, Fe 10.7 µM</td>
<td>10.6 ± 2.9</td>
</tr>
<tr>
<td>pH 6.2, Fe 1.1 µM</td>
<td>7.4 ± 1.5</td>
</tr>
<tr>
<td>pH 6.2, Fe 0.1 µM</td>
<td>7.9 ± 2.6</td>
</tr>
<tr>
<td>pH 6.2</td>
<td>7.5 ± 4.0</td>
</tr>
<tr>
<td>pH 8.0, Fe —</td>
<td>8.5 ± 0.8</td>
</tr>
<tr>
<td>pH 6.2, Fe —</td>
<td>13.9 ± 8.3</td>
</tr>
</tbody>
</table>

a Plants were cultured as described in Experimental procedures. The values for pH and Fe supplementation refer to the medium that was used for daily watering.

b Numbers are means values ± standard errors from three different bacteroid preparations, which were assayed in duplicate.

The quartz sand was initially washed with 1 N HCl.

**Competition assay with the wild type and a ΔhmuT-hmuR mutant in soybean nodules**

To determine the competitiveness of a heme uptake mutant, soybean seedlings were infected with a 1:1 mixture of B. japonicum wild type and ΔhmuT-hmuR mutant 8640. After 22, 26, 29, 33 and 38 days the ratio of the two strains in re-isolated bacteroids was determined (see Experimental procedures). No significant deviation from the assigned 1:1 ratio was observed indicating that the mutant strain had no competition defect. The same result was obtained with plants induced for senescence by treatment with DCMU.

**The effect of heme on hmuT-lacZ and hmuR-lacZ expression**

We investigated the effect of heme on hmu expression by assaying hmuT-lacZ and hmuR-lacZ expression at increasing heme concentration. Due to its hydrophobicity, heme tends to precipitate in PSY medium or to adhere to bacterial cells, which interferes with conventional β-galactosidase assays. Therefore, cells were grown on solid medium containing variable heme concentrations and used for activity assays as described in Experimental procedures. The results are shown in Fig. 6.1.1. Increasing
concentration of heme led to a comparable decrease of hmuR-lacZ and hmuT-lacZ expression. Only basal activity was measured in cells grown with \( \geq 50 \mu\text{M} \) heme.

![Graph showing expression of hmuT-lacZ and hmuR-lacZ in dependence of heme concentration.](image.png)

**Fig. 6.1.1 Expression of hmuT-lacZ and hmuR-lacZ in dependence of heme.** Colonies of the indicated strains grown for 8 or 9 days at 30°C on PSY agar plates supplemented with increasing amounts of heme were washed off and used for \( \beta \)-galactosidase activity tests as described in Experimental procedures.

### 6.1.3 Discussion

**No symbiotic role of the hmu system**

Taken together, from the results of the plant infection test described in Chapter 5 and the competition assay presented here, we conclude that the heme uptake system is fully dispensable for symbiosis under laboratory conditions. The low expression level of hmuT-lacZ and hmuR-lacZ in bacteroids indicates that bacteroids face an iron-replete environment in nodules. We asked the question whether the plant and bacteroids compete for iron under severe iron-limiting conditions. Unfortunately, we were not able to clearly induce iron starvation in the soybean plants. It is known from recent works that certain soybean cultivars are highly resistant to Fe-chlorosis by successfully scavenging iron from the soil (Fleming et al., 1984).
We speculated about a role of the heme uptake system during nodule senescence when *B. japonicum* cells might be able to exploit leghemoglobin as an iron source before re-entering the soil habitat (see Discussion Chapter 5). Again, our attempts to induce nodule senescence by treatment of the plants with DCMU were not successful. Possibly additional nitrate feeding could improve induction of nodule senescence like it has been applied to *Phaseolus vulgaris* nodules (Matamoros *et al*., 1999). In conclusion, we cannot exclude a role of the heme uptake system in senescent nodules.

**What is the role of heme in the expression of the hmu system?**

Many uptake systems for siderophores are up-regulated in the presence of the respective iron chelator. The receptor protein itself or a specific regulator sense the respective iron chelator (Crosa, 1997). It is tempting to speculate about a similar regulation mechanism for heme uptake. Indeed, expression studies with BhuR, the heme receptor from *Bordetella bronchiseptica*, indicated such a control mechanism. The production of BhuR was markedly enhanced in heme-supplemented medium (Vanderpool and Armstrong, 2001). Similarly, a two-component regulatory system controls the expression of a heme oxygenase (HmuO) via a heme-responsive mechanism in *C. diphteria* (Schmitt, 1999). We did not observe a positive effect of heme on *hmu* expression in *B. japonicum*. By contrast, high heme concentrations negatively affected *hmu* expression. Similar observations have been reported for the expression of a heme uptake system in *P. aeruginosa* (Ochsner *et al*., 2000) and in *H. influenza* (Jin *et al*., 1996). It should be noted, however, that our experiments cannot clearly discriminate between regulatory effects of heme itself and those exerted by iron which is probably released from heme in the cytoplasm.

**6.1.4 Experimental procedures**

**Media and growth conditions**

Peptone-salts-yeast extract (PSY) medium (Regensburger and Hennecke, 1983) supplemented with 0.1% L-arabinose was used for routine aerobic cultures of *B. japonicum*. Concentrations of antibiotics were as follows (µg/ml): spectinomycin, 100; kanamycin, 100, tetracycline 35. For growth on different heme concentrations, PSY
agar was used supplemented with 0.1% arabinose, 5 μM FeSO₄ and heme as indicated. Heme solutions were always prepared freshly (1mM in 50% EtOH, 0.1 N NaOH). For growth on heme agar plates, a 3-old *B. japonicum* culture was diluted and 10³ or 10² cells were plated (assuming that OD₆₀₀ = 4 x 10⁹ cells/ml).

