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Genetic regulation of environmental stress responses in *Bradyrhizobium japonicum*

A. Heat shock regulatory network

B. Genes involved in phosphate metabolism

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Table of contents

Abstract

This work presents six genes of *Bradyrhizobium japonicum*, the nitrogen-fixing root-nodule symbiont of soybean, whose products are involved in cellular strategies to cope with changing environmental conditions as different as heat shock, phosphate starvation or symbiosis.

Part A of this work presents a general overview of bacterial heat shock gene regulation, and a detailed description of the complex regulatory network in *B. japonicum* that controls the cellular response to a sudden temperature upshift. To further elucidate this network, the four heat-inducible genes *dnaKJ*, *grpE* and *hrcA* were analysed is detail. The chaperones encoded by the *dnaKJ* operon and the *grpE* gene form the so-called DnaK chaperone machinery, which is involved in intracellular protein folding and translocation, oligomeric protein assembly and protein degradation. *In vivo* and *in vitro* transcript mapping demonstrated that both gene loci are transcribed from strongly heat-inducible promoters, which are specifically recognised by the heat shock sigma factor RpoH (σ^{32}). However, a significant level of DnaK is also produced at normal growth temperatures. All attempts to knock out *dnaK* or *grpE* by mutation failed. By contrast, *dnaJ* null mutants were easily obtained.

The *B. japonicum hrcA* gene codes for a protein, which has been identified in several organisms as a repressor that controls the expression of heat shock genes by binding to a DNA element called CIRCE. *In vivo* and *in vitro* transcript mapping indicated that *B. japonicum hrcA* itself is transcribed from an RpoH-dependent promoter. The supposed negative regulatory function of HrcA was confirmed *in vivo* in an *hrcA* mutant, which showed an increased transcription of the CIRCE-associated chaperonin operons *groESL*$_4$ and *groESL*$_5$ as well as elevated β-galactosidase activity derived from corresponding *groEL-lacZ* fusions even at physiological temperatures. Specific binding of HrcA to the CIRCE element was confirmed *in vitro* by gel retardation experiments using a histidine-tagged version of the protein purified under non-denaturing conditions. The DNA binding activity was not improved by the GroE or the DnaK chaperone machinery. Rather, the addition of GroESL led to a reduction of HrcA-CIRCE complexes *in vitro*. This result is in apparent conflict with *in vivo* data, which suggested a positive effect of GroESL on HrcA activity.

Part B of this work introduces two *B. japonicum* genes, *phoB* and *pmtA*, whose products are involved in the phosphate metabolism and which were shown to be necessary under phosphate limited conditions and during symbiosis, respectively.

The *phoB* gene codes for the central phosphate regulatory protein PhoB, which induces the expression of the so-called *pho* regulon under low phosphate conditions. The fortuitously cloned *phoB* gene of *B. japonicum* was identified on the basis of the pronounced similarity of the deduced protein to the PhoB protein of *Sinorhizobium meliloti*, *Escherichia coli* and other bacterial species. Mutation of *B. japonicum phoB* led to impaired growth of the mutant strain in media containing low phosphate concentrations, but it had no effect on the symbiotic interaction of the mutant with the soybean host plant.

The *B. japonicum pmtA* gene is located downstream of the heat shock operon *dnaKJ*. Its corresponding gene product was identified as a phospholipid *N*-methyltransferase, which is involved in the synthesis of phosphatidylcholine, the major membrane component in rhizobia. *PmtA* mutants show slightly reduced growth rates in rich media under aerobic and microaerobic conditions. Interestingly, this growth defect was alleviated by the addition of choline to the medium suggesting the presence of an alternative, choline-dependent pathway of phosphatidylcholine biosynthesis. Soybean root nodules of plants infected with *B. japonicum pmtA* mutants showed a strongly reduced nitrogen fixation activity, which is most probably caused by the reduced number of bacteroids within infected plant cells. Accordingly, the *pmtA* gene product seems to be necessary for a functional symbiosis between *B. japonicum* and soybean.

Kurzfassung

Die vorliegende Arbeit beschreibt sechs verschiedene Gene von *Bradyrhizobium japonicum*, dem Stickstoff-fixierenden Wurzelknöllchen-Symbionten der Sojabohne, deren Genprodukte für die Anpassung der Zelle an so unterschiedliche Umweltbedingungen wie Hitzeschock, Phosphatmangel oder Symbiose von Bedeutung sind.

Der **erste Teil** dieser Arbeit vermittelt einen generellen Überblick über die Regulation von bakteriellen Hitzeschock-Genen und geht anschliessend detailliert auf das komplexe Regulationsnetzwerk von *B. japonicum* ein, welches die Reaktion der Zelle auf einen plötzlichen Temperaturanstieg kontrolliert. Als Beitrag zur Aufklärung dieses Netzwerkes wurden die vier hitzeinduzierbaren Gene *dnaKJ*, *grpE* und *hrcA* genauer untersucht. Die drei Chaperone, die vom *dnaKJ* Operon resp. dem *grpE* Gen kodiert werden, bilden zusammen die sogenannte 'DnaK Chaperon-Maschine'. Dieser Proteinkomplex spielt eine wichtige Rolle in der Proteinfaltung und dem Proteintransport innerhalb der Zelle, bei der Assemblierung von oligomeren Proteinkomplexen, sowie beim Abbau von Proteinen. *In vivo* und *in vitro* Transkriptionsanalysen ergaben, dass sowohl *dnaKJ* als auch *grpE* von einem stark hitzeinduzierbaren Promotor aus transkribiert werden, welcher spezifisch vom Hitzeschock-Sigmafaktor RpoH (σ^{32}) erkannt wird. Eine signifikante Menge von DnaK wird allerdings auch bei normalen Wachstumstemperaturen synthetisiert. Alle Versuche, *dnaK* oder *grpE* durch Mutationen auszuschalten, schlugen fehl. Eine Nullmutation konnte dagegen problemlos in *dnaJ* eingeführt werden.

Das *hrcA* Gen von *B. japonicum* kodiert für ein Protein, welches bereits in mehreren Organismen als Repressor identifiziert wurde. Dieses Protein kontrolliert die Expression von Hitzeschock-Genen durch Bindung an ein DNA Element (CIRCE), welches sich in der Promoterregion dieser Gene befindet. *In vivo* und *in vitro* Transkriptionsanalysen von *B. japonicum hrcA* ergaben, dass dieses Gen ausgehend von einem RpoH-abhängigen Promotor transkribiert wird. Die vorausgesagte Funktion von HrcA als negativer Regulator wurde in einer *hrcA* Mutante überprüft. Dieser Bakterienstamm zeigte bei normalen Wachstumstemperaturen eine verstärkte Transkription der beiden CIRCE-abhängigen Operons *groESL*$_4$ und *groESL*$_5$ sowie eine

erhöhte β-Galactosidase Aktivität aufgrund der verstärkten Expression der entsprechenden *groEL-lacZ* Fusionen. Die spezifische Bindung von HrcA an das CIRCE Element wurde *in vitro* in Gelretardationsexperimenten nachgewiesen. Dabei wurde eine mit einem Polyhistidin-Schwanz (His_6-Tag) versehene Variante des HrcA Proteins verwendet, die bei nicht-denaturierenden Bedingungen gereinigt wurde. Die gefundene DNA-Bindungsaktivität von HrcA konnte durch Zugabe der Chaperone GroESL oder DnaKJ nicht verbessert werden. Im Gegenteil, die Zugabe von GroESL führte vielmehr zu einer Verminderung der HrcA-CIRCE Komplexen im *in vitro* Experiment. Dieser Befund steht im Moment im Widerspruch zu den *in vivo* Daten, welche einen positiven Effekt von GroESL auf HrcA zeigen.

Der **zweite Teil** dieser Arbeit stellt die beiden *B. japonicum* Gene *phoB* und *pmtA* vor, deren Genprodukte am Phosphatmetabolismus beteiligt sind und eine wichtige Rolle bei Phosphatmangel resp. in der Symbiose spielen.

Das *phoB* Gen kodiert für das zentrale Regulationsprotein PhoB, welches bei tiefen Phosphatkonzentrationen die Expression des sogenannten *pho* Regulons aktiviert. Das zufälligerweise klonierte *phoB* Gen von *B. japonicum* konnte aufgrund der grossen Ähnlichkeit des davon abgeleiteten Proteins mit den Regulator PhoB von *Sinorhizobium meliloti*, *Escherichia coli* und anderen Bakterienarten identifiziert werden. Die Mutation von *B. japonicum phoB* führte zu einem verlangsamten Wachstum der Bakterien bei Phosphatmangel, hatte aber keinen Einfluss auf deren symbiotisches Verhalten.

Das *pmtA* Gen von *B. japonicum* befinden sich unmittelbar unterhalb der beiden Hitzeschock-Gene *dnaKJ*. Das davon abgeleitete Protein (PmtA) wurde als Phospholipid *N*-Methyltransferase identifiziert. Dieses Enzym ist an der Biosynthese von Phosphatidylcholin beteiligt, dem wichtigsten Baustein der Zellmembran von Rhizobien. Mutationen im *pmtA* Gen verursachen ein reduziertes Wachstum der Zellen sowohl unter aeroben als auch unter mikroaeroben Bedingungen. Interessanterweise konnte dieser Wachstumsdefekt durch Zugabe von Cholin ins Nährmedium ausgeglichen werden, was auf einen alternativen, Cholin-abhängigen Biosyntheseweg zur Herstellung von Phosphatidycholin schliessen lässt. Wurzelknöllchen von Sojabohnen, die mit *B. japonicum pmtA* Mutanten infiziert wurden, zeigten eine

erheblich reduzierte Stickstofffixierungsaktivität, was sehr wahrscheinlich auf die geringere Anzahl Bakteroide in den infizierten Pflanzenzellen zurückzuführen ist. Aus dieser Beobachtung lässt sich schliessen, dass das durch *pmtA* kodierte Protein für eine effektive Symbiose von *B. japonicum* mit seiner Wirtspflanze notwendig ist.

PART A

Heat shock regulatory network

The following chapters are dealing with the genetic regulation of the heat shock response in bacteria and, more specifically, in *Bradyrhizobium japonicum*.

Chapter 1 introduces the two principally different strategies involved in the control of bacterial heat shock gene expression. Transcription of these genes is either induced by an alternative sigma factor (positive regulation) or repressed by binding of a repressor protein to a DNA element in the promoter region (negative regulation).

Chapter 2 describes the characterisation of the *B. japonicum dnaKJ* operon. These heat shock genes are coding for two major chaperones, DnaK and DnaJ, which are ubiquitously present in probably all organisms and which play a critical role under physiological and non-physiological growth conditions. In co-operation with GrpE they form the DnaK chaperone machinery which is involved in intracellular protein folding and translocation, oligomeric protein assembly and protein degradation. According to the data presented in this work, the *dnaKJ* operon belongs to the class of positively regulated heat shock genes in *B. japonicum* whose transcription depends on the heat shock sigma factor RpoH.

The HrcA-CIRCE system is a widespread negative regulatory mechanism controlling the expression of bacterial heat shock genes. The temperature-dependent gene regulation is achieved by a *cis*-acting DNA element called CIRCE and the cognate, *trans*-acting protein HrcA which acts as a repressor under non-heat shock conditions. The identification and characterisation of the *B. japonicum hrcA* gene and the biochemical analysis of purified HrcA protein are described in **chapter 3**. Interestingly, the heat-inducible synthesis of HrcA is positively regulated by the heat shock sigma factor RpoH. Thus, a link between positive and negative regulatory mechanisms exists in *B. japonicum*.

Chapter 4 summarises the present knowledge about the heat shock response in *B. japonicum*. To date three different regulatory mechanisms are known, and it became evident that these mechanisms do interact with each other to form a complex network.

CHAPTER 1

Heat shock regulation in bacteria

The heat shock response is a protective and homeostatic cellular response to cope with damaged proteins caused by elevated temperatures or other stress factors. Many bacterial heat shock proteins (Hsps) are molecular chaperones (e.g. DnaK, GroEL and their cohorts) or ATP-dependent proteases (e.g. Lon, ClpPA, FtsH) which play major roles in protein folding, assembly, transport, repair and turnover under stress and nonstress conditions (for recent reviews see Bukau & Horwich, 1998; Herman & D'Ari, 1998). Their induced synthesis after a heat shock is guaranteed by two principally different regulatory strategies. (i) Positive regulatory mechanisms are based on the specific recognition of heat shock gene promoters by an alternative sigma factor. The cellular concentration of these sigma factors strongly increases after a heat shock, which results in elevated transcription of heat shock genes. (ii) Negative regulatory mechanisms depend on the specific binding of a repressor protein to a DNA element in the promoter region of heat shock genes. Under normal growth conditions, this protein-DNA interaction prevents transcription of the associated genes. At elevated temperatures, repression is relieved by unknown mechanisms which leads to increased heat shock gene transcription (for recent reviews see Mager & De Kruijff, 1995; Narberhaus, 1999; Yura & Nakahigashi, 1999). An overview of the currently known mechanisms responsible for regulating the bacterial heat shock response is presented below.

1.1 Positive regulation

1.1.1 Regulation of the σ^{32} (RpoH) regulon

The currently best studied alternative sigma factor in the context of heat shock regulation is σ^{32} (RpoH) of *Escherichia coli*. An increase in the cellular concentration of this sigma factor after a sudden temperature upshift to 42°C results in a transiently elevated transcription of more than 30 genes constituting the σ^{32} regulon. The raised level of σ^{32} is mainly caused by enhanced translation of *rpoH* mRNA in combination with increased activity and stability of the corresponding gene product (for recent reviews see Gross, 1996; Storz, 1999; Yura & Nakahigashi, 1999).

Two promoter proximal coding regions (A and B) of *rpoH* mRNA are involved in translational control of σ^{32} synthesis. Region A (downstream box) is complementary to the 3' region of 16S rRNA and acts as a positive element by enhancing translation, whereas region B is a negative element, which forms an extensive secondary structure with region A. While the formation of this structure prevents efficient translation of the *rpoH* mRNA under normal growth conditions (Morita *et al.*, 1999a), its thermal melting at elevated temperatures enhances ribosome entry and translational initiation without involvement of other cellular components (Morita *et al.*, 1999b). This finding indicates that the *rpoH* mRNA itself serves as a built-in thermosensor.

The activity and stability of σ^{32} are subject to feedback-control by the DnaK machinery (consisting of DnaK, DnaJ and GrpE) which sequesters the sigma factor under low-temperature conditions (Tomoyasu *et al.*, 1998). Binding of σ^{32} to the DnaK system prevents association with the RNA polymerase core enzyme (activity control) and permits efficient degradation of σ^{32} primarily by the FtsH protease (stability control). After a heat shock, denatured proteins presumably titrate the DnaK chaperones away from σ^{32}, leaving the latter stable and competent for complex formation with RNA polymerase. In the subsequent adaptation phase of the heat shock response, the σ^{32}-dependent production of DnaK, DnaJ and GrpE exceeds the amount of chaperones needed to cope with denatured proteins. As a consequence, the DnaK system becomes able to sequester σ^{32}, which finally leads to the shutdown of the heat shock response.

To date, *rpoH* genes from 18 different proteobacteria were isolated and characterised (summarised in Yura & Nakahigashi, 1999). While most organisms possess only a

single *rpoH* gene, *Bradyrhizobium japonicum* encodes three σ^{32}-like proteins with distinct features regarding their regulation and function (*cf.* chapter 4 and Narberhaus *et al.*, 1997). All known RpoH homologs contain the highly conserved RpoH box, a stretch of nine amino acids located in region C, which is thought to be involved in the DnaK-mediated negative feedback-control of the heat shock response (McCarty *et al.*, 1996). However, recent data indicate that region C does not bind DnaK but instead RNA polymerase (Arsène *et al.*, 1999). The region A and B-mediated secondary structures found in *E. coli rpoH* mRNA were only predicted for *rpoH* transcripts from gamma proteobacteria (Nakahigashi *et al.*, 1995). In accordance with this structure prediction, enhanced RpoH synthesis after heat shock was found to be primarily controlled on the translational level in these organisms (Nakahigashi *et al.*, 1998). In some alpha proteobacteria, the potential lack of translational control is compensated by a σ^{32}-dependent promoter which controls the heat-induced RpoH synthesis on the transcriptional level, e.g. in *Caulobacter crescentus* (Wu & Newton, 1997). Another strategy is used by *Rhodobacter sphaeroides* and *B. japonicum*. These bacteria encode two or three RpoH proteins, respectively, whose levels vary under different conditions (Narberhaus *et al.*, 1997; Karls *et al.*, 1998). Thus, the priciple of enhancing the σ^{32} level to cope with heat stress appears to be well conserved, but detailed mechanisms differ considerably among bacteria species.