**Strains and plasmids**
The bacterial strains and plasmids used in this part of the work are listed in Table 6.1.3.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype or properties</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>110spc₄</td>
<td>Sp⁰ wild type</td>
<td>Regensburger and Hennecke (1983)</td>
</tr>
<tr>
<td>8640</td>
<td>Sp⁰ Km⁴ hmuT::aphII::hmuR (aphII and hmuT oriented in the same direction)</td>
<td>This work</td>
</tr>
<tr>
<td>8664</td>
<td>Sp⁰ Tc⁰ hmuR-lacZ chromosomally integrated in <em>B. japonicum</em> 110spc₄</td>
<td>This work</td>
</tr>
<tr>
<td>8666</td>
<td>Sp⁰ Tc⁰ hmuT-lacZ chromosomally integrated in <em>B. japonicum</em> 110spc₄</td>
<td>This work</td>
</tr>
</tbody>
</table>

**β-Galactosidase assay with isolated bacteroids**
For the determination of β-galactosidase activity in bacteroids, we collected nodules from at least 1 plant (approximately 150-200 mg nodule fresh weight). Isolation of root nodule bacteroids and β-galactosidase assay were performed as described previously (Gubler and Hennecke, 1988; Fischer et al., 1993). At least two samples of each bacteroid preparation were measured in parallel.

For the β-galactosidase assay with *B. japonicum* cells grown on heme agar, the plates were incubated for 8 or 9 days at 30°C. Colonies were washed off and resuspended in 5 ml 0.9% NaCl. 1:5 dilutions of the cell suspension were used in a standard β-galactosidase assay.

**Plant infection tests**
Unless otherwise indicated plant infection tests were performed with soybean [Glycine max (L.) Merr. cv. Williams] as host plant and nitrogenase activity was measured in an
acetylene reduction assay (Hahn and Hennecke, 1984; Göttfert et al., 1990). DCMU [3-(3,4-dichloro-phenyl)-1,1-dimethyl-urea]-treated plants were grown for 32 d and treated with 10 ml DCMU-solution (1:2000 dilution in H$_2$O of 1% DCMU dissolved in methanol) after 18 and 25 days. DCMU was purchased from Sigma-Aldrich.

For the plant infection test under different Fe conditions, we used larger plastic pots with a perforated bottom (15 cm height and 7 cm diameter). The pots were filled with approximately 200 ml quartz sand (grain size 0.7 to 1.2 mm). The quartz sand was washed with deionized water for group I to VI except for group VII which was treated with 1 N HCl (1 volume) to remove Fe$^{3+}$ and subsequently washed several times with deionized water until the pH of the rinsing solution reached 5.5 (see Table 6.1.2). The soybean seedlings were inoculated with 1 ml of a *B. japonicum* cell suspension (OD$_{600}$ = 2). Starting one day after the infection, the plants were watered 2 times per day with 150 ml of N-free nutrient solution (Hammer et al., 1978), whose pH was adjusted to 6.2 or 8 with 0.5 N KOH. The nutrient solution was supplemented with Fe-EDTA (13% Hauert iron fertilizer) to different final iron concentrations (light: 16h, 26$^\circ$C, 80% relative humidity; dark: 8h, 22$^\circ$C, 90% relative humidity). The light period started and ended by a ramp of 8 different light intensity levels each lasting 15 min. For extended growth of more than 3 weeks, the plants were poured after the third week with tap water at intervals appropriate for preventing desiccation of the quartz sand.

**Competition assay**

A standard soybean infection test was performed with a 1:1 mixture of wild-type strain 110$spc$4 and 8640 ($\Delta$hmu$T$-hmu$R$). At the specified time points 2 nodules of 2 plants were used for bacteroid isolation. The nodules were surface-sterilized with EtOH in a sterile tube and subsequently washed with sterilized water. The nodules were crushed with blunt tweezers and the cell debris was resuspended in 1 ml 0.9% NaCl. Subsequently, 50 µl of a $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilutions, were plated in parallel on 2-3 PSY agar plates containing cycloheximide (100 µg/ml) plus either spectinomycin (100 µg/ml) for determination of total cell number or spectinomycin and kanamycin (each at 100 µg/ml) for determination of 8640 cells. The colony number was counted 12 days after incubation of the plates at 30$^\circ$C.
6.2 Evidences for a second Ton system in *B. japonicum*

6.2.1 Introduction

Our observation that the Δ*exbDtonB* mutant was not affected in the uptake of iron citrate and ferrichrome indicated the existence of a second Ton system in *B. japonicum* (see Chapter 5). In order to verify the presumed second copy of tonB and/or *exbBD*, we performed Southern blot hybridizations of *B. japonicum* chromosomal DNA under low-stringency conditions.

6.2.2 Results

The probe for *exbBD* hybridized to fragments of the *exbBD* locus as expected (Fig. 6.2.1; see also Fig. 5.1). At least one hybridizing fragment of lower intensity was detected in each lane, which indicated the additional existence of paralogous *exbBD*-like genes in *B. japonicum*. Generally, ExbB and ExbD proteins are highly conserved in bacteria. Indeed, the *B. japonicum* ExbB or ExbD proteins are more than 50% identical to their best orthologues (Table 6.2.1). Notably, two *exbBD* paralogues are present also in the genome of *M. loti* and *S. meliloti*.

Unlike in the hybridization with *exbBD*, we did not observe additional genomic regions hybridizing to the tonB probe. In contrast to ExbB and ExbD, TonB proteins show only weak similarities when compared among different bacteria. The *B. japonicum* TonB protein is less than 35% identical to its best orthologues (Fig. 6.2.2), and thus it also may display only low similarity to a (hypothetical) second TonB protein in this organism.