1.1.2 Regulation of the σ^{E} (RpoE) regulon in *Escherichia coli*

A second heat shock regulon in *E. coli* is controlled by σ^{E}, which belongs to the class of sigma factors that responds to extracytoplasmic stimuli. Such ECF sigma factors control the expression of genes that are devoted to extracytoplasmic functions (Lonetto *et al.*, 1994). In the case of σ^{E}, the signals were identified as misfolded outer membrane and periplasmic proteins which are formed during exposure of the cells to extreme temperatures (50°C) or ethanol (for recent review see Raivio & Silhavy, 1999). Under nonstress conditions, σ^{E} is inactivated through membrane sequestration via interaction with the membrane-spanning anti-sigma factor RseA (regulator of sigma E; De Las Peñas *et al.*, 1997; Missiakas *et al.*, 1997). This negative interaction is further reinforced through periplasmic interactions between RseB and RseA (Fig. 1.1B).

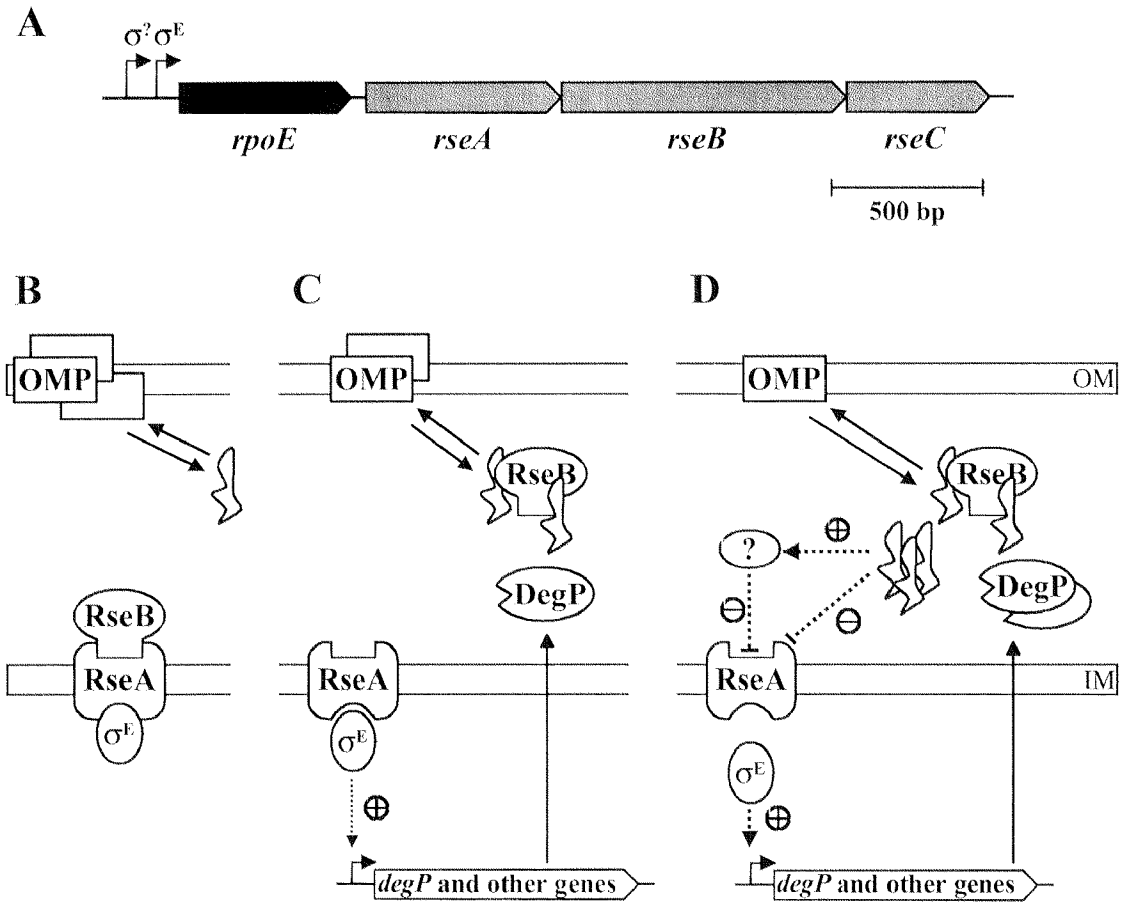

Fig. 1.1 A. Physical map of the *E. coli rpoErseABC* operon. Arrows mark the identified transcription start sites. σ^E indicates the promoter that is recognised by the *E. coli* extreme heat shock sigma factor RpoE. The RNA polymerase recognising the upstream promoter ($\sigma^?$) is currently unknown. **B, C, D.** Model of signal transduction to σ^E. (+) symbolises activation, and (–) repression. The figure is adapted from (De Las Peñas *et al.*, 1997). Details are explained in the text.

Envelope perturbations that disrupt the folding pathway of outer membrane proteins (OMPs) alleviate the negative interaction between σ^E and RseA. This is accomplished at two levels: first, RseB may be titrated away from RseA by misfolded OMPs, weakening the interaction between RseA and σ^E (Fig. 1.1C). Secondly, misfolded OMPs further weaken this interaction by direct or indirect association with RseA, leading to full activation of the σ^E-dependent pathways (Fig. 1.1D). σ^E is released from RseA, associates with RNA polymerase core enzyme and thereby activates transcription of the σ^E regulon, which includes genes encoding the OMP folding and degrading factors FkpA and DegP, the *rpoH* gene, the *rpoErseABC* operon itself, virulence genes and other genes yet to be identified. Autoregulation of σ^E amplifies the

response and permits a rapid shut-off through overproduction of the negative regulators RseA and RseB once the misfolded OMPs have been refolded or degraded (Fig. 1.1A; De Las Peñas *et al.*, 1997; Missiakas *et al.*, 1997). Synthesis of the periplasmic DegP protease is also controlled by the Cpx two-component signal transduction system indicating that at least two partially overlapping regulatory mechanisms are involved in the cellular response to extracytoplasmic (periplasmic) stress situations (for recent reviews see Pallen & Wren, 1997; Raivio & Silhavy, 1999).

1.1.3 Regulation of the σ^B (SigB) regulon in *Bacillus subtilis*

In *Bacillus subtilis*, a complex network of at least four different regulatory mechanisms is responsible for the control of heat-inducible genes. One of these mechanisms depends on an alternative sigma factor, σ^B, which induces transcription of more than 60 genes (class II heat shock genes). Induction of the σ^B-dependent genes is not limited to heat shock but also triggered by various types of stress and starvation conditions, which makes σ^B a general stress sigma factor (for recent review see Hecker & Völker, 1998).

The activity of σ^B is regulated by the anti-sigma factor RsbW (regulator of sigma B; Fig. 1.2B). During exponential growth, RsbW binds to σ^B, which prevents its association with RNA polymerase core enzyme. In response to stress or starvation, RsbW is captured by the dephosphorylated form of its antagonist protein RsbV, thereby releasing σ^B to direct transcription of its target genes. The phosphorylation state of RsbV is controlled by opposing kinase (RsbW) and phosphatase (RsbU) activities. ATP depletion as a result of energy starvation inhibits the phosphorylation of RsbV by the RsbW kinase activity. Consequently, RsbV remains in its dephosphorylated state, which binds to RsbW, causing the induction of the σ^B-dependent heat shock response. The regulatory system consisting of a phosphatase (RsbU), an antagonist protein (RsbV) and a kinase (RsbW) form a so-called 'partner-switching module' (downstream module). In contrast to the direct influence of starvation on this regulatory system, heat shock or other physical stresses are perceived by an additional partner-switching module consisting of RsbX, RsbS and RsbT (upstream module), which functions in a similar manner as the downstream module. In response to these stress situations, the regulator protein RsbT, which is released from its antagonist RsbS, directly binds to

RsbU and thereby stimulates its phosphatase activity. Consequently, RsbV is dephosphorylated and binds to RsbW, resulting in the release of σ^B and induction of the σ^B-dependent heat shock response. An additional control within the upstream partner-switching module is exerted by RsbR. Depending on its phosphorylation state, RsbR directly interacts with the RsbS antagonist and thereby prevents the formation of complexes between RsbS and RsbT (Akbar *et al.*, 1997). The stress response is further amplified as the downstream module of the *sigB* operon itself is transcribed from a σ^B-dependent promoter (Fig. 1.2A; summarised in Hecker & Völker, 1998; Helmann, 1999). Although the knowledge on this complex signal transduction cascade is constantly increasing, the cellular signal, which is ultimately triggered by physical stress factors such as heat shock, remains to be elucidated.

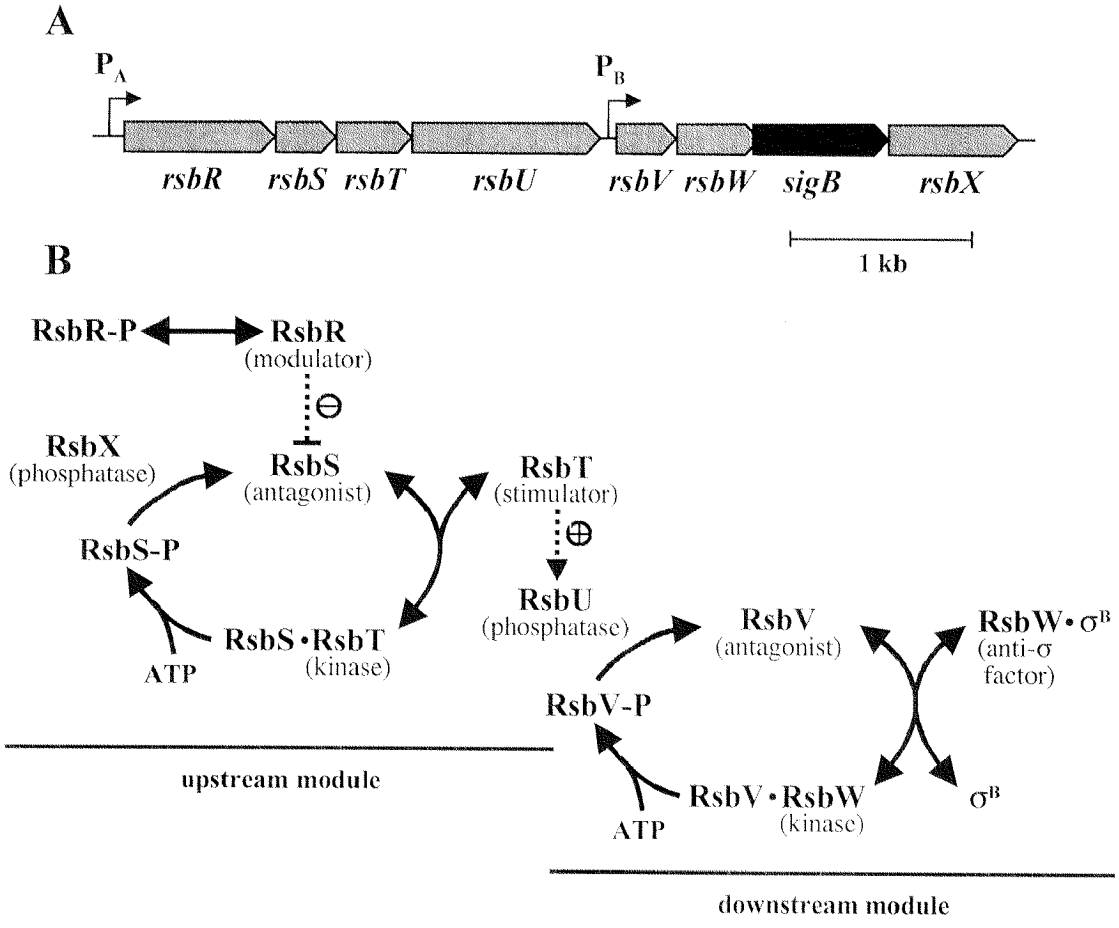

Fig. 1.2 A. Physical map of the *B. subtilis sigB* operon. Arrows mark the identified transcription start sites. P_A indicates a housekeeping promoter and P_B an internal, σ^B-dependent heat shock promoter. **B.** Model of σ^B regulation by two coupled partner-switching modules. (+) symbolises activation, and (–) repression. The figure is adapted from (Helmann, 1999). Details are explained in the text.

1.2 Negative regulation

1.2.1 Regulation of the CIRCE regulon

The CIRCE system is the currently best-understood negative regulatory mechanism of heat shock gene expression. CIRCE (for controlling inverted repeat of chaperone expression; Zuber & Schumann, 1994) comprises a widespread and highly conserved inverted repeat which so far was identified in the promoter regions of chaperone operons from more than 40 different eubacteria (Hecker *et al.*, 1996; Narberhaus, 1999) and whose consensus sequence was established as TTAGCACTC-N_9-GAGTGCTAA (Hecker *et al.*, 1996). Recent studies attributed a dual role to CIRCE, namely as a DNA element reducing transcription and as a RNA element promoting the rapid mRNA turnover under normal growth conditions.

CIRCE serves as a binding site for a repressor protein designated HrcA (for heat regulation at CIRCE; Roberts *et al.*, 1996; Schulz & Schumann, 1996). Binding of HrcA to the CIRCE element under normal growth conditions prevents expression of the genes located downstream of the element. By analogy with the feedback-control of σ^{32} by the DnaK machinery in *E. coli* (see above), the activity of HrcA was found to be modulated by the GroE chaperonin system (Mogk *et al.*, 1997). A direct correlation between the cellular GroESL level and HrcA activity suggested that the repressor requires chaperonins for proper function *in vivo* (Babst *et al.*, 1996; Mogk *et al.*, 1997). After a heat shock, the chaperonins become engaged in refolding heat-denatured proteins, which depletes the GroESL pool and presumably causes the formation of inactive HrcA. The response is self-limiting when the heat-induced production of chaperonins exceeds the amount of GroESL needed to cope with denatured proteins, and the chaperonins become available again to modulate the HrcA activity. The assumption that non-native proteins might act as signals triggering the heat shock response of CIRCE-dependent genes was further supported by the fact that the HrcA-CIRCE regulon is not only induced by a heat shock but also by the presence of ethanol, puromycin or the amino acid analogue *para*-fluoro-phenylalanine, which all cause the accumulation of misfolded proteins within the cell (Babst *et al.*, 1996; Mogk *et al.*, 1998).

In addition to its role as a repressor binding site, the CIRCE element is involved in the posttranscriptional regulation of heat shock gene expression. CIRCE elements,

which are positioned close to the ribosome binding site, might induce at the mRNA level a secondary structure which impairs the interaction of ribosomes with their binding site (Homuth *et al.*, 1999). Consequently, the translation of the downstream genes is significantly impeded. Moreover, the ribosome-mediated protection of mRNA against degradation by RNases is reduced, which causes a destabilisation of mRNA transcribed from CIRCE-containing DNA templates as observed previously (Yuan & Wong, 1995a; Segal & Ron, 1996a). Both mechanisms contribute to the repression of CIRCE-dependent genes under non-heat shock conditions.

A more detailed description concerning the role of CIRCE and HrcA in the heat shock regulatory network of *B. japonicum* is presented in chapter 3 and 4.

1.2.2 Regulation of the CtsR regulon in Gram-positive bacteria

Expression of the class III heat shock genes of *B. subtilis*, which encode subunits of the Clp ATP-dependent protease (ClpP, ClpC and ClpE), are negatively regulated by the repressor protein CtsR (for class three stress gene repressor), the product of the first gene of the *clpC* operon (*ctsRorf2orf3clpCorf5orf6*) (Krüger & Hecker, 1998; Derré *et al.*, 1999). A hepta-nucleotide direct repeat located within the *clpP* and *clpC* promoter regions was identified as the likely CtsR recognition sequence. The consensus motif was established as $^{A}/_{G}$GTCAAANAN$^{A}/_{G}$GTCAAA (Derré *et al.*, 1999). The fact that these direct repeats overlap or are near the σ^{A}-dependent promoters of the *clpP* and *clpC* genes strongly suggests that CtsR acts as a negative regulator by interfering with the binding of the RNA polymerase to these promoters (Derré *et al.*, 1999). The identification of similar CtsR binding sites upstream from *clp* and other heat shock genes of many Gram-positive bacteria indicates that the regulation of gene expression by CtsR is a highly conserved and widespread mechanism of bacterial heat shock response.

Purified CtsR binds to DNA at 37°C but not at 48°C which might indicate that the repressor acts as a direct heat sensor with intrinsic temperature sensitivity leading to inactivation when exposed to elevated temperatures (Derré *et al.*, 1999). However, it cannot be excluded that other factors might be required *in vivo* for CtsR to obtain or loose its active conformation. Interestingly, the expression of the CtsR-dependent *clpP* gene was partially derepressed even at normal temperature in a *clpC* mutant, indicating

a possible chaperone function of the ClpC ATPase in (re)folding CtsR into an active conformation (Derré *et al.*, 1999). As the *ctsR* and *clpC* genes are coexpressed, the suggested modulation of the CtsR binding activity by ClpC would be another example of a feedback-control of heat shock gene expression.

1.2.3 Regulation of the HspR regulon

Analysis of the *dnaK* gene expression in *Streptomyces coelicolor* and *Streptomyces albus* led to the identification of yet another negative regulatory mechanism. Although transcription of the *dnaKgrpEdnaJhspR* operon initiates from a housekeeping promoter, its expression is repressed under normal growth conditions and strongly heat-inducible. This heat shock control mechanism is mediated by the autoregulatory repressor HspR, which binds specifically to the promoter region of the *dnaK* operon under normal growth conditions and thereby prevents transcription of the downstream genes (Bucca *et al.*, 1997; Grandvalet *et al.*, 1997). A highly conserved inverted repeat motif was identified as the HspR binding site (Bucca *et al.*, 1995). The consensus motif (CTTGAGT-N_7-ACTCAAG) has been designated HAIR (for HspR associated inverted repeat; Grandvalet *et al.*, 1999). This regulatory mechanism is not specific to *Streptomyces* as similar inverted repeat motifs were found in the promoter regions of heat shock genes from 9 different Gram-positive and Gram-negative bacterial species (Grandvalet *et al.*, 1999).

Sequence analysis of the HspR protein from *S. albus* predicts the existence of a putative helix-turn-helix DNA binding motif and a coiled-coil structure which might act as a protein-protein interaction site. These structural elements are consistent with the assumption that the HspR repressor binds to the HAIR motif as part of a multimeric complex (Grandvalet *et al.*, 1999). As the *hspR* gene is coexpressed with the genes encoding the DnaK machinery, it is tempting to speculate that the chaperones modulate the activity of HspR by facilitating, directly or indirectly, its multimerisation or its binding to the HAIR motif. Such feedback control of HspR activity would allow a rapid repression of transcription of the HspR-regulated *dnaK* operon once a sufficient level of the DnaK machinery had been attained and could explain the transient character of the HspR-regulated heat shock response (Narberhaus, 1999).

1.2.4 Regulation of the OrfY regulon in *Streptomyces albus*

Expression of *S. albus hsp18*, encoding a small heat shock protein, is controlled by temperature-dependent regulation of both gene transcription and translation.

Heat-inducible transcription of *hsp18* from its vegetative promoter is mediated by OrfY, the gene product of an open reading frame (*orfY*) located 150 bp upstream of *hsp18* in the opposite orientation (Servant & Mazodier, 1996). Disruption of *orfY* resulted in elevated *hsp18* transcript levels at normal temperatures. Complementation of the *orfY* mutant with a plasmid overproducing OrfY restored the repression of *hsp18*. These findings led to the suggestion that OrfY might act as a repressor protein, which binds specifically to the promoter region of *hsp18*. Moreover, this putative repressor is assumed to be inactive or unstable at elevated temperatures, causing the observed increase of the steady-state level of Hsp18 protein after heat shock (Servant & Mazodier, 1996). In support of a repressor-mediated regulation of *hsp18*, two similar inverted repeat sequences, one (GTCATC-N_5-GATGAC) overlapping the –35 region of the *hsp18* promoter and one (GTCGTC-N_5-GATGAC) located 60 bp upstream of the *orfY* start codon, were identified as possible repressor binding sites (Servant & Mazodier, 1996).

In addition to the transcriptional control, thermally induced expression of *hsp18* is also subject to posttranscriptional regulation. Although *hsp18* transcript was synthesised at high level in an *orfY* mutant even at low temperatures, Hsp18 protein was only detectable after heat shock (Servant & Mazodier, 1996). This temperature-dependent regulation is most likely based on a translational control of *hsp18*, involving the formation of mRNA secondary structures under normal growth conditions, which might prevent binding of the ribosome to the translation initiation region of *hsp18* transcript.

1.2.5 Regulation of the ROSE regulon in *Bradyrhizobium japonicum*

ROSE (for repression of heat shock gene expression; Narberhaus *et al.*, 1998a) is a highly conserved *cis*-acting DNA element of approximately 100 bp which is positioned between the transcriptional and the translational start sites of several heat shock operons and whose occurrence seems to be restricted to the genus of *Bradyrhizobium*. The current model assigns two different regulatory functions to the ROSE element.

First, it might act as a binding site for a putative repressor protein under normal growth conditions. Second, the formation of a specific secondary structure by the ROSE-containing mRNA at normal temperature might provide a posttranscriptional control for the repression of ROSE-regulated genes.

A more detailed description of the ROSE-dependent heat shock gene regulation in *B. japonicum* is presented in chapter 4.

1.3 Heat shock regulatory networks

Current data demonstrate that most bacteria use more than one strategy to control heat shock gene expression. The heat shock response may involve combinations of positive and/or negative regulatory systems. In *E. coli*, for example, transcription of heat shock genes is induced by positively acting alternative sigma factors (σ^{32} and σ^{E}; Yura & Nakahigashi, 1999), whereas the heat shock response in *S. albus* involves at least three different repressor systems (HrcA-CIRCE, HspR-HAIR and OrfY; Servant & Mazodier, 1996; Bucca *et al.*, 1997; Grandvalet *et al.*, 1997; Grandvalet *et al.*, 1998). Combinations of positive and negative regulatory systems within a single organism were identified in *B. subtilis* (σ^{B}, HrcA-CIRCE and CtsR; Hecker *et al.*, 1996; Krüger & Hecker, 1998; Derré *et al.*, 1999) and *B. japonicum* (RpoH, HrcA-CIRCE and ROSE; Narberhaus, 1999). In addition, even a single gene or operon can be controlled simultaneously by two different heat shock regulatory systems, as examplified by the *dnaK* operon in *C. crescentus* (σ^{32} and HrcA-CIRCE; Baldini *et al.*, 1998) and in *Staphylococcus aureus* (HrcA-CIRCE and CtsR; Derré *et al.*, 1999) and the *clpC* operon in *B. subtilis* (σ^{B} and CtsR; Krüger & Hecker, 1998; Derré *et al.*, 1999). Most interestingly, some of the regulatory mechanisms that coexist within a single organism depend on each other as demonstrated by the fact that the central mediator of one regulatory system is provided by a second regulatory system, e.g. the RpoH-dependent synthesis of HrcA in *C. crescentus* (Roberts *et al.*, 1996) and the ROSE-dependent production of $RpoH_1$ in *B. japonicum* (Narberhaus *et al.*, 1996). These findings indicate the existence of complex regulatory networks controlling the overall heat shock response in some bacteria. The current model of the heat shock regulatory network in *B. japonicum* is presented in detail in chapter 4 (*cf.* Fig. 4.1). It is known from *B. subtilis* and *B. japonicum* that different classes of heat shock genes display different induction kinetics upon heat shock and respond to common as well as to distinct inducers. It appears as if the formation of a complex heat shock regulatory network may guarantee an optimal adjustment of the heat shock protein level to the given environmental condition and thereby ensure the survival of the cell.

CHAPTER 2

The *dnaKJ* operon belongs to the σ^{32}-dependent class of heat shock genes in *Bradyrhizobium japonicum*

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2.1 Abstract

The *dnaKJ* genes of *Bradyrhizobium japonicum* were cloned and sequenced. They map adjacent to each other, as in other proteobacteria of the α and γ subgroups. Primer extension experiments identified two strongly heat-inducible transcripts starting 99 bp (T1) and 204 bp (T2) upstream of *dnaK*. Synthesis of the shorter transcript T1 in *Escherichia coli* required the presence of a recently characterised σ^{32} homologue ($RpoH_1$) of *B. japonicum*. The –35 and –10 regions of the promoters associated with the transcription start sites T1 and T2 displayed nucleotide sequence motifs characteristic for σ^{32}-dependent promoters in *E. coli* and α-proteobacteria. Heat shock regulation of *dnaK* expression was confirmed by immunoblot analysis of DnaK protein. All of these results put *dnaK* into the σ^{32}-dependent class, not the CIRCE-dependent class, of heat shock genes in *B. japonicum*. At normal growth temperature *dnaK* was expressed at a significant basal level. All attempts to eliminate *dnaK* function by insertion or deletion mutagenesis failed. By contrast, *dnaJ* null mutants and insertions in the *dnaKJ* intergenic region were easily obtained. The growth rate of *dnaJ* mutants was reduced but the final cell density reached in rich medium and their symbiotic properties were indistinguishable from the wild type.

Key words: heat shock proteins; Hsp40 Hsp70; molecular chaperone; transcriptional control

2.2 Introduction

The DnaK and DnaJ proteins are members of two distinct families of heat shock proteins, Hsp70 and Hsp40. They are ubiquitously present in probably all organisms and play a critical role under physiological and non-physiological growth conditions. The ability of DnaK and DnaJ to stabilise nascent, unfolded or partially denatured polypeptides has led to their classification as molecular chaperones (for review see Hartl, 1996). By the controlled binding and release of a target polypeptide, the DnaK/J chaperones assist in intracellular protein folding and translocation, oligomeric protein assembly, and protein degradation (Hendrick & Hartl, 1993; Georgopoulos *et al.*, 1994). At elevated temperatures, living cells have to cope with increased amounts of denatured proteins. Accordingly, heat shock inducible regulatory systems have evolved to meet the varying demands for cellular chaperones. Studies on the heat shock response in bacterial model systems revealed a surprising variety of different mechanisms involved in this type of control (for reviews see Yura *et al.*, 1993; Hecker *et al.*, 1996; Schumann, 1996).

In *Escherichia coli*, the so-called heat shock sigma factor σ^{32} mediates the rapid heat shock response in the cell (Bukau, 1993; Yura *et al.*, 1993). This sigma factor directs the RNA polymerase to the specific promoter sequence of heat shock genes and subsequently induces transcription of associated genes or operons (Grossman *et al.*, 1984; Cowing *et al.*, 1985). Upon heat shock the σ^{32} concentration in the cell is greatly increased due to enhanced σ^{32} synthesis and stability (Straus *et al.*, 1987). During the shut-off phase of the heat shock response, the free chaperones DnaK, DnaJ and GrpE, which are no longer involved in the refolding or degradation of denatured proteins, form a complex with σ^{32} and deliver it to a proteolytic system. This interaction causes a rapid inactivation of the heat shock sigma factor ('DnaK/DnaJ-titration model'; Bukau, 1993; Gamer *et al.*, 1996).

A completely different heat shock regulation system has been demonstrated for the *Agrobacterium tumefaciens groESL* operon (Segal & Ron, 1996a), the *Bacillus subtilis dnaK* operon (Zuber & Schumann, 1994) and the *Lactococcus lactis dnaJ* gene (van Asseldonk *et al.*, 1993). The expression of these chaperone genes is controlled by a promoter that requires a housekeeping sigma factor in concert with a highly conserved *cis*-acting regulatory element located in the transcribed, non-translated 5' region of the

corresponding genes. Because of its regulatory function this inverted repeat sequence has been designated the CIRCE element (controlling inverted repeat of chaperone expression; Zuber & Schumann, 1994). Meanwhile this element has been identified in the promoter regions of more than 50 chaperone-encoding genes or operons in Gram-positive as well as in several Gram-negative bacteria (Hecker *et al.*, 1996). Moreover, the gene for the putative repressor protein which, under non-inducing conditions, binds to the CIRCE element has been identified in *B. subtilis* (Yuan & Wong, 1995b; Schulz & Schumann, 1996).

Recent studies with *A. tumefaciens* and *Caulobacter crescentus* revealed the existence of both the CIRCE- and the σ^{32}-dependent heat shock regulatory systems within a single bacterial organism (Mantis & Winans, 1992; Avedissian & Gomes, 1996; Reisenauer *et al.*, 1996; Segal & Ron, 1996a; Wu & Newton, 1996). Likewise, in *Bradyrhizobium japonicum*, the nitrogen-fixing root-nodule symbiont of soybean, both regulatory systems were found to be involved in the heat shock regulation of a multigene family consisting of five *groESL* operons (Fischer *et al.*, 1993; Babst *et al.*, 1996). Three members of this gene family are heat shock inducible. In the case of $groESL_4$ (and probably also $groESL_5$) transcription of the operon is negatively regulated by a CIRCE element. On the other hand, the promoter of the $groESL_1$ operon shows a σ^{32} consensus sequence and is recognised in *E. coli* by the RNA polymerase containing the heat shock sigma factor σ^{32} (Babst *et al.*, 1996).

The purpose of this study was to gain a more detailed insight into the complex heat shock regulatory system of *B. japonicum* by characterising the *dnaKJ* genes. As shown in this work, the absence of a CIRCE element implied and promoter analysis confirmed that they belong to the σ^{32}-dependent class of heat shock genes in this organism.

2.3 Materials and methods

2.3.1 Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 2.1. *E. coli* cells were grown in Luria-Bertani (LB) medium (Miller, 1972) supplemented with ampicillin (200 μg/ml), chloramphenicol (20 μg/ml), kanamycin (30 μg/ml), or tetracycline (10 μg/ml) if required. The growth temperature for *E. coli* strains was 37°C, except for *E. coli* KY1603, which was grown at 28°C. *B. japonicum* strains were propagated aerobically at 28°C in PSY medium (Regensburger & Hennecke, 1983) supplemented with 0.1 % (w/v) arabinose. If appropriate, antibiotics were added at the following concentrations (μg/ml): chloramphenicol, 20 (for counterselection against *E. coli* donor strains); kanamycin, 100; spectinomycin, 100; and tetracycline, 50.

Table 2.1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype	Reference or origin
E. coli		
DH5α	*supE44* Δ*lacU169* (ψ80*lacZ*ΔM15) *hsdR17 recA1 gyrA96 thi-1 relA1*	Gibco-BRL, Gaithersburg, MD, USA
MC4100	F⁻ *araD139* Δ*(argF-lac)U169 rpsL150 relA1 deoC1 ptsF25 flbB5301 rbsR*	(Casadaban, 1976)
GW4813	Cm^r *dnaK52*	(Paek & Walker, 1987)
KY1603	Km^r *rpoH::kan*	(Kusukawa & Yura, 1988)
JM101	*supE thi* Δ*(lac-proAB)* F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15]	(Messing, 1983)
S17-1	Sm^r Sp^r *hdsR* RP4-2 *kan*::Tn*7 tet*::Mu, integrated in the chromosome	(Simon *et al.*, 1983)
B. japonicum		
110*spc*4	Sp^r (wild type)	(Regensburger & Hennecke, 1983)
8168	Sp^r Km^r, *aphII* inserted into the *dnaKJ* intergenic region in the same orientation as *dnaKJ*	This study

(continued)

(Table 2.1 continued)

8170	Sp^r Km^r, *aphII* inserted into the *dnaKJ* intergenic region in the opposite orientation to *dnaKJ*	This study
8201	Sp^r Km^r *dnaJ::aphII*, *dnaJ* and *aphII* oriented in the same direction	This study
8202	Sp^r Km^r *dnaJ::aphII*, *dnaJ* and *aphII* oriented in opposite directions	This study
Plasmids		
M13mp18		(Norrander *et al.*, 1983)
M13mp19		(Norrander *et al.*, 1983)
pBluescript II KS+	Ap^r	Stratagene, La Jolla, CA, USA
pMCL210	Cm^r P15A *ori*	(Nakano *et al.*, 1995)
pUC18	Ap^r	(Norrander *et al.*, 1983)
pSUP202	Ap^r Cm^r Tc^r *oriT* from RP4	(Simon *et al.*, 1983)
pSUP202pol4	Tc^r (pSUP202) part of polylinker from pBluescript II KS+ between *Eco*RI and *Pst*I sites	(Fischer *et al.*, 1993)
pSUP202pol6K	Tc^r (pSUP202pol4) *Sma*I site altered to *Kpn*I site	(Zufferey *et al.*, 1996)
pNM482	Ap^r (pUC8) *'lacZ*	(Minton, 1984)
pSUP482	Tc^r (pSUP202pol4) *'lacZ* (5.1 kb *Eco*RI-*Stu*I fragment from pNM482)	H.M. Fischer, unpublished
pUC4-KIXX-PSP	Ap^r Km^r (pUC4-KIXX) *aphII* cassette with *Pme*I-*Swa*I-*Pac*I linker in *Sma*I site	(Kündig *et al.*, 1993)
pDM38	Ap^r (pWSK29) 7 kb *Bam*HI fragment containing *htpY*, *dnaK* and *dnaJ* from *E. coli*	(Missiakas *et al.*, 1993)
pRJ5000	Ap^r (pUC18) *B. japonicum* $rpoH_1$, $rpoH_1$ in α*lacZ* orientation	(Narberhaus *et al.*, 1996)
pEC5007	Ap^r (pUC18) *E. coli rpoH*, *rpoH* in α*lacZ* orientation	F Narberhaus, unpublished
pRJ8144[a]	Ap^r (pUC18) 2.9 kb *Eco*RI fragment containing *B. japonicum 'dnaKJ'* opposite to α*lacZ* orientation	This study
pRJ8163	Ap^r (pUC18) 8.1 kb *Bam*HI fragment containing *B. japonicum dnaKJ* in α*lacZ* orientation	This study
pRJ8164	Ap^r (pUC18) 8.1 kb *Bam*HI fragment containing *B. japonicum dnaKJ* opposite to α*lacZ* orientation	This study

(continued)

(Table 2.1 continued)

pRJ8165[a]	Km^r Tc^r (pSUP202pol4) 3.09 kb *Eco*RI fragment with *'dnaK'::aphII dnaJ'*; *dnaK* and *aphII* in the same direction	This study
pRJ8168	Km^r Tc^r (pSUP202pol4) 2.91 kb *Eco*RI fragment from pRJ8144 with *aphII* inserted into *Nru*I site; *'dnaK* and *aphII* oriented in the same direction	This study
pRJ8170	Km^r Tc^r (pSUP202pol4) 2.91 kb *Eco*RI fragment from pRJ8144 with *aphII* inserted into *Nru*I site; *'dnaK* and *aphII* oriented in opposite directions	This study
pRJ8174	Tc^r (pSUP482) *dnaK'-'lacZ*, 1.3 kb *Eco*RI *dnaK'* fragment from pRJ8163	This study
pRJ8197[a]	Tc^r (pSUPpol6K) 3.2 kb *Pst*I-*Kpn*I *B. japonicum* *'dnaKJ'* fragment	This study
pRJ8201	Tc^r Km^r (pRJ8197) *dnaJ::aphII*, *dnaJ* and *aphII* oriented in the same direction	This study
pRJ8202	Tc^r Km^r (pRJ8197) *dnaJ::aphII*, *dnaJ* and *aphII* oriented in opposite directions	This study
pRJ8203	Cm^r (pMCL210) *dnaK'-'lacZ*, 6 kb *Bgl*II-*Xba*I fragment from pRJ8174	This study

[a] The inserts of these plasmids are depicted in Fig. 2.1.