<table>
<thead>
<tr>
<th>Organism</th>
<th>ExbB Accession</th>
<th>ExbB ID/SMa</th>
<th>ExbD Accession</th>
<th>ExbD ID/SMa</th>
<th>TonB Accession</th>
<th>TonB ID/SMa</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. loti</em></td>
<td>mll4168b</td>
<td>59 / 64</td>
<td>mll4167b</td>
<td>61 / 69</td>
<td>mll4165b</td>
<td>33 / 41</td>
</tr>
<tr>
<td></td>
<td>mll3891b</td>
<td>40 / 49</td>
<td>mll3890b</td>
<td>40 / 50</td>
<td>mll1158b</td>
<td>32 / 41</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>Q05605</td>
<td>56 / 72</td>
<td>Q05606</td>
<td>64 / 76</td>
<td>Q05605</td>
<td>30 / 50</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>P18783</td>
<td>52 / 60</td>
<td>P18784</td>
<td>56 / 64</td>
<td>BAB35175</td>
<td>27 / 35</td>
</tr>
<tr>
<td><em>S. meliloti</em></td>
<td>SMc02085c</td>
<td>47 / 54</td>
<td>SMc02084c</td>
<td>57 / 66</td>
<td>CAC47004</td>
<td>30 / 38</td>
</tr>
<tr>
<td></td>
<td>SMc03958c</td>
<td>36 / 46</td>
<td>SMc03957c</td>
<td>39 / 47</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. leguminosarum</em></td>
<td>na</td>
<td></td>
<td>na</td>
<td></td>
<td>AJ310723</td>
<td>24 / 31</td>
</tr>
<tr>
<td><em>Pasteurella multicoda</em></td>
<td>AAK03270</td>
<td>34 / 73</td>
<td>AAK03271</td>
<td>27 / 42</td>
<td>AAC83227</td>
<td>28 / 38</td>
</tr>
</tbody>
</table>

a ID = % amino acid sequence identity; SM = % similarity  
b http://www.kazusa.or.jp/rhizobase/  
c http://sequence.toulouse.inra.fr/meliloti.html  
d na = gene not available
Fig. 6.2.1 Southern blot hybridization with chromosomal B. japonicum DNA. For hybridization under low stringency, DIG-labeled PCR probes of the complete exbBD genes (A) or of the tonB gene (B) were used. Genomic DNA was digested with the following restriction enzymes: BamHI (B), BglII (Bg), EcoRI (E), NcoI (N), SacI (S) and XhoI (X). The sizes of the marker DNA fragments that cross-hybridized with the DIG-labeled probe are indicated. Unmarked, strongly hybridizing bands correspond to the genuine exbBD (A) and tonB (B) fragments. Asterisks in panel A mark DNA fragments of additional genomic regions that hybridize with the exbBD probe.

6.2.3 Discussion

Search for the putative second tonB

The hybridization experiment yielded no evidence for a second tonB gene in B. japonicum. However, the additional DNA region, hybridizing to the exbBD probe might be useful to identify tonB2 because tonB genes are often located adjacent to exbBD gene clusters. A more global approach to search for the putative tonB2 gene could include a random transposon mutagenesis of B. japonicum and subsequent selection of mutants that fail to grow under iron-deplete conditions. Complementation of an E. coli tonB mutant with a genomic library of B. japonicum represents yet another strategy.
Once a tonB2 gene is identified, growth assays on different siderophores with mutants lacking tonB1 and/or tonB2 should reveal evidence for specific and redundant functions of the respective TonB proteins in *B. japonicum*. Eventually, analysis of the symbiotic phenotype of those mutants could reveal whether high-affinity iron uptake is of significance for symbiosis.

**TonB proteins with specificity for heme uptake**

The main question is whether TonB1 functions are restricted to heme uptake and, if so, what the determinants are for such specificity. TonB proteins with specificity for heme receptors were investigated in *V. cholerae* and in *P. aeruginosa* (Zhao and Poole, 2000; Seliger et al., 2001; see Discussion Chapter 5). Another example is HasB in *S. marcescens*, a TonB homologue that belongs to the ‘has’ (heme acquisition) gene cluster (C. Wandersman, personal communication). Very strikingly, two tonB genes have been identified also in *M. loti*. One of these paralogues (msr1158) is located in a genomic region that very much resembles the hmu cluster in *B. japonicum*. The deduced protein Msr1158 comprises only 77 amino acids that are homologous to the C-terminal part of *B. japonicum* TonB (Fig. 6.2.2). Because of the small size it is questionable whether msr1158 codes for a functional TonB protein. Interestingly, an open reading frame of 194 codons (msr1157) was identified immediately upstream of msr1158, which codes for a proline-rich protein. We speculate that the deduced proteins of msr1157 and msr1158 represent two domains of a TonB protein with a combined size similar to that of the entire *B. japonicum* TonB protein. Taken together, specialized TonB proteins are often found in the context of heme acquisition systems in different bacteria. Probably, unique structural features in the periplasmic portion of heme receptors require specific TonB proteins.

**Structure-function predictions for TonB proteins**

Based on amino acid sequence analyses, structural and functional predictions were made for TonB proteins (Postle, 1993; Moeck and Coulton, 1998; Braun et al., 1999). All TonB proteins known can be divided in 3 functional domains (Fig. 6.2.2).

i.) The N terminus of TonB is anchored in the cytoplasmic membrane by a single transmembrane domain constituted by an uncleaved signal sequence (Postle and Skare, 1988). Using the programs SignalP V1.1 (www.cbs.dtu.dk) and Predictprotein
(dodo.cpmc.columbia.edu) the first 49 amino acids of *B. japonicum* TonB were identified as a putative signal peptide including a putative α-helix (H₉ to M₃⁹). Another typical feature of TonB N termini is an SXXXH motif that was shown in *E. coli* to be essential for TonB function and to be required for the coupling of TonB to the cytoplasmic membrane electrochemical gradient (Larsen and Postle, 2001). We identified this motif (SQPEH₄⁶) in the N terminus of *B. japonicum* TonB but not in all TonB proteins compared in Fig. 6.2.2. Thus, this motif may not be absolutely required for TonB function.

ii.) All known TonB proteins have a distinct proline-rich region in common that spans the periplasm. Often, this proline-rich region contains Glu-Pro and Lys-Pro repeats, but its overall conservation is rather low. These specific repeats are particularly frequent in the *Pasteurella multocida* TonB protein whereas in *B. japonicum*, for example, this region seems to represent a random accumulation of Glu, Pro and Lys residues (Fig. 6.2.2). An *E. coli* mutant of TonB lacking the proline-rich region was still able to interact with the receptor under conditions of low osmolarity. Therefore, it was postulated that this proline-rich region does not play any role in energy transduction but rather provides a physical extension sufficient to reach the outer membrane (Larsen et al., 1993).

iii.) The C-terminal part is the most conserved region in TonB proteins and includes sites that directly contact outer membrane transport proteins (Moeck et al., 1997). *In vivo* cross-linking experiments demonstrated a direct interaction between *E. coli* TonB and the vitamin B₁₂ receptor BtuB (Cadieux and Kadner, 1999). The residues that were cross-linked to BtuB are located in a highly conserved region in enterobacterial TonB proteins, which is followed by a predicted amphiphatic α-helix (Larsen et al., 1996). Recently, the crystal structure of the C-terminal domain from *E. coli* TonB was solved (Chang et al., 2001). Thereby, the predicted amphipathic α-helix was nicely confirmed. It was postulated that the region at the beginning of this α-helix constitutes a binding cleft, which could accommodate an element of the receptor as the ligand. Notably, an α-helix is predicted also for *B. japonicum* TonB between positions V₂₃⁵ and A₂₅₀, which succeeds a conserved Tyr-Pro₂₀₃ motif. Evidently, these conserved amino acids are promising candidates for further studies of TonB-receptor interactions, e.g. by site-directed mutagenesis.
Fig. 6.2.2 Amino acid sequence comparison of the *B. japonicum* (Bj) TonB protein with its best homologues. Residues conserved in all proteins are highlighted on a solid background and represented by uppercase letters in the consensus line; residues conserved in at least six proteins are emphasized by white letters on a grey background, those conserved in five proteins by black letters on a light grey background. The proteins (accession no.) included are as follows: *M. loti* (Ml) TonB (mll4165 in http://www.kazusa.or.jp/rhizobase/), *S. marcescens* (Sm) HasB (data from C. Wandersman, Paris), *S. meliloti* (Si) TonB (CAC47004), *P. putida* (Pu) TonB (Q05605), *Pasteurella multicauda* (Pm) TonB (AAC83227), *R. leguminosarum* bv. *viciae* (Rl) TonB (CAC34389), *M. loti* (Ml*) TonB (msrl 158 in http://www.kazusa.or.jp/rhizobase/). The horizontal bar indicates the proline-rich domain with a high content of the amino acids P, K and E. An open box points to an SXXXH motif that might be involved in ExbBD interaction. For details see text.
6.2.4 Experimental procedures

Southern blot hybridization was performed according to standard protocols (Sambrook et al., 1989). Low-stringency conditions were applied by hybridization at 58°C in 5x SSC. The *B. japonicum* exbBD and tonB probes were PCR-amplified from plasmid pRJ8633 (Table 5.4). For synthesis of the exbBD probe, the following oligonucleotides were used: Exbfor (5'-CAAGTCCAGGCTTCGGCAC14237-3'; numbering of nucleotides refers to accession no. AJ311165) and Exbrev (5'-GACCAGGGCGACCTTCAAGTAG15395-3'), which resulted in a PCR fragment of 1194 bp. For synthesis of the tonB probe, primers: TonBfor (5'-GCCTTTGCCCTGCACGAG15466-3') and TonBrev (5'-TACCTCACCCTAACTCCACCG16245-3') were used, which resulted in a PCR product of 817 bp. The purified DNA fragments were DIG (digoxigenin-11-dUTP)-labeled by nick translation using random hexameric primers according to standard protocols (Roche Diagnostics, Rotkreuz, CH). About 100 to 300 ng of labeled DNA were used in individual hybridization experiments.
Chapter 6.3

6.3 Gel retardation experiments with the ICE (iron control element) and the hmu promoter region

6.3.1 Introduction
With a gel retardation assay using B. japonicum crude extracts, we attempted to examine whether the cis-acting iron control element (ICE) of the hmu promoter region represents a binding site for a regulatory protein. Foremost, we addressed the question whether the iron response regulator Irr is able to bind specifically to ICE. As reported in Chapter 5, hmuR-lacZ regulation by iron was retained in an irr mutant background; yet the maximal expression level was reduced to 50-75% as compared to the wild type. This finding left it open whether Irr interacts directly with the hmuR promoter or whether it exerts its regulatory role via an indirect mechanism.

6.3.2 Results

Binding of purified Irr to the A/T-rich iron control element
Using 5 µM Irr protein and a 35-bp DNA fragment (HmuR5) comprising the A/T-rich ICE in a gel retardation experiments, we observed a protein-dependent retardation in the band shift experiment (Fig. 6.3.1, lane 3 and 4). Possibly due to distinct oligomeric forms of Irr, we detected a total of three retarded bands (Fig 6.3.1). As a control for the specificity of DNA binding by Irr, we used a heterologous, 36-bp DNA fragment (FRPwt) with a (G+C):(A+T) ratio of 1:1. The FRPwt fragment originates from the fixR promoter region and was used earlier in our laboratory for binding studies with RegR (Emmerich et al., 2000). Irr also bound to FRPwt, but probably weaker because poly(dIdC) was more effective in competing with FRPwt than with HmuR5 (compare lanes 4 and 5 in Fig. 6.3.1). Binding of Irr to either labeled fragment was drastically reduced by an excess of unlabeled DNA (lanes 2 and 7). In order to compare specific and unspecific DNA binding of Irr, competitive gel retardation experiments were performed by using unlabeled homologous (HmuR5) or heterologous DNA (FRPwt or poly(dIdC)) in addition to radiolabeled HmuR5. Homologous competition reduced binding of Irr to radiolabeled HmuR5 significantly more than competition with heterologous DNA (Fig. 6.3.2). The relative binding efficiencies were calculated as described in the legend of Fig. 6.3.2. The calculation was done separately for the upper
and the lower complex. The results are displayed in the graphs below corresponding gels (Fig. 6.3.2). Compared to the homologous competition with HmuR5, more than 10-fold and 100-fold amounts of FRPwt and poly(dIdC), respectively, were required to decrease binding efficiency by 50%. This behavior was observed with both the lower and the upper complex.