2.3.2 DNA manipulations

Recombinant DNA techniques were performed according to standard protocols (Sambrook *et al.*, 1989). Chromosomal DNA from *B. japonicum* was isolated as described previously (Hahn & Hennecke, 1984). DNA was sequenced by the chain termination method (Sanger *et al.*, 1977) with a model 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Both double-stranded plasmid DNA and single-stranded DNA originating from bacteriophage M13 derivatives were used. The DNA region sequenced and the deduced proteins were analysed using the software package of the UWGCG (Genetics Computer Group of the University of Wisconsin, Madison, USA; version 8.0) or the NCBI (National Center for Biotechnology Information) BLAST network server. For the 'CODONPREFERENCE' program the previously established *B. japonicum* codon usage served as a reference (Ramseier & Göttfert, 1991).

2.3.3 DNA probes and hybridisation conditions

For probing *B. japonicum* genomic DNA as well as a cosmid library, a 3 kb *Acc*I fragment from plasmid pDM38 containing *E. coli dnaK'* was radioactively labelled by nick translation. In subsequent hybridisation experiments, either the nick-translated 2.9 kb *Eco*RI insert of pRJ8144 containing *B. japonicum 'dnaKJ'* or a 1.1 kb PCR product containing the *B. japonicum dnaJ* gene was used. The latter DNA probe was synthesised by PCR using primers from the 5' and the 3' regions of *dnaJ*, and labelled non-radioactively by incorporation of digoxigenin-11-dUTP. Homologous hybridisations were performed at 67°C in 2 x SSC (1 x SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7), 0.02% (w/v) SDS, 20 mM sodium phosphate buffer (pH 6.5), 1% (w/v) blocking reagent for nucleic acid hybridisation (Boehringer Mannheim, Mannheim, Germany), 200 µg sonicated salmon sperm DNA per ml, followed by three washes in 2 x SSC plus 0.1% (w/v) SDS at 67°C. Heterologous hybridisations were performed at 56°C in 5 x SSC, 0.1% (w/v) SDS, followed by three washes in 5 x SSC plus 0.1% (w/v) SDS at 56°C. Hybridisation signals were detected on Fuji RX medical X-ray films or with a PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA).

2.3.4 Transcript mapping

RNA isolation and primer extension analysis was performed as described (Babst *et al.*, 1996). The following oligonucleotides were used to determine the transcription start sites upstream of *dnaK*:

DnaK6 (1361) 5'-GGTGACGGCGACGATCGAAGGCGTCGTGCG-3'

DnaK12 (1323) 5'-CGGAATTCTCGATGACTTTGGCGTTCTTGC-3'

DnaK28 (1311) 5'-TGACTTTGGCGTTCTTGCCATCCATCAC-3'

The numbers in parentheses indicate the position of the 5' end based on the numbering used in the nucleotide sequence shown in Fig. 2.2.

2.3.5 Construction of *B. japonicum dnaJ* mutant strains

For the construction of *dnaJ* insertion mutations the 1.7 kb *Xho*I fragment of pUC4-KIXX-PSP containing the *aphII* cassette (Km^r) was inserted into the *Xho*I site located in the *dnaJ* gene present on plasmid pRJ8197. Both orientations of the *aphII* cassette with respect to the *dnaJ* gene were obtained (pRJ8201 and pRJ8202). Similarly, the

aphII cassette was inserted as a 1.7 kb *Sma*I fragment in both orientations into the *Nru*I site located between *dnaK* and *dnaJ* on plasmid pRJ8144. The *Eco*RI inserts of the resulting plasmids were then cloned into pSUP202pol4 yielding plasmids pRJ8168 and pRJ8170. Finally, a *dnaK* deletion was constructed by replacing the 1.52 kb *Eco*RV-*Nru*I fragment on pRJ8144 with the *aphII* cassette present on the 1.7 kb *Sma*I fragment derived from pUC4-KIXX-PSP. The *Eco*RI insert of the resulting plasmid was cloned into pSUP202pol4 yielding plasmid pRJ8165. All five plasmids (pRJ8165, pRJ8168, pRJ8170, pRJ8201 and pRJ8202) were mobilised from *E. coli* S17-1 into *B. japonicum* 110*spc*4 as described previously (Hahn & Hennecke, 1984). Marker exchange mutants were differentiated from clones containing the mobilised plasmid as a co-integrate in the chromosome by their Tc^S and Tc^r phenotypes, respectively. The genomic structure of the mutant strains was analysed by Southern blot hybridisations of genomic DNA digested with appropriate restriction enzymes. The physical structure of relevant plasmid inserts and of *B. japonicum* mutants is shown in Fig. 2.1.

2.3.6 Western blot analysis

Crude extracts of *E. coli* and *B. japonicum* cells were prepared, separated on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes as described previously (Babst *et al.*, 1996). Proteins were detected by binding of rabbit anti-*E. coli* DnaK serum (kindly provided by B. Bukau; 3,000-fold dilution) and using a chemiluminescence Western blot detection kit (Boehringer Mannheim, Mannheim, Germany).

2.3.7 Plant infection test

The symbiotic phenotype of the *B. japonicum dnaJ* mutants was determined in a soybean plant infection test as described previously (Hahn & Hennecke, 1984; Göttfert *et al.*, 1990).

2.4 Results

2.4.1 Cloning of the *dnaKJ* gene region of *B. japonicum*

In order to identify the *dnaKJ* gene region of *B. japonicum*, a radioactively labelled *E. coli dnaK* probe was hybridised to total genomic DNA digested with *Eco*RI, and to a cosmid library of *B. japonicum* chromosomal DNA (see Materials and methods). Subsequent Southern blot analysis of three selected cosmids digested with *Eco*RI led to the identification of a strongly hybridising 2.9 kb *Eco*RI fragment that was also observed in the genomic DNA. This fragment was subcloned into the *Eco*RI-linearised pUC18 vector, yielding plasmid pRJ8144 (Fig. 2.1). The amino acid sequence deduced from partial DNA sequence analysis at each end of the 2.9 kb *Eco*RI fragment revealed

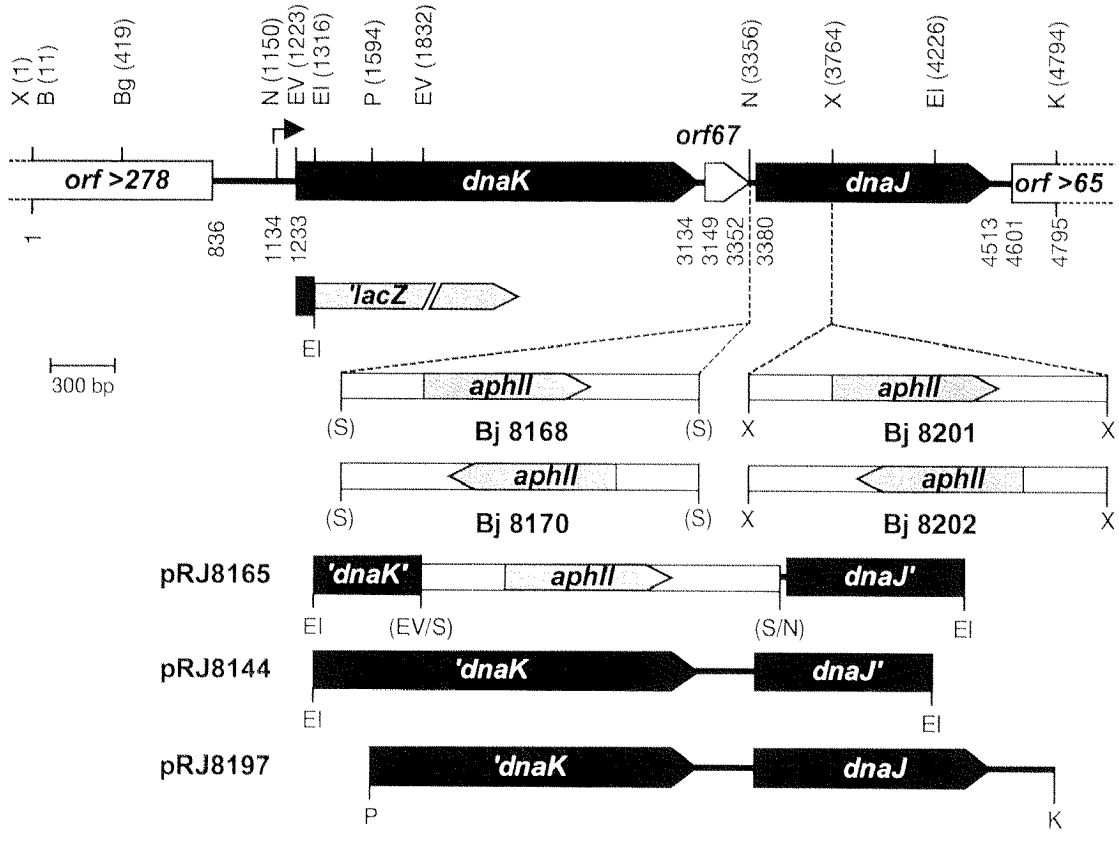


Fig. 2.1 Physical map of the *B. japonicum dnaKJ* gene region. Numbers indicate start and stop codon positions of ORFs, the transcription start site (horizontal arrow; 1,134), the beginning (1) and the end (4,795) of the sequenced gene region, and recognition sites of the following restriction enzymes: B, *Bam*HI; Bg, *Bgl*II; EI, *Eco*RI; EV, *Eco*RV; K, *Kpn*I; N, *Nru*I; P, *Pst*I; X, *Xho*I. Restriction sites in parentheses were destroyed during the cloning procedures. The structure of the *B. japonicum* insertion mutant strains 8168, 8170, 8201 and 8202, the *dnaK'-'lacZ* fusion and the inserts of plasmids pRJ8144, pRJ8165 and pRJ8197 are shown below the physical map.

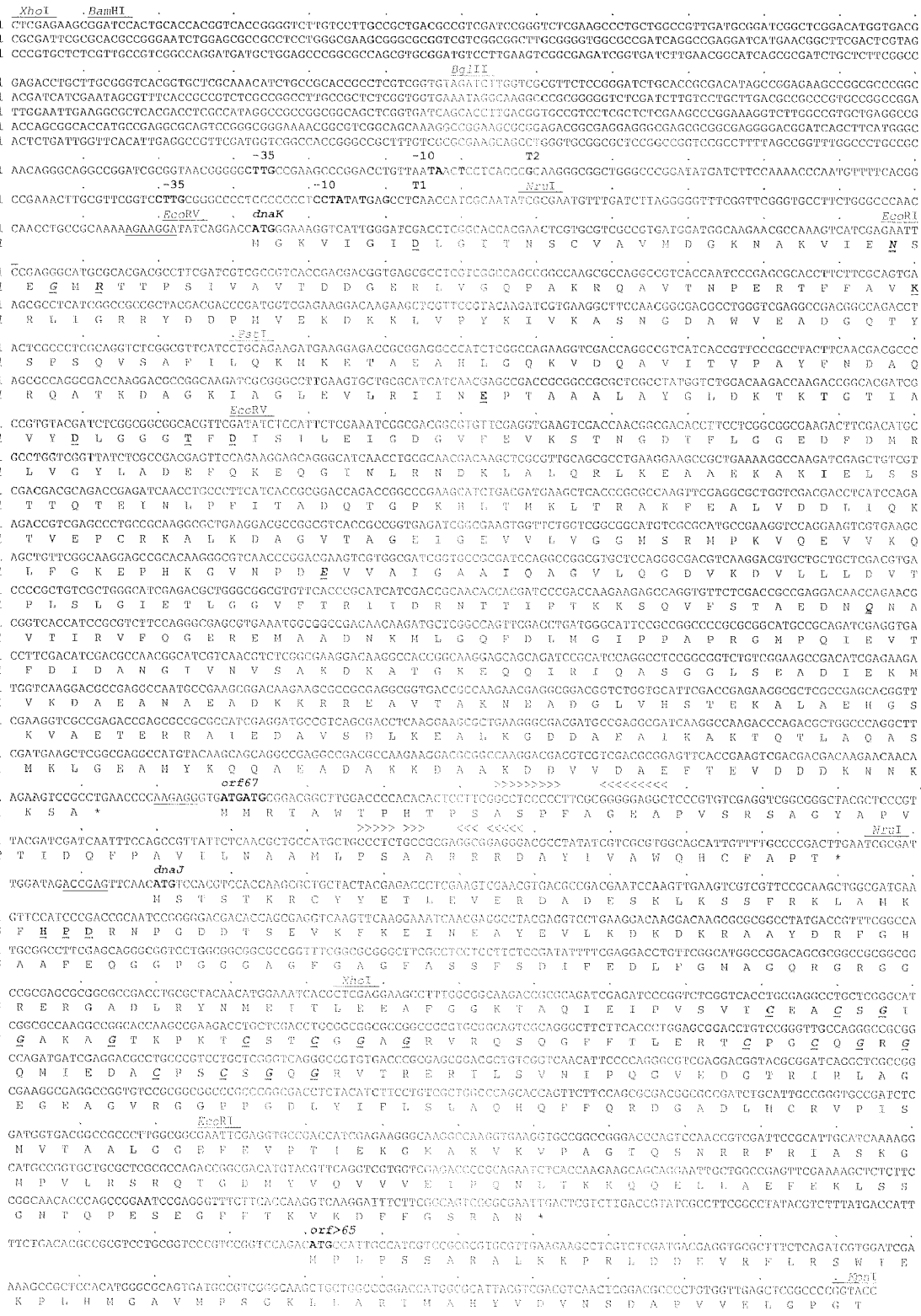
a high degree of similarity to the amino acid sequence of *E. coli* DnaK and DnaJ. Using the insert of pRJ8144 as a probe, the complete *B. japonicum dnaKJ* region was subcloned in plasmids pRJ8163 and pRJ8164 as a 8.1 kb *Bam*HI fragment originating from one of the hybridising cosmids.

To address the question whether there is more than one copy of the *dnaK* gene in the *B. japonicum* chromosome, as is the case for *groESL* (Fischer *et al.*, 1993) and *rpoH* (Narberhaus *et al.*, 1996), the 2.9 kb *Eco*RI insert of pRJ8144 was hybridised under low stringency conditions (5 x SSC, 56°C) to *B. japonicum* chromosomal DNA digested with several different restriction enzymes. No evidence for the existence of another *dnaKJ*-homologous region was obtained (data not shown).