**Fig. 6.3.1 Gel retardation experiments with purified *B. japonicum* Irr.** DNA-binding reactions were performed with a DNA fragment (HmuR5) of 35 bp derived from the *hmu* promoter region (A) or with a 36-bp DNA fragment (FRPwt) originating from the *fixR* promoter region, which served as a heterologous control (B). Binding of Irr to radiolabeled HmuR5 or FRPwt was competed with either poly(dIdC) or the respective unlabeled DNA fragment as indicated at the top. 330 ng unlabeled DNA corresponds to approximately 700-fold excess of labeled DNA.
Chapter 6.3

A

HmuR5

FRPwt

unlabeled DNA

Irr-DNA complex

Free DNA

Quantification of complex u

Quantification of complex l

B

HmuR5

poly(dIdC)

28.1 5.6 1.1 0.2 0.05 0.5 2 11 56 281 ngDNA

poly(dIdC) HmuR5

Irr-DNA complex

Quantification of complex u

Quantification of complex l
**Fig. 6.3.2 Specificity of the Irr-HmuR5 binding.** The interaction between the radiolabeled HmuR5 fragment and Irr (1.5 μM) was challenged by variable amounts of different competing DNAs: unlabeled HmuR5 and FRPwt (A) or HmuR5 and poly(dIdC) (B). The graphs below the gels show the quantification of the competition experiments. The amount of unlabeled competing DNA ranges from 0.05 to 28 ng (A) or is indicated above each lane in (B). Quantification was done separately for both the upper complex (u) and the lower complex (l). Rectangles labeled with A, B or C indicate exemplarily the area that was integrated for quantification of the band shifts in lane 4. Binding activity was calculated as the ratio of radioactivity in B / radioactivity in A for the upper band or area C/A for the lower band, respectively. For calculation of relative values, binding activities of individual complexes were normalized to those of the corresponding complexes in lane 6, which were defined as 100%.

Gel retardation assay with crude extracts from *B. japonicum* and ICE

Crude extract of *B. japonicum* cells was prepared from cultures grown under iron-limited or iron-replete conditions and tested in a gel retardation assay with radiolabeled HmuR5. We detected two distinct retarded complexes when using crude extracts from wild-type cells. Different iron conditions used for cultivation of the cells had no effect on the pattern of retarded bands (Fig. 6.3.3). Unexpectedly, the same complexes were observed using the control DNA, FRPwt (Fig. 6.3.3, lane 12). Thus, we concluded that the interaction between HmuR5 and the unknown component in the *B. japonicum* crude extract was not specific. In parallel, we also tested crude extracts prepared from the *fur* mutant GEM4, the *irr* mutant LODTm5 and the respective wild-type strain USDA122DES. The gel retardation patterns observed with these extracts resembled that obtained with the wild type 110spc4 (data not shown).

Instead of HmuR5, we further used a 200-bp fragment (Hmu6/7) that spans the promoter region between *hmUT* and *hmUR*. There might be further factors required for the acitvation of *hmU* expression, which bind outside the ICE locus. However, the preliminary results of a gel retardation experiment did not allow any conclusions because observed Hmu6/7 complexes might be similarly unspecific as presumed for the complexes revealed with crude extracts and HmuR5 (data not shown).
Fig. 6.3.3 Gel retardation experiments with crude extract from wild-type *B. japonicum*. DNA-binding reactions were performed with radiolabeled, 35-bp DNA fragment HmuR5 (lanes 1–11) and for control, with FRPwt (lane 12). Crude extract was prepared from *B. japonicum* 110spec4 (wild type) grown under the iron conditions indicated. Applied protein amounts are indicated above the lanes.

### 6.3.3 Discussion

The gel retardation experiments revealed that Irr binds to the ICE element with distinct specificity. In conclusion, we cannot exclude any direct involvement of this regulator in *hmu* expression, although it remains difficult to correlate binding specificity of Irr with its requirement for *hmu* regulation. The retained induction of *hmu* genes in the *irr* mutant grown under low-iron conditions rules out a strict dependence on Irr. Also, alteration of ICE has a more pronounced effect on *hmu* activation than the absence of Irr (Chapter 5). Notwithstanding this fact, it is tempting to speculate about a contribution of Irr in the activation of heme uptake genes given the following particularities of this regulatory protein in iron homeostasis: i.) Next to its function as a repressor Irr is able to mediate positive regulation. The phenotype of an *irr* mutant led to the assumption that Irr activates an uptake system for iron citrate via a positive regulation mechanism (Hamza *et al.*, 1998). In contrast, the Fur protein, which directly senses iron, regulates iron uptake in a negative manner.
ii.) Irr regulates the intra cellular heme content. The best characterized role of Irr is its function in heme biosynthesis. Irr negatively regulates hemB, encoding the δ-aminolevulinic acid dehydratase, and thereby coordinates the synthesis of protoporphyrins with iron availability (Hamza et al., 1998).

iii.) Heme is an effector of the Irr protein. The primary control of Irr levels is mediated by the cellular heme content. Recently, Qi and coworkers (1999) have proven that degradation of Irr involves direct binding of heme to the protein by the heme regulatory motif (HRM). In conclusion, Irr is definitely playing an important role in heme homeostasis; yet, its contribution to the activation of the heme uptake system hmu in B. japonicum remains unclear.

The question arises whether Irr and Fur can replace each other. Our attempts to construct an irr mutation in the fur mutant background failed. Although Irr and Fur are structurally similar they are not functional homologues because respective mutant strains have distinct phenotypes and they can act independently of each other (Hamza et al., 2000). Yet, they are linked at the regulatory and the functional level. First, this is documented by Fur-dependent regulation of irr. Fur represses irr expression by directly binding to the irr promoter region under iron-replete conditions. Secondly, Fur regulates hemA, the gene encoding δ-aminolevulinic acid synthase (Hamza et al., 2000), and thus not only Irr but also Fur coordinates heme biosynthesis with iron availability. To date, very little is known about DNA binding specificity of Fur in B. japonicum. The binding site of Fur in the irr promoter region is dissimilar to the E. coli Fur consensus box, although B. japonicum Fur is able to bind to the Fur box of E. coli (Hamza et al., 2000). No information is available about Irr binding sites. Thus, without more precise knowledge about the sequence determinants required for DNA binding by Fur and Irr, it is not possible to evaluate whether or not ICE represents a target for any of these regulators.

Alternatively, one has to consider the involvement of another regulator in hmu regulation. Unfortunately, gel retardation experiments using crude extract from B. japonicum were not suitable to detect a specific interaction between the ICE and the presumed regulatory protein. Obviously, alternative methods are required for identifying the putative factor interacting with ICE (see Chapter 7).
6.3.4 Experimental procedures

Strains and plasmids

The bacterial strains and plasmids used in this part are listed in Table 6.3.1.

Table 6.3.1 Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or properties</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td>B. japonicum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>110spc4</td>
<td>Sp' wild type</td>
<td>Regensburger and Hennecke (1983)</td>
</tr>
<tr>
<td>USDA DES122</td>
<td>wild type</td>
<td>O'Brian et al. (1987)</td>
</tr>
<tr>
<td>GEM4</td>
<td>Sp' fur::Ω parental strain USDA 1110</td>
<td>Hamza et al. (1999)</td>
</tr>
<tr>
<td>LODTM5</td>
<td>Km' Sm' irr::Tn5 in strain LO</td>
<td>Hamza et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>(LO = spontaneously nalidixic acid resistant derivative of USDA DES122)</td>
<td></td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRJ8638</td>
<td>Ap' (pUC18) 6.9-kb EcoRI-BamHI insert with hmuT', hmuR, orf110, orf167, exbBD and tonB</td>
<td>This work</td>
</tr>
</tbody>
</table>

Gel retardation assay

The DNA-binding activity of Irr was tested in band shift experiments using the 35-bp DNA fragment HmuR5. Irr protein was kindly provided by M. O'Brian (State University of NY at Buffalo; Qi et al., 1999). Unlabeled DNA or poly(dIdC) was mixed with purified Irr protein in DNA-binding buffer (Stratagene) as indicated. The labeled fragment (25 000 c.p.m.; approximately 10–20 fmoles) was added in a final volume of 25 μl. The DNA-protein samples were incubated for 5 min at room temperature, mixed with 5 μl loading dye (30% glycerol, 0.02% bromphenol blue in water) and loaded onto a 6% non-denaturing polyacrylamide gels (cross-linker ratio 29:1; gels prepared with 1x TBE buffer (89 mM Trisbase, 89 mM boric acid, 2.5 mM EDTA; pH 8) containing 1 mg/ml Triton X-100). Gels were run in 1x TBE buffer at 4°C, dried under vacuum and exposed on a phosphoimager screen. Signal intensities of free DNA and retarded bands were quantified with a phosphoimager and the program IMAGEQUANT (version 3.3, Molecular Dynamics, Sunnyvale, CA, USA). Relative binding activities were calculated as described in the legend of Fig. 6.3.2. Gel retardation experiments with crude extract were performed similarly with the following modifications: each reaction contained 1.5 μg poly(dIdC) in a final volume of 50 μl.
DNA fragments used in gel retardation experiments

FRPwt was described previously by Emmerich et al. (2000) and was prepared similarly as HmuR5. HmuR5 was generated by annealing of two single-stranded oligonucleotide: HmuR-F5 (5'-cgccggaAATTTACAATCGATATAAACTgcaacca-3') and HmuR-R5 (5'-tggttgcAGTTTATATCGATTGTAATTTcgcg-3'); capital letters refer to the 21-bp ICE of the hmu promoter region. The annealing was performed in a thermo cycler using the following conditions: 10' 95°C, 10' 90°C, 10' 85°C, 30' 80°C, 1h 75°C, 1h 70°C, 1h 65°C, 1h 60°C, 1h 55°C, 1h 50°C, 1h 45°C, 4°C. The double-stranded oligonucleotides HmuR5 and FRPwt were endlabeled with [γ-32P]ATP using T4 polynucleotide kinase (MBI Fermentas) followed by purification over a NAP-10 (Sephadex G-25) column (Amersham Pharmacia Biotech, UK). For PCR amplification of the 200-bp Hmu6/7 fragment, we used the primers Hmu-rev7 (5'-GCAAGGCGAACGACTGGC10796-3'; the nucleotide position refers to accession AJ311165) and Hmu-for6 (5'-CCGTGTGATGCGCCTTCGi063o-3') and plasmid pRJ8638 as template. PCR reactions were done in the presence of 10% DMSO in a volume of 100 µl (cycling conditions: 30'' 95°C, 30'' 53°C, 50'' 75°C). The Hmu6/7 fragment (500 ng) was end labeled with [γ-32P]ATP and purified by phenol extraction.