2.4.2 Nucleotide sequence of the *dnaKJ* gene region

The DNA sequence of the *B. japonicum dnaKJ* gene region extending from the *Xho*I site at position 1 to the *Kpn*I site at position 4,794 was established (Figs 2.1 and 2.2). Comparisons between the deduced amino acid sequences and those of *E. coli* DnaK and DnaJ (Bardwell & Craig, 1984; Bardwell *et al.*, 1986) led to the assignment of the open reading frames (ORFs) of *B. japonicum dnaK* (position 1,233 to 3,134) and *dnaJ* (position 3,380 to 4,513). The *dnaK* gene encodes a predicted protein of 633 amino acids (M_r 68,292) whereas the deduced gene product of *dnaJ* consists of 377 amino acids with a molecular weight of 40,945. Upstream of both genes potential ribosome binding sites were found which, in the case of *dnaJ*, showed only marginal similarity to the consensus sequence in *E. coli* (Shine & Dalgarno, 1974). Further investigation of

Fig. 2.2 (following page) Nucleotide sequence and relevant restriction sites of the 4,795 bp *Xho*I-*Kpn*I fragment harbouring the *dnaKJ* gene region of *B. japonicum*. Start codons of *dnaK*, *orf67*, *dnaJ* and *orf>65* are marked by bold letters and stop codons by asterisks. Putative ribosome binding sites are underlined. Arrowheads above the DNA sequence denote regions of dyad symmetry. The two transcription start sites (T1 and T2) identified by primer extension analysis (Fig. 2.4) and conserved nucleotides in the –10 and –35 regions of the corresponding promoters are marked by bold letters (see also Fig. 2.7). The deduced amino acid sequences (single-letter code; numbers in italics) of *dnaK*, *dnaJ*, and other *orfs* are indicated below the DNA sequence. Amino acids presumably involved in the interaction of DnaK with Mg-ADP and the residues of the DnaJ protein thought to interact with DnaK are underlined and printed in bold letters. The amino acids of DnaK which are proposed to interact with the co-chaperone GrpE or target substrates (Q at position 428) as well as the residues forming the DnaJ zinc finger motifs are underlined and printed in bold italics (see also Discussion). The nucleotide sequence shown has been deposited in the GenEMBL database under accession number Y09633.


XhoI
BamHI
BglII
-35
-10
T2
-35
-10
T1
NruI
EcoRV
dnaK
EcoRI
PstI
EcoRV
NruI
orf67
dnaJ
XhoI
orf>65
NruI

the sequenced DNA region using the 'CODONPREFERENCE' program revealed three additional ORFs. The first ORF (*orf>278*; not shown in Fig. 2.2), divergently oriented to *dnaKJ*, starts at position 836 and codes for the N-terminal end of a polypeptide of at least 278 amino acids. The second ORF (*orf67*) is located in the intergenic region between *dnaK* and *dnaJ* (position 3,149 to 3,352; Fig. 2.2). The corresponding gene product consists of 67 amino acids and has a predicted molecular weight of 7,229. Upstream of *orf67* a well conserved, presumptive ribosome binding site was detected (AAGAGG; position 3,140 to 3,145). The products of both ORFs, *orf>278* and *orf67*, showed no significant similarity to any known protein sequence in the database. There are two inverted repeats located in *orf67* which may represent rho-independent transcription termination signals (see Fig. 2.2 and Discussion). The third ORF (*orf>65*) starts 87 bp downstream of *dnaJ* (position 4,601) and is oriented in the same direction as the *dnaKJ* genes. The deduced 65 amino acids of this protein fragment showed similarity to the amino acid sequence encoded by the 5' end of a putative ORF located in the *dnaJ* downstream region in *Brucella ovis* (36.8% identity in 38 amino acids; Cellier *et al.*, 1992).

2.4.3 Heat-induced synthesis of DnaK

Heat-shock regulation of DnaK synthesis was studied by Western blot analysis of total protein extracts of *B. japonicum* cells using an antiserum raised against *E. coli* DnaK (kindly provided by B. Bukau; Fig. 2.3). The protein extracts were made from cells harvested before and at different time points after a temperature shift from 28°C to 43°C. Extracts prepared from *E. coli* MC4100 cells (heat-shocked for 4 min at 42°C)

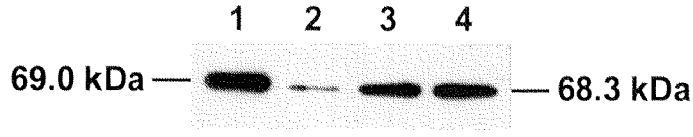


Fig. 2.3 Immunoblot analysis of *E. coli* and *B. japonicum* extracts using anti-*E. coli* DnaK serum. *B. japonicum* 110*spc*4 cells were grown to mid-exponential phase at 28°C. After a reference sample was taken (lane 2), the culture was shifted to 43°C and samples were collected at 2 (lane 3) and 5 min (lane 4) after the temperature shift. As a control, *E. coli* MC4100 cells were also grown to mid-exponential phase at 28°C. Some 4 min after shift to 42°C a sample was collected (lane 1). Crude cell extracts of all samples were prepared, separated on a 12% SDS polyacrylamide gel, and subjected to Western blot analysis. The calculated molecular masses of the *E. coli* and *B. japonicum* DnaK proteins are indicated at the left and right margins, respectively.

served as a control (Fig. 2.3, lane 1). Starting with a clearly detectable basal level of DnaK protein in *B. japonicum* cells grown at 28°C, a strong increase in the amount of DnaK was detected within 2 min after the temperature shift, indicating a rapid heat shock response in *B. japonicum*. The slightly lower apparent molecular mass of the *B. japonicum* DnaK protein compared with the *E. coli dnaK* gene product is in agreement with their distinct, calculated molecular weights of 68,292 (*B. japonicum*) and 68,983 (*E. coli*) derived from the respective gene sequences. Further experiments revealed that under continuous heat shock conditions, the amount of *B. japonicum* DnaK was kept at an elevated level for at least 5 h (data not shown). To assess whether the heat induction of DnaK occurs at the level of transcription, synthesis of *dnaK* mRNA was then analysed.

2.4.4 Transcriptional analysis of the *dnaKJ* gene region

The transcription start site of *B. japonicum dnaK* was determined by primer extension analysis with primers DnaK6 and DnaK12 which are complementary to the 5' end of the *dnaK* gene (results for DnaK12 shown in Fig. 2.4). A major signal (T1) corresponding to C_{1134} was detected 99 nucleotides upstream of the translational start of *dnaK* (see also Fig. 2.2). In addition, a second, minor signal (T2) corresponding to G_{1029}

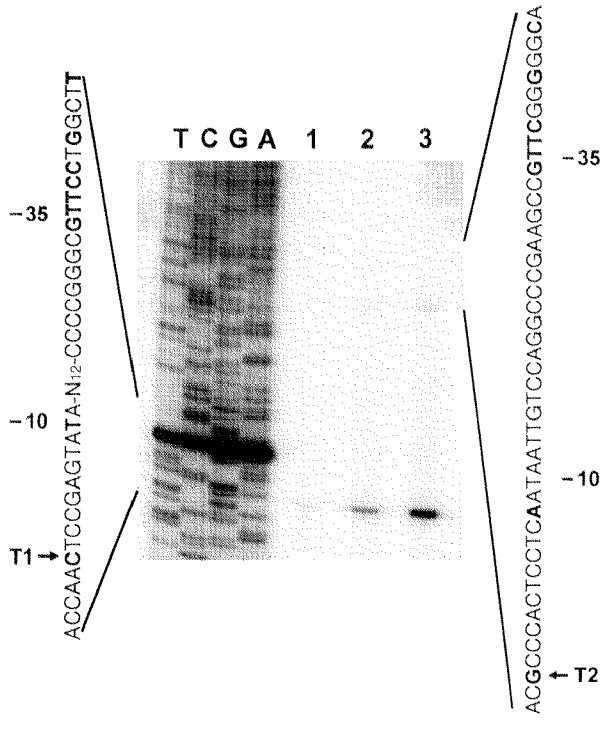


Fig. 2.4 Determination of the transcription start site of *dnaK* by primer extension mapping. Total RNA was isolated from *B. japonicum* 110*spc*4 cells harvested before (lane 1) and 30 min after a heat shock from 28°C to 39°C (lane 2) and to 43°C (lane 3). Identical amounts of RNA (5.8 µg) were used for the extension reactions with the DnaK12 primer. The same primer and an appropriate template DNA were used to produce the sequence ladder. The products of the primer extension and sequencing reactions were run side by side on the same gel. Both transcription start sites (T1 and T2) and conserved positions in the corresponding –10 and –35 promoter regions are emphasised in bold letters in the nucleotide sequence of the coding strand shown at the margins. The nucleotides in the strong compression zone upstream of T1 are known from higher resolution sequencing gels (*cf.* Fig. 2.2).

was observed 204 bp upstream of *dnaK*. Both transcripts were clearly heat inducible as indicated by the approximate 30- and 10-fold increase in intensity of the start signals T1 and T2, respectively, after a temperature shift from 28 to 43°C (Fig. 2.4, lane 3). Primer extension analysis in the *dnaKJ* intergenic region did not uncover a separate transcription start site for *dnaJ*, suggesting that the *dnaKJ* genes are organised in an operon (data not shown).

The –10 and –35 regions of the putative promoter associated with the start of transcript T1 includes motifs characteristic for σ^{32}-dependent promoters (see Fig. 2.7 and Discussion). Moreover, no CIRCE-like element could be detected around the transcriptional start sites of T1 and T2, indicating that the heat shock regulation of *B. japonicum dnaKJ* does not follow the CIRCE-dependent mechanism (see Introduction). Unfortunately, no *B. japonicum* mutant lacking σ^{32} is currently available, which makes it impossible to verify the presumptive σ^{32}-dependent transcription of *dnaKJ* in *B. japonicum* (see also Discussion). Therefore, the activity of the *B. japonicum dnaK* promoter was studied in *E. coli* in the presence of either *E. coli* RpoH or *B. japonicum* $RpoH_1$. *E. coli* strain KY1603 (*rpoH*⁻) was transformed with plasmid pRJ8203 (*dnaK*'-'*lacZ*) plus either pRJ5000 (*B. japonicum rpoH*$_1$), pEC5007 (*E. coli rpoH*), or pUC18

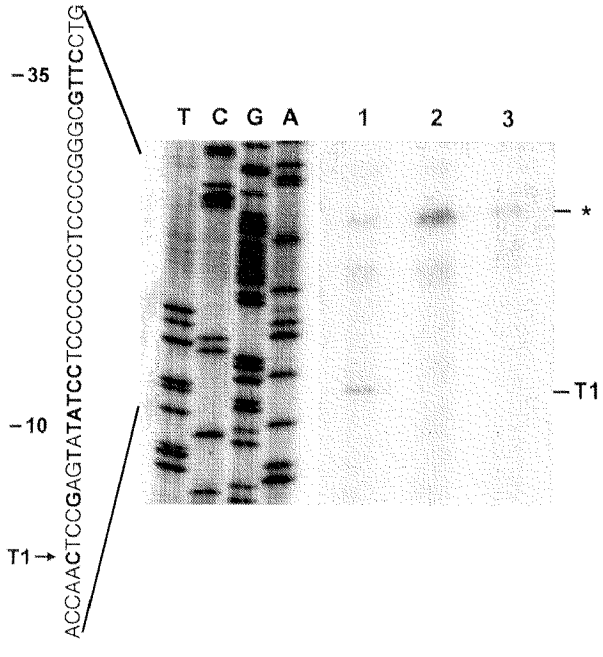


Fig. 2.5 *B. japonicum* $RpoH_1$-dependent transcription from the *B. japonicum dnaK* promoter in *E. coli*. RNA was isolated from *E. coli* KY1603 strains harbouring *B. japonicum dnaK*'-'*lacZ* (pRJ8203) plus either *B. japonicum rpoH*$_1$ (pRJ5000; lane 1), *E. coli rpoH* (pEC5007; lane 2), or pUC18 (control; lane 3). IPTG (0.5 mM) was present in the cultures for induction of $RpoH_1$ synthesis which is under the control of the *lac* promoter present on the vector pUC18. Twelve micrograms of RNA were used for the extension reactions with the DnaK28 primer. DnaK28 was also used as primer for preparation of the sequence ladder. T1 refers to the primer extension product in lane 1 which is specifically dependent on *B. japonicum* $RpoH_1$. This transcription start site T1 (C_{1134}) and conserved positions in the corresponding –10 and –35 promoter regions are emphasised in bold letters in the nucleotide sequence of the coding strand shown at the left margin. The identity of the *rpoH*-independent primer extension product marked with an asterisk is not known.

(vector control). Only colonies of *E. coli* KY1603/pRJ8203 plus pRJ5000 turned slightly blue when grown on solid medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 30 µg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM; data not shown). To further substantiate this observation, primer extension experiments were performed using RNA from the same three *E. coli* strains and primers DnaK12 and DnaK28 (results shown for primer DnaK28 in Fig. 2.5). In agreement with the result of the X-Gal plate test, a transcript (T1) was detected in the *E. coli* strain expressing *B. japonicum* $rpoH_1$ (present on pRJ5000; Fig. 2.5, lane 1) but neither in the *E. coli* strain expressing *rpoH* of *E. coli* (pEC5007) nor in the control strain containing the vector pUC18. Most notably, the transcript T1 started at the same position (C_{1134}) as the *dnaK* transcript observed in *B. japonicum* (see above).

2.4.5 Mutational analysis of the *dnaKJ* region

B. japonicum dnaJ mutants were constructed by insertion of a kanamycin resistance cassette into the *Xho*I site located in the *dnaJ* gene (see Fig. 2.1 and Materials and methods). The genomic structure of *B. japonicum* strains 8201 and 8202, which differ with respect to the orientation of the inserted resistance gene, was verified by Southern blot hybridisation with a *B. japonicum dnaJ* probe (see Materials and methods). Unfortunately, the effect of the mutations on DnaJ synthesis could not be analysed by Western blot hybridisation because no suitable anti-DnaJ serum was available.

The symbiotic properties of the *dnaJ* mutants 8201 and 8202 were tested in a plant infection test. None of the mutant strains differed from the wild type with respect to the ability to nodulate soybean roots and fix nitrogen under symbiotic conditions (data not shown). This indicates that the *dnaJ* gene product is not essential for symbiosis. The mutants were further analysed for their growth behaviour at 26 and 33°C (Fig. 2.6). Aerobic growth of the mutants in rich medium (PSY medium) was significantly slowed as compared with the wild type. In the wild type, the doubling times at 26 and 33°C were 14 and 10 h, respectively, while in the *dnaJ* mutant strains 8201 and 8202 the corresponding values were between 20 and 25 h.

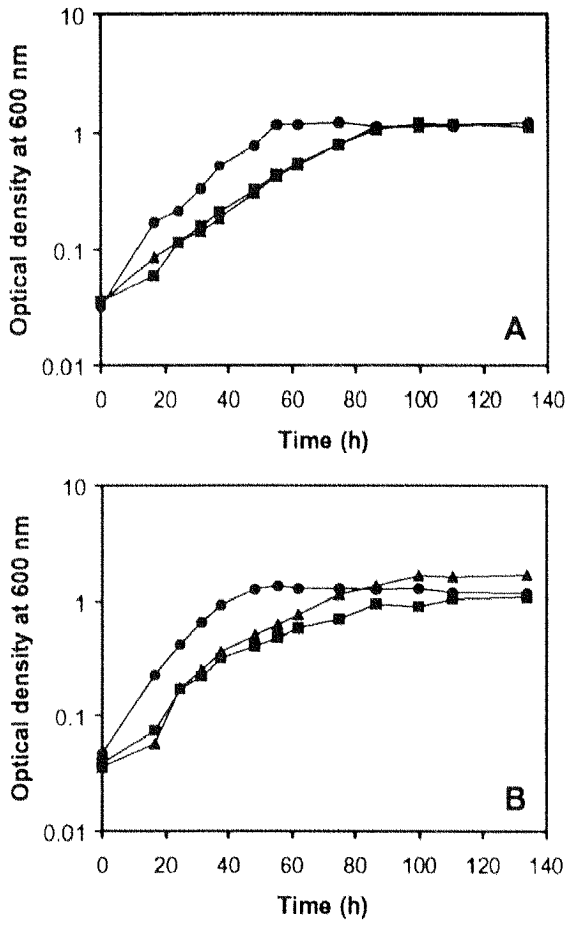


Fig. 2.6 A, B. Growth of *B. japonicum* wild type (●) and *dnaJ* mutants 8201 (■) and 8202 (▲) in rich medium (PSY) under aerobic conditions at 26°C (**A**) and 33°C (**B**). The growth curves are based on mean values from two parallel cultures.