Preparation of crude extracts

*B. japonicum* cultures were grown aerobically in 100 ml PSY medium for at least 40 h to an OD<sub>600</sub> of approximately 1. For iron-limited growth we used PSY (Regensburger and Hennecke, 1983) supplemented with 0.5 µM FeSO<sub>4</sub> (see Chapter 5.5), for iron-replete conditions 50 µM FeSO<sub>4</sub> were added. Cells were harvested by centrifugation and washed in 4°C cold French press buffer (50 mM Tris-HCl, pH 8; 1 mM EDTA; 1 mM phenylmethylsulfonylfluorid; 2 mM DTT; 25 mM MgCl₂; 100 mM KCl). Cells were resuspended in 3 ml buffer and passed three times through a cooled French pressure cell at 16 000 psi. Cell debris were separated by centrifugation at 10 500 rpm for 30 min at 4°C. The supernatant was subjected to ultra centrifugation at 35 000 rpm for 60 min at 4°C. The supernatant was termed crude extract and stored at −20°C with 10% glycerol added. Protein concentration of crude extracts was determined with the Bradford assay (BioRad, Glattbrugg, CH).
Chapter 7

Future perspectives

In this chapter, information about heme uptake systems in various bacteria is summarized with the aim to deduce strategies for the further functional and regulatory characterization of the heme uptake system in *B. japonicum*. 
7.1 How do Hmu proteins contribute to iron acquisition?

**Binding and transport of heme by HmuR**

While a wealth of genetic data is available for bacterial heme receptors, relatively little is known at the biochemical level about the receptor-heme interaction. The best-studied heme receptor is HemR of *Y. enterocolitica* (Bracken *et al.*, 1999). The ability of HemR to bind to heme or hemoglobin was assessed by affinity purification using heme- and hemoglobin-agarose. Analogous experiments could be performed with outer membrane proteins of *B. japonicum* wild-type and *hmuR* mutant cells to obtain evidence for binding of heme to HmuR.

Although the phenotype of the *hmuR* mutant points to a function of HmuR in heme acquisition (Chapter 5), direct transport of heme by HmuR has not been demonstrated. To do so, uptake studies with $^{14}$C-hemin could be performed as it was described by Stojiljkovic and Hantke (1994) for *Y. enterocolitica*. An alternative method to study heme uptake by outer membrane receptors was described by Bracken and colleagues (1999) who used small heme-peptide conjugates with peroxidase activity. These microperoxidases inhibit growth upon entering the cell. By using microperoxidases in growth assays, the uptake of heme via HemR of *Y. enterocolitica* was confirmed. Furthermore, this assay was used for the analysis of HemR mutant variants.

With the aim to investigate *B. japonicum* HmuR functions, specific *hmuR* mutations are required. Attractive targets for site-directed mutagenesis are amino acids of the RYDXY motif or the FRAP/NPLN motif (see Fig. 5.6; Wandersman and Stojiljkovic, 2000), which may be surface exposed and involved in heme binding.

Rhizobial heme receptors, including HmuR of *B. japonicum*, show maximal similarity to receptors that interact with a HasA-like hemophore (Chapter 4), including the HasR from *S. marcescens* and *P. aeruginosa* (see Fig. 5.6). Although the *B. japonicum hmu* gene cluster does probably not include a gene for a hemophore-like protein, and no HasA homologue was annotated in the genome sequence of *S. meliloti* or *M. loti*, we cannot exclude its existence in *B. japonicum*. Further information about the presence or absence of a HasA-like protein could be obtained from Western blot analysis with supernatant of a *B. japonicum* culture grown under iron–limited conditions. A polyclonal antibody against the HasA protein from *S. marcescens* was made available to us by C. Wandersman (Institute Pasteur, Paris).
Chapter 7

The function of HmuTUV

The HmuTUV transport system is not absolutely required for heme acquisition in *B. japonicum* (Chapter 5) and we concluded either that heme can be transported via an alternative transport system in *B. japonicum* (Chapter 5) or passively diffuses through the inner membrane due to its pronounced hydrophobicity (Light and Olson, 1990). In *Y. enterocolitica*, HemU and HemV enhanced iron-limited growth at low heme concentration but HemT, the periplasmic heme-binding protein, was not essential for growth under these conditions (Stojiljkovic and Hantke, 1994). Thus, subtle defects of the *B. japonicum* hmuTUV mutant studied in this work may have escaped our attention.

Similar to the distribution of heme receptor genes, hmuTUV-like genes have been described in numerous bacteria including the Gram-positive species *C. diphtheriae* (Drazek et al., 2000). Most of the respective gene products were assigned to function in heme transport on the basis of their similarity to HemTUV of *Y. enterocolitica* and mutant analysis. For testing the heme binding ability of *B. japonicum* HmuT, the protein could be overexpressed (e.g. as a polyhistidine-tagged variant) and used for binding studies with hemin agarose. Furthermore, interactions of hemin with purified HmuT could be studied photometrically, similarly as it was performed with the Irr protein of *B. japonicum* (Qi et al., 1999). The best conserved region of HmuT includes four residues that are conserved not only in heme-binding proteins but also in other iron-complex-binding proteins (Fig. 7.1; Stojiljkovic and Hantke, 1994). In general, heme proteins use a variety of axial ligands including histidine, tyrosine and cysteine (Poulos, 1996). Recently, a novel mode of heme binding was proposed for HbpA, the periplasmic heme-binding protein of *H. influenzae*. A structure of HbpA has been modeled by computational comparison with the periplasmic binding protein for dipeptides in *E. coli* (Dunten and Mowbray, 1995). Strikingly, the proposed heme binding sites do not include axial ligands. It was proposed that the propionic acid moiety of heme would form hydrogen bonds with Tyr, Ser, and with the side-chain amide nitrogen of Gln and Arg. Additional non-polar residues provide van der Waals interactions with the rest of the heme group. Interestingly, the highly conserved amino acids in HmuT include such amino acids (Fig. 7.1).
Fig. 7.1 Amino acid sequence comparison of the B. japonicum HmuT protein and its best homologues. Residues conserved in all proteins are highlighted on a solid background and represented by uppercase letters in the consensus line; residues conserved in six proteins are emphasized by white letters on a grey background, those conserved in five proteins by black letters on a light grey background. The proteins included are as follows: R. leguminosarum bv. viciae (Rl) HmuT (CAC34393.1), M. loti (Ml) Mill 151 (BAB48590.1), S. meliloti (Sm) HmuT (SMc01512), V. cholerae (Vc) HutB (AAB94547), P. aeruginosa PhuT (AAC13287), Y. pestis (Yp) HmuT (AAC64868) and Y. enterocolitica HemT (CAA54866). The residues marked with a dot are also conserved in other iron-complex binding proteins (Stojiljkovic and Hantke, 1994).
Proteins involved in heme degradation and iron acquisition

Little is known about the fate of the heme molecule after it has entered the cytoplasm. It is clear that some sort of heme degradation must occur prior to its use as an iron source. Stojiljkovic and Hantke (1994) have proposed that the Y. enterocolitica cytoplasmic protein HemS degrades heme because it prevents the accumulation of heme to toxic levels when it is co-expressed with the heme receptor protein HemR in E. coli. Homologues of HemS have been detected in P. aeruginosa (PhuS), S. dysenteriae (ShuS), Y. pestis (HmuS), and B. pertussis (BhuS). Recently, bacterial heme oxygenases have been identified: HmuO in C. diphtheriae (Schmitt, 1997), and HemO in N. meningitidis (Zhu et al., 2000). The latter was biochemically characterized and shown to be involved in the oxidative degradation of heme. HemO cleaves the porphyrin ring resulting in the release of CO, iron and biliverdin. The hemO gene is located upstream of the heme receptor gene hmbR in N. meningitides, and mutant analysis showed that hemO is required for the release of iron from acquired heme.

Although the B. japonicum hmu gene cluster does probably not encode a heme oxygenase, one has to postulate that B. japonicum possesses at least one mechanism to acquire iron from heme. Recently, an hmuO gene (AF182374), coding for a putative heme oxygenase, has been identified in the photosynthetic B. japonicum strain ORS278 next to the puf operon. The puf genes code for components of the light-harvesting complex and the reaction center. Mutant analysis indicated that hmuO in B. japonicum is not required for photosynthesis or symbiosis; unfortunately, the hmuO mutant was not investigated with respect to heme acquisition (Giraud et al., 2000).

An alternative mechanism for the removal of iron from heme has been suggested for H. influenzae (Loeb, 1995). It includes reverse ferrochelatase activity leading to the release of iron from protoporphyrin IX. Indications for reverse ferrochelatase activity in B. japonicum could be obtained from the examination of a hemA-hemH double mutant. A hemA-hemH mutant is unable to synthesize protoporphyrin and requires heme for growth. If the ferrochelatase HemH of B. japonicum were a prerequisite for the use of heme as an iron source, the mutant would presumably fail to grow on heme under iron-limited conditions.

In addition to the known bacterial strategies for heme iron acquisition, one also might think about novel mechanisms. Although highly speculative, one may consider a function of the products of orf110 or orf167 in heme iron acquisition (see Discussion in
Chapter 3). To test this hypothesis, non-polar mutations should be introduced into orf110 or orf167.

7.2 How is the hmu system regulated by iron?

Possible mechanisms of positive regulation

In many bacteria, iron uptake systems, although commonly regulated by Fur, underlie additional regulation by system-specific control mechanisms. Known positive regulators of iron acquisition systems belong to three mechanistic classes (reviewed by Crosa, 1997): (i) alternative sigma factors, exemplified by E. coli FecI involved in ferric citrate utilization (Kim et al., 1997), (ii) classical two-component regulatory systems such as the PfeR-PfeS system of P. aeruginosa controlling enterobactin utilization (Dean and Poole, 1993), and (iii) AraC-like transcriptional regulators like YbtA of Y. pestis which is required for maximal expression of genes encoding the Psn yersiniabactin system (Fetherston et al., 1996). Common to the regulators of all three classes is their activation upon sensing of the respective siderophore followed by transcriptional activation of target genes.

Unlike for siderophore systems, positive control of heme acquisition systems has not been described to date. Most recently, however, a homologue of FecI, named HasI, was found to be encoded in the has operon of S. marcescens. Preliminary results indicated that overexpression of HasI resulted in a 10-fold increase of hasR expression (C. Wandersman, personal communication).

In principle, the hypothetical regulator acting via the ICE motif of the B. japonicum hmu divergon could belong to any class of the above-mentioned regulatory proteins. Yet, an iron-regulated σ factor seems very unlikely because of the location of ICE relative to the transcriptional start of hmuT and hmuR, which suggest the involvement of a transcriptional activator. Notably, orf319 located downstream of hmuV encodes an AraC-type regulator which may be worthwhile to be tested for its potential role in hmu regulation.
Methods to identify the presumed regulator protein

Regardless of the type of regulation mechanism, different strategies are conceivable for the identification of a \textit{B. japonicum} protein that presumably binds to ICE and activates \textit{hmu} expression. Our attempts to demonstrate specific binding of a protein with crude extracts failed (Chapter 6.3), possibly due to unfavourable \textit{in vitro} conditions or limiting abundance or low binding activity of the ICE-binding protein. A more sensitive approach could be provided by a so-called "one-hybrid-system" which is based on \textit{in vivo} protein-DNA interactions (Wei \textit{et al.}, 1999). Alternatively, one may try to activate e.g. an \textit{hmuR-lacZ} reporter fusion in \textit{E. coli} by transforming a suitable strain with a genomic \textit{B. japonicum} expression library. Obviously, this approach depends on the functional interaction of the regulatory protein of interest with the \textit{E. coli} transcription machinery.
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