In order to mutate the *B. japonicum dnaK* gene several attempts were made to create an insertion and a deletion derivative of *dnaK*. However, our efforts to insert a kanamycin resistance cassette into the central *Eco*RV site of *dnaK* or to replace the 1.5 kb *Eco*RV-*Nru*I fragment containing the 3' portion of *dnaK* and *orf67* by the kanamycin resistance gene (see pRJ8165 in Fig. 2.1) were unsuccessful, even when the matings and the subsequent screening procedure were performed at 25°C to allow growth of potentially temperature-sensitive *dnaK* mutants. By contrast, we were able to insert the kanamycin resistance gene cassette in both orientations into the *Nru*I site located just 223 bp downstream of *dnaK* in the *dnaKJ* intergenic region (mutant strains 8168 and 8170; see Fig. 2.1). In the mutagenesis experiments performed to construct *B. japonicum* mutants 8168 and 8170 the mating resulted in 28% Km^r Tc^s (potential marker exchange mutants) and 72% Km^r Tc^r clones (potential co-integrates). The corresponding values derived from the matings with pRJ8165 (*dnaK::aphII*) were 2.8% Km^r Tc^s and 97.2% Km^r Tc^r clones. The low proportion of Km^r Tc^s clones reflects the number of spontaneously occurring Km^r *B. japonicum* clones which appeared with

about the same frequency in mating controls lacking the *E. coli* donor strain. Thus, in the matings with pRJ8165 apparently only co-integrates were obtained. In fact, four clones tested by Southern blot hybridisation were shown to harbour pRJ8165 integrated in the chromosome in such a manner that an intact copy of the *dnaK* gene was retained (data not shown; see also Discussion).

2.4.6 Complementation of an *E. coli dnaK* mutant strain with *B. japonicum dnaK*

The function of the *B. japonicum* DnaK protein in *E. coli* was studied by complementation tests using the *E. coli dnaK* deletion mutant GW4813, which is unable to grow at temperatures higher than 37°C. Two plasmids, both containing the complete *B. japonicum dnaKJ* region in either the same (pRJ8163) or in opposite orientation (pRJ8164) relative to the *lac* promoter of the pUC18 vector, were transformed individually into *E. coli* GW4813. The resulting strains were tested for growth at 30, 37 and 42°C in the absence or presence of IPTG (for induction of the *lac* promoter). Unlike the control plasmid pDM38 (*E. coli dnaKJ*$^+$), none of the *B. japonicum dnaKJ* plasmids could restore growth at 42°C of the *E. coli dnaK* mutant. Western blot analysis proved that DnaK protein was indeed synthesised in cells containing pRJ8163 but not in those harbouring pRJ8164, indicating that expression from the *lac* promoter did occur (data not shown).

2.5 Discussion

Previous studies on the expression of a *groESL* multigene family revealed that heat shock regulation in *B. japonicum* is more complex than in most other bacteria (Fischer *et al.*, 1993; Babst *et al.*, 1996). To gain more detailed insight into the heat shock control systems of *B. japonicum* we have investigated the molecular organisation and regulation of the *dnaKJ* genes encoding the evolutionarily highly conserved heat shock proteins DnaK and DnaJ (Gupta & Golding, 1993; Boorstein *et al.*, 1994).

The analysis of the sequenced *B. japonicum* DNA region revealed five ORFs. Two of them were identified as the *dnaK* and *dnaJ* homologues, which are probably organised in an operon, as in most α- and γ-proteobacteria (Segal & Ron, 1996b). In contrast to the case in many other bacteria, the gene encoding the co-chaperone GrpE is not associated with the *dnaKJ* genes. Interestingly, a short ORF (*orf67*) was found in the unusually large *dnaK-dnaJ* intergenic region. Two putative stem-loop structures located in *orf67* (see Fig. 2.2) might cause at least a partial transcription termination, thereby attenuating expression of *orf67* and *dnaJ*. Such a mechanism has already been proposed for the *E. coli dnaKJ* operon to explain the ten-fold lower level of DnaJ protein as compared with DnaK (Bardwell *et al.*, 1986; Ohki *et al.*, 1986).

The pronounced conservation of bacterial chaperones is reflected by the high degree of identity in amino acid sequence between the DnaK and DnaJ proteins of *B. japonicum* and those of the selected bacterial species listed in Table 2.2 (64.1-81.6% and 44.8-63.6% identity for DnaK and DnaJ, respectively). Accordingly, it was easy to identify in the *B. japonicum* DnaK protein those amino acid residues which have been shown to be involved in the interaction of the *E. coli* DnaK protein with Mg-ADP (McKay *et al.*, 1994), the co-chaperone GrpE (Buchberger *et al.*, 1994) and target substrates (Zhu *et al.*, 1996) (see Fig. 2.2). Similarly, in the DnaJ protein both the N-terminal HPD motif presumed to interact with DnaK (Wall *et al.*, 1994) and the central cysteine-rich region likely to be involved in the formation of two zinc finger motifs (Szabo *et al.*, 1996) are present.

Table 2.2 Amino acid sequence similarity matrix of DnaK and DnaJ proteins from different bacteria

Organism[a]	DnaK (% identity)					DnaJ (% identity)		
	B	C	D	E	F	D	E	F
(A) *Bradyrhizobium japonicum*	81.6	81.4	79.2	68.5	64.1	63.6	54.6	44.8
(B) *Agrobacterium tumefaciens*		89.3	83.1	67.7	64.6			
(C) *Rhizobium meliloti*			83.0	68.5	64.3			
(D) *Brucella ovis*				67.3	62.4		58.2	45.9
(E) *Escherichia coli*					58.5			52.6
(F) *Bacillus subtilis*								

[a] References are: *A. tumefaciens* DnaK (Segal & Ron, 1995); *R. meliloti* DnaK (Falah & Gupta, 1994); *B. ovis* DnaK and DnaJ (Cellier *et al.*, 1992); *E. coli* DnaK (Bardwell & Craig, 1984); *E. coli* DnaJ (Bardwell *et al.*, 1986); *B. subtilis* DnaK and DnaJ (Wetzstein *et al.*, 1992).

The *B. japonicum dnaKJ* genes were functionally analysed by mutagenesis and complementation experiments. They were not able to rescue the growth defect at 42°C of the *E. coli dnaK* mutant strain GW4813, even when transcription was forced from the *lac* promoter. Although at least some *B. japonicum* DnaK protein was immunologically detectable, the expression level was possibly too low for successful complementation. Alternatively, it is possible that the *B. japonicum* DnaK or DnaJ proteins differs from the corresponding *E. coli* chaperone with regard to function during growth at high temperature. At least partial complementation of an *E. coli dnaK* mutant has been reported with *dnaK* of *Zymomonas mobilis* (Michel, 1993), *Francisella tularensis* (Zuber *et al.*, 1995) and *Brucella ovis* (Cellier *et al.*, 1992), whereas this was not possible with the *dnaK* genes of *Bacillus megaterium* (Sussman & Setlow, 1987), *Mycobacterium tuberculosis* (Mehlert & Young, 1989) or *Borrelia burgdorferi* (Tilly *et al.*, 1993).

All our attempts to knock out the *dnaK* gene in *B. japonicum* failed, even when the mutagenesis and selection procedures were performed at a low temperature (25°C) to enable survival of potentially temperature-sensitive *dnaK* mutants. By contrast, we easily obtained viable insertion mutants close to the 3' end of the *dnaK* gene (*Nru*I site at position 3,356). Also, we were able to isolate co-integrate structures resulting from chromosomal integration of plasmid pRJ8165 (*dnaK::aphII*) via a single cross-over

event in the *dnaK* region. In principle, two different types of co-integrates were expected depending on where the cross-over takes place. Cross-overs occurring upstream of the kanamycin resistance cassette present on pRJ8165 would lead to two disrupted *dnaK* genes in the chromosome, whereas cross-overs downstream of the cassette would result in one intact and one disrupted *dnaK* gene. Interestingly, all of the co-integrates we tested by Southern blot analysis showed the latter structure. Hence, this finding, together with the fact that insertions in the *dnaKJ* intergenic region were readily obtained, indicated that the mutagenesis procedure *per se* worked. We therefore postulate that the failure to isolate *dnaK* knock-out mutants is due to the fact that the *dnaK* gene product has an essential function. This interpretation is compatible with our finding that significant amounts of DnaK protein are synthesised under normal (i.e., non-heat shock) conditions and that hybridisation experiments do not indicate the presence of a second *dnaK*-like gene that might substitute for the *dnaK* gene we attempted to mutagenise. Interestingly no *dnaK* null mutants have been obtained in *Streptomyces coelicolor* A3(2) (Brans *et al.*, 1996) either, whereas such mutants are available in *E. coli* (Bukau & Walker, 1989) and *Brucella suis* (Köhler *et al.*, 1996); however, these latter mutants are extremely temperature sensitive and display several additional defects. Only *dnaK*-deficient *B. subtilis* strains have been reported to be viable over a wide temperature range (16-52°C; Schulz *et al.*, 1995).

The analysis of *B. japonicum dnaJ* mutants indicated that the DnaJ protein is not absolutely required for free-living growth and symbiotic nitrogen fixation, although the mutation resulted in a reduced growth rate, particularly at elevated temperatures. Such an effect was also observed in *dnaJ* null mutants of *E. coli*, in which DnaJ is not essential for growth at temperatures up to 42°C (Sell *et al.*, 1990). This has been explained by the existence of a DnaJ analogue, named CbpA, which can compensate for loss of DnaJ (Ueguchi *et al.*, 1994; Ueguchi *et al.*, 1995). Although Southern hybridisations using a *B. japonicum dnaJ* probe provided no evidence for a second *dnaJ* homologue, the existence of a functional analogue of DnaJ cannot be ruled out in *B. japonicum*.

Primer extension experiments revealed two transcription start sites (T1 and T2) located upstream of *dnaK*, both of which are used under normal growth conditions and strongly induced upon heat shock. The nucleotide sequences surrounding these start

sites did not display any similarity to the conserved inverted repeat sequence (CIRCE) that is located in the promoter regions of many *groE* and *dnaK* operons (Zuber & Schumann, 1994; Hecker *et al.*, 1996; Schumann, 1996). Thus, heat shock regulation of the *B. japonicum dnaKJ* genes does not follow the CIRCE-dependent mechanism which was previously shown to control *groESL*$_4$ expression in this organism (Babst *et al.*, 1996). This is further supported by the observation that maximal induction of *dnaK* transcription occurs in cells shifted to 43°C; this differs from the CIRCE-dependent transcription of *B. japonicum groESL*$_4$ which reaches its maximum at 39°C (Babst *et al.*, 1996). Moreover, the kinetics of DnaK protein synthesis upon heat treatment were very similar to those observed for the heat shock σ factor RpoH$_1$ (Narberhaus *et al.*, 1996; see below) but clearly differed from the transiently enhanced expression of *groESL*$_4$.

Promoter	**–35 Region**		**–10 Region**	**+1**
B. japonicum dnaK P1	TTCGGTCCTTGCG	GGCCCCTCCCCCCC	TCCTATATGAG	CCTC
B. japonicum dnaK P2	ACGGGGGCTTGCC	GAAGCCCGGACCTGT	TAATAACTCCT	CACCCG
B. japonicum groESL$_1$	AAGACCTCTTGAA	CGGCCGTTTTTCGGA	TCCTAGGTCGT	TTTCGC
A. tumefaciens dnaK	CTTGCGCCTTGCA	GCAGGAAAAATCGCT	CCTTATATACG	CCGC
C. crescentus dnaK P1	TTATGGCCTTGCG	TGGCGGCCCCTATC	CCCCATATCCG	GCTTCG
α-purple proteobacteria heat shock consensus:	CTTG	17-18 bp	CYTAT-T--G	
E. coli σ^{32} consensus:	TCTC-CCCTTGAA	13-17 bp	CCCCAT-TA	

Fig. 2.7 Comparison of the *B. japonicum dnaK* promoter sequences with the promoters of *B. japonicum groESL*$_1$ (Babst *et al.*, 1996), *A. tumefaciens dnaK* (Segal & Ron, 1995) and *C. crescentus dnaK* (P1; Gomes *et al.*, 1990). Nucleotides matching the –35/–10 consensus sequence proposed for heat shock promoters in α-proteobacteria (Segal & Ron, 1995) and transcriptional start sites are emphasised in bold letters. Additional positions occupied by identical nucleotides as in the *E. coli* σ^{32} consensus promoter (Yura *et al.*, 1993) are underlined. The abbreviation Y stands for pyrimidine.

The transcription start site T1 of *dnaK* is preceded by the promoter P1, whose –35 and –10 regions perfectly match the consensus sequence proposed for heat shock promoters in proteobacteria of the α subdivision (Segal & Ron, 1995; Fig. 2.7). Very similar heat shock promoters are present upstream of the *dnaKJ* operons of *A. tumefaciens* and *C. crescentus* (Fig. 2.7). By contrast, the –10 region of promoter P2 associated with the transcription start site T2 is less well conserved, which may account

for its rather weak activity. The similarity of the consensus heat shock promoter in α-purple bacteria to σ^{32}-dependent promoters of *E. coli* implies that this promoter type is recognised by a form of the RNA polymerase which contains a σ factor similar to σ^{32}. In fact, it was demonstrated that the σ^{32}-like protein encoded by the *C. crescentus rpoH* gene specifically recognises the P1 promoter of the *C. crescentus dnaK* gene (Wu & Newton, 1996). Recently, the existence of a σ^{32} homologue was also documented in *B. japonicum* by the molecular characterisation of the $rpoH_1$ gene (Narberhaus *et al.*, 1996). Immunological and genetic data indicated that *B. japonicum* harbours multiple *rpoH*-like genes whose detailed analysis is currently in progress (F. Narberhaus, manuscript submitted). In this work we have demonstrated that in the *E. coli* background, the product of *B. japonicum* $rpoH_1$ is required for specific transcription from the *B. japonicum dnaK* promoter P1. It will be interesting to find out in future studies whether the products of the alternative *B. japonicum rpoH* genes are able to initiate transcription from the *dnaK* promoter P1, as $RpoH_1$ does. In contrast to our previous results from expression studies on the *B. japonicum* $groESL_1$ promoter (Babst *et al.*, 1996), RNA polymerase of *E. coli* associated with its own σ^{32} factor (RpoH) failed to initiate detectable transcription from the P1 promoter of *B. japonicum dnaK*. It is tempting to speculate that the disparate activities of *E. coli* σ^{32}-RNA polymerase at the *B. japonicum dnaK* and $groESL_1$ promoters are related to the differences in their respective –35 promoter regions which make the $groESL_1$ promoter more similar to the *E. coli* σ^{32} consensus than is the *dnaK* promoter (Fig. 2.7).

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CHAPTER 3

The role of HrcA and CIRCE in the heat shock regulatory network of *Bradyrhizobium japonicum*

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3.1 Abstract

A large number of bacteria regulate chaperone gene expression by the CIRCE-HrcA system in which the DNA element called CIRCE serves as binding site for the repressor protein HrcA under non-heat shock conditions. We have cloned the two consecutive genes *hrcA* and *grpE* of *Bradyrhizobium japonicum* by using a complementation approach that screened for GrpE function. *In vivo* and *in vitro* transcript mapping demonstrated that both genes are transcribed separately from RpoH (σ^{32})-dependent promoters. To investigate the supposed negative regulatory function of HrcA, we compared the expression of putative target genes in the wild type and in a *hrcA* mutant. Transcription of the CIRCE-associated chaperonin operons *groESL*$_4$ and *groESL*$_5$ as well as the β-galactosidase activity derived from corresponding *groE-lacZ* fusions was strongly elevated in the *hrcA* mutant even at physiological temperatures. Expression of other heat shock regulons (RpoH- or ROSE-dependent) was not affected. To study the activity of HrcA *in vitro*, we purified a histidine-tagged version of the protein under non-denaturing conditions. Specific binding to the CIRCE element was obtained with a soluble fraction of HrcA in gel retardation experiments. This DNA binding activity was neither improved by the GroE nor by the DnaK chaperone machinery. Rather, the addition of GroESL led to a reduction of HrcA-CIRCE complexes *in vitro*. This result is in conflict with data obtained *in vivo* which suggested a positive effect of GroESL on HrcA activity. Possible reasons for this apparent discrepancy are discussed.

Key words: CIRCE; heat shock; *hrcA*; regulatory network; repressor

3.2 Introduction

The survival of a cell depends on its ability to adapt to changing environmental conditions. In the case of a sudden temperature upshift, all organisms cope with the increased amount of misfolded proteins by an enhanced synthesis of so-called heat shock proteins (Hsps). This collection of proteins consists mainly of chaperones and proteases, which are involved in protein (re)folding and protein degradation, respectively (for reviews see Bukau & Horwich, 1998; Herman & D'Ari, 1998).

Both eukaryotic and prokaryotic cells have established a number of complex regulatory strategies to tightly control the transcription of heat shock genes under any given condition (Mager & De Kruijff, 1995; Morimoto, 1998; Narberhaus, 1999). Coordinate expression of heat shock genes in *Escherichia coli* is mediated by alternative sigma factors, which direct the RNA polymerase to specific promoter sequences upstream of these genes. An increase in the cellular concentration of σ^{32} (RpoH) after a sudden temperature upshift results in elevated transcription of more than 30 genes of the σ^{32} regulon. Activity and stability of σ^{32} are subject to feedback-control by the DnaK machinery, which sequesters the sigma factor under low-temperature conditions. Binding of σ^{32} to the DnaK system prevents association with the RNA polymerase core enzyme and promotes degradation of σ^{32} by the FtsH protease. After a heat shock, denatured proteins presumably titrate the DnaK chaperones away from σ^{32} leaving the latter stable and competent for complex formation with RNA polymerase (for recent reviews see Gross, 1996; Yura & Nakahigashi, 1999).

In contrast to the positive regulation by alternative sigma factors, heat shock expression in a majority of bacteria was found to be controlled by negative regulation (summarised in Narberhaus, 1999). Our knowledge of such systems is by far less advanced than that of the *E. coli*-type regulation. The regulatory principle is often based on a specific interaction between a repressor protein and a DNA element that is located in the promoter region of heat shock genes. Repression is relieved upon a temperature upshift, thereby allowing transcription of the downstream genes. A widespread negative control mechanism consists of the repressor protein HrcA and a DNA element called CIRCE (for Controlling Inverted Repeat of Chaperone Expression; Zuber & Schumann, 1994). First evidence that this highly conserved inverted repeat might act as a negative *cis* element was obtained by the observation that mutations in one or both arms of the

inverted repeat resulted in elevated transcription of the downstream genes even at normal growth temperatures (van Asseldonk *et al.*, 1993; Zuber & Schumann, 1994). Chromosomal mutations in *Bacillus subtilis* which affected the expression of CIRCE-dependent genes were localised in *orf39*, the first gene of the *dnaK* operon (Schulz *et al.*, 1995; Yuan & Wong, 1995b). Disruption of *orf39* in *B. subtilis* and of the equivalent gene in *Caulobacter crescentus* confirmed the function of the Orf39 protein as negative regulator of CIRCE-dependent genes and led to its designation as HrcA (for Heat Regulation at CIRCE) (Roberts *et al.*, 1996; Schulz & Schumann, 1996). By analogy with the feedback-control of σ^{32} activity and stability by the DnaK machinery in *E. coli*, the activity of *B. subtilis* HrcA was found to be modulated by the GroE chaperonin system (Mogk *et al.*, 1997). A direct correlation between the cellular GroESL level and HrcA activity suggested that the repressor requires chaperonins for proper function *in vivo* (Babst *et al.*, 1996; Mogk *et al.*, 1997). Accordingly, heat stress would render HrcA inactive because GroES and GroEL become engaged in the refolding of denatured proteins.

Unfortunately, attempts to confirm the proposed HrcA-CIRCE and HrcA-GroEL interactions *in vitro* were hampered by the insolubility of overproduced HrcA, which tends to form inclusion bodies, requiring purification under denaturing conditions. Despite this shortcoming, it has been possible to visualise the binding of purified HrcA of *Staphylococcus aureus* to the CIRCE element by atomic force microscopy (Ohta *et al.*, 1996) and to document CIRCE binding of purified *Bacillus stearothermophilus* HrcA in gel retardation experiments (Mogk *et al.*, 1997).

In *Bradyrhizobium japonicum*, the nitrogen-fixing root-nodule symbiont of soybean, an unusually complex network of both positive and negative regulatory mechanisms controls transcription of heat shock genes (Narberhaus, 1999). Three different regulatory systems have been identified so far. One class of heat shock genes is transcribed from σ^{32}-dependent promoters. The search for the corresponding sigma factor revealed the existence of three disparately regulated *rpoH* genes whose σ^{32}-like products have different promoter specificities (Narberhaus *et al.*, 1997; Narberhaus *et al.*, 1998b). The expression of a second class of heat shock genes is negatively controlled by a highly conserved DNA element called ROSE (for Repression Of heat Shock gene Expression) (Narberhaus *et al.*, 1998a). Evidence for a third class of heat

shock genes was deduced from the presence of a CIRCE element in the promoter region of the heat shock-inducible chaperonin operons *groESL*$_4$ and *groESL*$_5$ (Babst *et al.*, 1996). Elevated transcription from the *groESL*$_4$ promoter at normal temperatures, caused either by a deletion of 4 bp within the corresponding CIRCE element or by disruption of *groEL*$_4$, had clearly demonstrated the regulatory function of CIRCE and, moreover, indicated the presence of a feedback regulatory mechanism. According to these observations, the existence of a CIRCE-specific repressor protein whose stability and/or activity is modulated by chaperonins was suggested.

Here, we report the cloning of the *B. japonicum hrcA* gene and the assignment of its position in the complex heat shock network of this organism. The function of the HrcA protein as a CIRCE-specific repressor protein is demonstrated by a complementary set of *in vivo* and *in vitro* experiments.

3.3 Materials and Methods

3.3.1 Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 3.1. *E. coli* cells were grown in Luria-Bertani (LB) medium (Miller, 1972) supplemented with ampicillin (200 µg/ml), chloramphenicol (20 µg/ml), kanamycin (30 µg/ml), or tetracycline (10 µg/ml) if required. The growth temperature for *E. coli* strains was 37°C except for *E. coli* DA259 (*grpE*^{ts}) which was grown at 30°C. *B. japonicum* strains were propagated aerobically at 30°C in PSY medium (Regensburger & Hennecke, 1983) supplemented with 0.1% (w/v) arabinose. If appropriate, antibiotics were added at the following concentrations (µg/ml): chloramphenicol, 20 (for counterselection against *E. coli* donor strains); kanamycin, 100; spectinomycin, 100; and tetracycline, 50.

Table 3.1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype	Reference or origin
E. coli		
DH5α	*supE44* Δ*lacU169* (ψ80*lacZ*ΔM15) *hsdR17 recA1* *gyrA96 thi-1 relA1*	Gibco-BRL, Gaithersburg, MD, USA
S17-1	Sm^r Sp^r *hdsR* RP4-2 *kan*::Tn*7 tet*::Mu, integrated in the chromosome	(Simon *et al.*, 1983)
C600	*supE44 hsdR thi-1 leuB6 lacY1 tonA21*	(Wu *et al.*, 1994)
DA258	Cm^r Km^r Tc^r (C600) *grpE*$^+$::Ω-Cm *thr*::Tn*10*	(Ang & Georgopoulos, 1989)
DA259	Cm^r Km^r Tc^r (C600) Δ*grpE*::Ω-Cm *thr*::Tn*10*	(Ang & Georgopoulos, 1989)
BL21/pLysS	Cm^r F$^-$ *ompT* r_B^- m_B^-, T7*lys*	(Studier *et al.*, 1990)
B. japonicum		
110*spc*4	Sp^r (wild type)	(Regensburger & Hennecke, 1983)
5400[a]	Sp^r Tc^r *hspA*'-'*lacZ* chromosomally integrated	(Narberhaus *et al.*,1998a)
5549	Sp^r Km^r *hrcA*::*kan*; *hrcA* and *kan* oriented in opposite directions	This study
5554[a]	Sp^r Tc^r *grpE*'-'*lacZ* chromosomally integrated	This study

(continued)

(Table 3.1 continued)

5559[a]	Sp^r Tc^r *hrcA*'-'*lacZ* chromosomally integrated	This study
7998[a]	Sp^r Tc^r *groEL*$_2$'-'*lacZ* chromosomally integrated	(Fischer *et al.*, 1993)
8067[a]	Sp^r Tc^r *groEL*$_1$'-'*lacZ* chromosomally integrated	This study
8092[a]	Sp^r Tc^r *groEL*$_5$'-'*lacZ* chromosomally integrated	This study
8174[a]	Sp^r Tc^r *dnaK*'-'*lacZ* chromosomally integrated	This study
8548[a]	Sp^r Tc^r *groEL*$_4$'-'*lacZ* chromosomally integrated	(Fischer *et al.*, 1993)
Plasmids		
pBluescript II KS+	Ap^r	Stratagene, La Jolla, CA, USA
pBluescript II SK+	Ap^r	Stratagene, La Jolla, CA, USA
pBSL15	Ap^r Km^r	(Alexeyev, 1995)
pUC18	Ap^r	(Norrander *et al.*, 1983)
pSUP202	Ap^r Cm^r Tc^r *oriT* from RP4	(Simon *et al.*, 1983)
pSUP202pol4	Tc^r (pSUP202) part of polylinker from pBluescript II KS+ between *Eco*RI and *Pst*I	(Fischer *et al.*, 1993)
pNM481	Ap^r (pUC8) '*lacZ*	(Minton, 1984)
pNM482	Ap^r (pUC8) '*lacZ*	(Minton, 1984)
pSUP481	Tc^r (pSUP202pol4) '*lacZ* (5.1 kb *Eco*RI-*Stu*I fragment from pNM481)	H.M. Fischer, unpublished
pSUP482	Tc^r (pSUP202pol4) '*lacZ* (5.1 kb *Eco*RI-*Stu*I fragment from pNM482)	H.M. Fischer, unpublished
pET28a(+)	Km^r	Novagene, Madison, WI, USA
pWSK29	Ap^r, low-copy-number vector	(Wang & Kushner, 1991)
pBW401	Ap^r (pWSK29) 1.3 kb *Sal*I-*Eco*RI fragment containing *E. coli grpE*	(Wu *et al.*, 1994)
pRJ5099	Ap^r (pRJ9519) 0.9 kb *Bgl*II-*Eco*RI fragment containing the *B. japonicum dnaKJ* promoter region	(Narberhaus *et al.*, 1998b)
pRJ5400	Tc^r (pSUP202pol4) *hspA*'-'*lacZ*	(Narberhaus *et al.*,1998a)
pRJ5523[b]	Ap^r (pUC18) 3.9 kb *Eco*RI fragment containing *B. japonicum* '*hrcA*, *grpE* and '*dnaK* opposite to α*lacZ* orientation	This study

(continued)

(Table 3.1 continued)

pRJ5530[b]	Ap^r (pBluescript II SK+) 3.0 kb *Not*I fragment containing *B. japonicum hrcA* and *grpE* opposite to α*lacZ* orientation	This study
pRJ5542	Ap^r (pRJ9519) 0.5 kb *Not*I-*Nru*I fragment from pRJ5530 containing the *B. japonicum hrcA* promoter region	This study
pRJ5543	Ap^r (pRJ9519) 0.7 kb *Sac*I-*Fsp*I fragment from pRJ5530 containing the *B. japonicum grpE* promoter region	This study
pRJ5549	Tc^r Km^r (pSUP202pol4) *hrcA::kan*; *hrcA* and *kan* oriented in opposite directions	This study
pRJ5552	Km^r (pET28a(+)) 1.1 kb *Not*I–*Nde*I amplification product containing *B. japonicum hrcA* gene, plasmid for overexpression of H_6-HrcA	This study
pRJ5554	Tc^r (pSUP482) *grpE*'-'*lacZ*, 348 bp *Msc*I-*Fsp*I *grpE*' fragment from pRJ5530	This study
pRJ5556	Ap^r (pUC18) 198 bp amplification product containing the promoter region of *B. japonicum* $groESL_4$	This study
pRJ5558	Ap^r (pUC18) 265 bp amplification product containing the promoter region of *B. japonicum* $groESL_5$	This study
pRJ5559	Tc^r (pSUP481) *hrcA*'-'*lacZ*, 937 bp *Eco*RI *hrcA*' fragment from pRJ5530	This study
pRJ7998	Tc^r (pSUP202pol4) $groEL_2$'-'*lacZ*	(Fischer *et al.*, 1993)
pRJ8067	Tc^r (pSUP202pol4) $groEL_1$'-'*lacZ*	(Babst *et al.*, 1996)
pRJ8092	Tc^r (pSUP202pol4) $groEL_5$'-'*lacZ*	(Babst *et al.*, 1996)
pRJ8174	Tc^r (pSUP202pol4) *dnaK*'-'*lacZ*	(*cf.* chapter 2)
pRJ8548	Tc^r (pSUP202pol4) $groEL_4$'-'*lacZ*	(Babst *et al.*, 1996)
pRJ9519	Ap^r (pBluescript II SK+) containing the *B. japonicum rrn* terminator	(Narberhaus *et al.*, 1998b)

[a] The same '*lacZ* fusions were also integrated into the chromosome of Bj5549. The resulting strains were given the same numbers preceded by "H"; see Table 3.3.
[b] The insert of this plasmid is depicted in Fig. 3.1.

3.3.2 DNA manipulations

Recombinant DNA techniques were performed according to standard protocols (Sambrook *et al.*, 1989). Construction of *B. japonicum* mutants by cointegrate formation or marker exchange mutagenesis, and isolation of chromosomal DNA from *B. japonicum* were performed as described previously (Hahn & Hennecke, 1984). Southern blot hybridisations using DIG (digoxigenin-11-dUTP)-labelled DNA probes were performed according to the manufacturer's instructions (Boehringer Mannheim). DNA was sequenced by the chain termination method (Sanger *et al.*, 1977) with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The DNA region sequenced and the deduced proteins were analysed using the software package of the UWGCG (Genetics Computer Group of the University of Wisconsin, Madison, USA; version 8.0) or the NCBI (National Center for Biotechnology Information) BLAST network server. The 'CODONPREFERENCE' program was applied using the *B. japonicum* codon usage table (Ramseier & Göttfert, 1991).

3.3.3 Construction of strains and plasmids

For the construction of *hrcA* deletion mutants the 2.0 kb *Not*I (position no. 2 in Fig. 3.1)-*Xmn*I (2,015) fragment of pRJ5530 was ligated into the 6.7 kb *Not*I-*Sma*I vector fragment of pSUP202pol4. In the resulting plasmid the 561 bp *Sal*I (706)-*Xho*I (1,267) internal fragment of *hrcA* was replaced by the 1.2 kb *Sal*I fragment of pBSL15 containing the neomycin phosphotransferase II cassette (Km^r). Both orientations of the resistance cassette relative to the *hrcA* gene were obtained, but for the subsequent work we used plasmid pRJ5549 in which the remaining portion of the *hrcA* gene and the resistance cassette are oriented in opposite directions. Plasmid pRJ5549 was mobilised from *E. coli* S17-1 into *B. japonicum* 110*spc*4 for marker replacement mutagenesis, yielding strain 5549 (Fig. 3.1).

In order to compare the expression of heat shock genes in a wild-type and a *hrcA* mutant background, suitable translational *lacZ* fusions present on pSUP202pol4 derivates were cointegrated via homologous recombination into the chromosome of *B. japonicum* wild type and 5549 (Table 3.1). The correct genomic structure of all mutant strains was confirmed by Southern blot hybridisation using appropriate DIG-labelled DNA probes.

Plasmids used as templates for *in vitro* transcription experiments were based on pRJ9519, which contains the *B. japonicum rrn* terminator and in which different promoter regions were introduced (Narberhaus *et al.*, 1998b). Plasmid pRJ5542 carries a 537 bp *Not*I (position no. 2 in Fig. 3.1)-*Nru*I (538) fragment containing the *B. japonicum hrcA* promoter region. The promoter region of *B. japonicum grpE* was introduced as a 691 bp *Sac*I (1,109)-*Fsp*I (1,796) fragment yielding plasmid pRJ5543.

To construct the overproduction plasmid pRJ5552, the *hrcA* gene was amplified from plasmid pRJ5530 by PCR with *Taq* DNA polymerase. The oligonucleotides used as primers were designed such that they introduced a *Nde*I recognition site overlapping the start codon and a *Not*I site immediately downstream of the stop codon of *hrcA*. The PCR-generated fragment was cut with both *Nde*I and *Not*I and ligated into pET28a(+) digested with the same enzymes. The encoded recombinant protein H_6-HrcA possesses a N-terminal extension of 20 amino acids adding 2.1 kDa to the molecular mass of HrcA (39.2 kDa). The correct nucleotide sequence of the PCR-amplified *hrcA* fragment was confirmed by sequencing plasmid pRJ5552.

3.3.4 Transcript mapping

RNA isolation and primer extension analysis was performed as described (Babst *et al.*, 1996). The following oligonucleotides were used to determine the transcription start sites upstream of *hrcA* or *grpE*:

HrcA10 (*hrcA*, 579) 5'-CAGCCGGGAAATATTGCGTGAGCCCACC-3'

HrcA11 (*hrcA*, 630) 5'-CAGATCGGCCATGACGTTGCGAACCGAG-3'

GrpE4 (*grpE*, 1,742) 5'-CTTCCTTCTGCAACAGCTCGACCGAGCC-3'

GrpE5 (*grpE*, 1,700) 5'-CGGGCATGATGTAGGGCTTCGACACCAC-3'

The numbers in parentheses indicate the position of the 5' end based on the numbering used in the physical map shown in Fig. 3.1.

3.3.5 *In vitro* transcription

Single-round transcription assays with *B. japonicum* RNA polymerase holoenzyme and core enzyme reconstituted with purified $RpoH_2$ were carried out as described previously (Beck *et al.*, 1997; Narberhaus *et al.*, 1998b). Suitable RNA size markers

were synthesised *in vitro* with T7 or T3 RNA polymerase and linearised pBluescript-based plasmids as templates.

3.3.6 β-Galactosidase assay

B. japonicum cells were grown aerobically to exponential phase at 30°C in PSY medium with spectinomycin as the only antibiotic. The β-galactosidase assay was performed as described previously (Miller, 1972).

3.3.7 Overproduction and purification of H_6-HrcA

Freshly transformed *E. coli* BL21/pLysS cells carrying pRJ5552 were used for overproduction of H_6-HrcA. The cells were grown at 30°C in 1 l LB medium containing chloramphenicol and kanamycin. When the cultures had reached an optical density at 600 nm of 0.5, production of the recombinant protein was induced by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 2 h, the cells were harvested, washed and resuspended in 20 ml TEPDM buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 25 mM $MgCl_2$, 100 mM KCl), and disrupted in a French pressure cell. The soluble protein fraction was obtained by removing the cell debris and membranes in two subsequent centrifugation steps at 4°C (1.5 h at 30,000 x g [Sorvall SS-34 rotor] and 1 h at 116,000 x g [Beckman SW55 Ti rotor]).

All of the following purification steps were performed at 4°C. 8x binding buffer was added to the supernatant fraction to a final concentration of 500 mM NaCl, before loading onto a 1.5 ml Ni-nitrilotriacetic acid-agarose column, (Qiagen, Basel, Switzerland) which had been equilibrated with 1x binding buffer (20 mM Tris-HCl [pH 7.9], 500 mM NaCl, 5 mM imidazole). The column was washed with 15 ml 1x binding buffer and 4.5 ml 1x binding buffer containing 120 mM imidazole. The H_6-HrcA protein was eluted with 4.5 ml 1x binding buffer containing 300 mM imidazole and collected in fractions of 0.5 ml. The fractions with the highest protein concentrations were pooled, dialysed for 2 h against storage buffer (20 mM Tris-HCl [pH 8], 200 mM NaCl, 10% glycerol) and centrifugated at 13,000 x g for 1 min. Aliquots of the supernatant were stored at –80°C. The concentration of purified H_6-HrcA protein was determined by the Bradford method (Bradford, 1976).

3.3.8 Gel retardation assay

The DNA-binding activity of H_6-HrcA was tested in gel shift experiments using a 269 bp *Pst*I-*Kpn*I fragment from pRJ5558 containing the promoter region and the CIRCE element of *groESL*$_5$, a 168 bp *Hpa*I-*Kpn*I fragment from pRJ5556 containing the promoter region and the CIRCE element of *groESL*$_4$, a 104 bp PCR-amplification product containing the 5' region of the *groESL*$_4$ promoter region, or a 94 bp PCR-generated fragment containing either the wild-type CIRCE element of *groESL*$_4$ (CTAGCACTCgcgggcacaGACTGCTAA) or a mutated DNA fragment in which the consensus sequence had been replaced (AGCTACAGAgcgggcacaAGACATCGA) (*cf.* Fig. 6B; CIRCE consensus sequence: TTAGCACTC-N_9-GAGTGCTAA (Hecker *et al.*, 1996)). These DNA fragments were purified from agarose gels and end labelled with [γ-^{32}P]ATP according to standard protocols. Labelled fragment (25,000 c.p.m.; approximately 10 to 20 fmol DNA) was mixed with purified H_6-HrcA protein in DNA binding buffer (12 mM HEPES [pH 7.9], 4 mM Tris-HCl [pH 8], 6 mM KCl, 3 mM $MgCl_2$, 0.5 mM dithiothreitol, 6 mM EDTA; Stratagene, La Jolla, CA, USA) in a final volume of 25 µl. If appropriate, the *E. coli* chaperones DnaK, DnaJ, and GrpE or GroES and GroEL (Epicentre Technologies, Madison, WI, USA) were added to H_6-HrcA and preincubated at room temperature for 10 min in DNA binding buffer containing ATP (1.2 mM) and magnesium acetate (23 mM) before the binding reaction was started by the addition of DNA. The DNA-protein mixtures were incubated for 5 or 10 min at room temperature, mixed with 5 µl loading dye (30% glycerol, 0.02% bromphenol blue in water), and then loaded onto 6% nondenaturing polyacrylamide gels (cross-linker ratio of 29:1 in 1x TBE buffer [pH 8; 89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA]) containing 1 mg/ml Triton X-100. Gels were run in 1x TBE buffer at 4°C, dried under vacuum, and exposed on a phosphorimager screen. Signal intensities of free DNA and retarded bands were quantified with a phosphorimager and the program 'IMAGEQUANT' (version 3.3, Molecular Dynamics, Sunnyvale, CA, USA).

3.3.9 Plant infection test

The symbiotic phenotype of the *B. japonicum hrcA* mutant was determined in a soybean plant infection test as described previously (Hahn & Hennecke, 1984; Göttfert *et al.*, 1990).

3.3.10 Nucleotide sequence accession numbers

The nucleotide sequence of the *B. japonicum hrcA* and *dnaK* gene region has been deposited in the GenEMBL database under accession number Y09633.

3.4 Results

3.4.1 Cloning the *B. japonicum hrcA* gene region

In contrast to several other organisms, the *B. japonicum hrcA* gene is not part of the *dnaK* operon (*cf.* chapter 2). Our initial attempts to identify the gene by heterologous hybridisation using DNA or oligonucleotide probes derived from *hrcA* homologs failed. The fact that the *C. crescentus hrcA* gene is closely linked to *grpE* (Roberts *et al.*, 1996) prompted us to search first for a *grpE* homolog of *B. japonicum* using a complementation approach. Assuming that *B. japonicum* GrpE would be functional in *E. coli*, we attempted to rescue the heat-sensitive *grpE*-deficient *E. coli* strain DA259 (kindly provided by D. Ang, Geneva, Switzerland) with *B. japoncium* DNA cloned into pUC18. The inability of this strain to grow at 39 or 42°C could be fully complemented by the presence of plasmid pRJ5523 which contains a 3.9 kb *Eco*RI fragment of *B. japonicum* (Table 3.2; Fig. 3.1). DNA sequence analysis revealed an open reading frame coding for a protein with high similarity to all known GrpE proteins. As we had hoped, an open reading frame (3'-end) coding for the C-terminus of a HrcA-like protein was present upstream of *grpE*. Incidentally, the 5'-end of *B. japonicum dnaK* was found only 2.4 kb downstream of the *hrcA-grpE* cluster (Fig. 3.1). The complete *B. japonicum hrcA* region was subcloned as a 3 kb *Not*I fragment originating from a suitable cosmid, resulting in plasmid pRJ5530 (Fig. 3.1).

Table 3.2 Temperature dependent growth of *E. coli* DA259 transformed with different plasmids

E. coli strain	Plasmid[a]	Growth temperature		
		30°C	39°C	42°C
DA258 (wild type)		+	+	+
DA259 (*grpE*$^-$)		+	–	–
DA259 (*grpE*$^-$)	pUC18	+	–	–
DA259 (*grpE*$^-$)	pBW401 (*Ec grpE*$^+$)	+	+	+
DA259 (*grpE*$^-$)	pRJ5523 (*Bj grpE*$^+$)	+	+	+

[a] The abbreviation *Bj* stands for *B. japonicum*, *Ec* for *E. coli*.

To address the question of whether there is more than one copy of the *hrcA* or *grpE* genes in the *B. japonicum* chromosome, as it is the case for *groESL* (Fischer *et al.*, 1993) and *rpoH* (Narberhaus *et al.*, 1996), suitable *hrcA* and *grpE* probes were hybridised under low stringency conditions (5 x SSC, 56°C) with *B. japonicum* chromosomal DNA that had been digested with different restriction enzymes. No evidence for a *hrcA* or *grpE* gene family was obtained.

3.4.2 Nucleotide sequence of the *hrcA* gene region

The DNA sequence of the *B. japonicum hrcA* gene region, extending from the *Not*I site at position 2 to the *Xho*I site at position 3,451, was established (Fig. 3.1), expanding the previously described sequence of the *dnaKJ* gene region (*cf.* chapter 2). Significant similarities of the deduced amino acid sequences to *C. crescentus* HrcA (54% identical amino acids) and GrpE (44%) (Roberts *et al.*, 1996) led to the more precise assignment of *B. japonicum hrcA* (position 433 to 1,521) and *grpE* (position 1,619 to 2,224). The *hrcA* gene begins with the alternative start codon GTG and

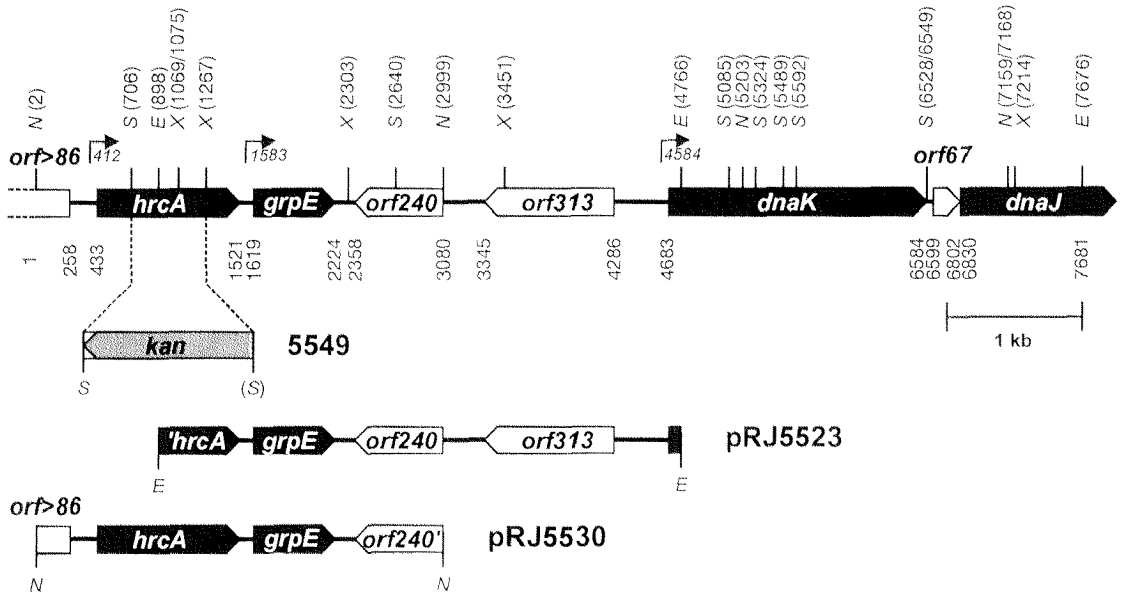


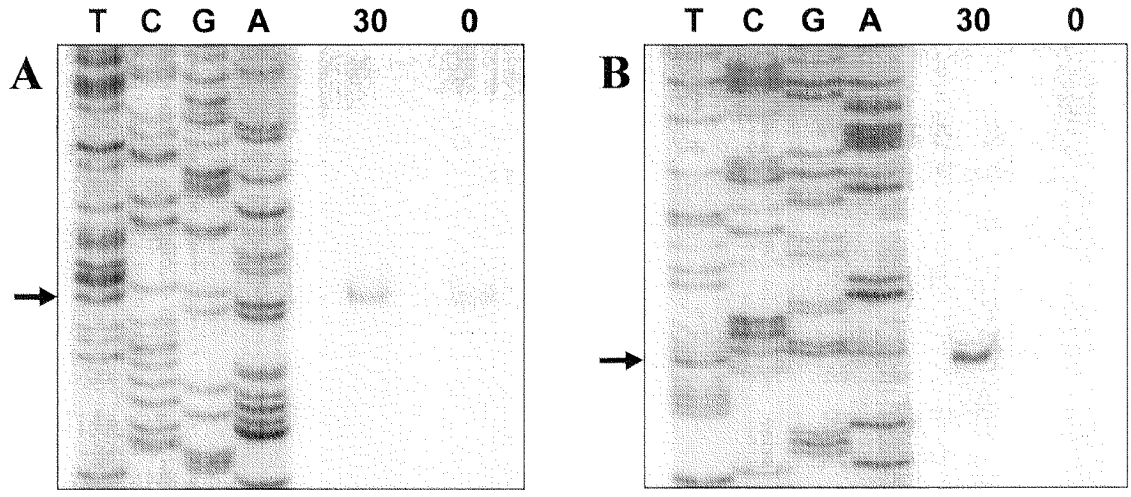
Fig. 3.1 Physical map of the *B. japonicum hrcA* gene region. Numbers indicate start and stop codon positions of open reading frames, the transcription start sites (horizontal arrows above gene map), the beginning (1) and the end (7,681) of the sequenced gene region, and recognition sites of the following restriction enzymes: *E*, *Eco*RI; *N*, *Not*I; *S*, *Sal*I; *X*, *Xho*I. The restriction site in parentheses was destroyed during the cloning procedure. The strategy to construct the *B. japonicum hrcA* deletion resulting in strain 5549 is indicated. The inserts of plasmids pRJ5523 and pRJ5530 are shown below the physical map.

encodes a predicted protein of 362 amino acids (M_r 39,165). An alignment of the *B. japonicum hrcA* product with 18 HrcA-like proteins deposited in the publicly available databases displayed only limited sequence similarity (overall sequence identity generally around 30% with the exception of HrcA proteins from related organisms such as *B. japonicum*, *Agrobacterium tumefaciens*, and *C. crescentus* that share about 50% identical amino acids). The similarity is restricted mainly to three previously described conserved regions (Schulz & Schumann, 1996).

The deduced gene product of *grpE* consists of 201 amino acids with a molecular weight of 21,655. Further investigation of the DNA region upstream of *dnaK* and *hrcA* revealed three additional open reading frames, all divergently oriented to *hrcA* and *dnaK*. The first open reading frame (*orf>86*) starts at position 258 and codes for the amino-terminal end of a polypeptide of at least 86 amino acids with high sequence similarity to the putative tRNA nucleotidyltransferase RnpH of *C. crescentus* (67% identical amino acids) encoded by the *rph* gene (Roberts *et al.*, 1996). Moreover, the genetic organisation of *orf>86*, *hrcA* and *grpE* is similar to the situation in *C. crescentus*. The gene product of *orf240* (M_r 25,406) shows a high sequence similarity to pyrazin-nicotinamidases (e.g. 41% identical amino acids shared with the putative *pncA* gene product of *Aquifex aeolicus* (Deckert *et al.*, 1998)). The *orf313* product (M_r 33,241) exhibits no significant similarity to any known protein sequence in the database.

3.4.3 Transcriptional analysis of the *hrcA* gene region

The transcription start sites within the *B. japonicum hrcA* gene region were determined by primer extension analysis with oligonucleotides complementary to the 5' ends of *hrcA* and *grpE*. A single start site was detected for each gene: 21 nucleotides upstream of the proposed translational start site of *hrcA* (Fig. 3.2A), and 36 nucleotides upstream of *grpE* (Fig. 3.2B). Both transcripts were clearly heat inducible as indicated by an 2.3- and 19-fold increase of the reverse transcription products for *hrcA* and *grpE*, respectively, after a 30-min temperature shift of the cells from 30 to 43°C. The deduced promoter regions displayed characteristic sequence motifs of heat-inducible σ^{32}-dependent promoters (Fig. 3.2C).



C

Promoter	−35 Region		−10 Region	+1
B. japonicum hrcA	AAC**CA**C**GCTTG**TC	AGGGACCTCCCGGATG	G**CCA**A**TTA**TA	AGC**A**
B. japonicum grpE	A**C**GGG**CGCTTGA**T	TTTCGGCGTCCGAAG	**CACGATA**T**CCG**	GGCC**A**
E. coli σ^{32} consensus	**TCTC-CCCTTGAA**	13-17 bp	**CCCCAT-TA**	

Fig. 3.2 Determination of the transcription start sites of *hrcA* (**A**) and *grpE* (**B**) by primer extension mapping. Total RNA was isolated from *B. japonicum* 110*spc*4 cells harvested before (0) and 30 min after a heat shock from 30°C to 43°C (30). The extension and sequencing reactions (TCGA) were performed with the primers HrcA11 (**A**) and GrpE4 (**B**). The transcription start sites are marked with an arrow and the deduced promoter sequences are shown below (**C**). Nucleotides matching the *E. coli* σ^{32} consensus promoter (Yura *et al.*, 1993) and transcriptional start sites are emphasized in bold letters.

The presumed σ^{32}-dependent transcription of *hrcA* and *grpE* was further confirmed by *in vitro* transcription of both genes using the *B. japonicum* core RNA polymerase reconstituted with purified $RpoH_2$-H_6 protein, a C-terminally histidine-tagged version of this protein. The $RpoH_2$ factor is responsible for expression of σ^{32}-dependent genes under normal growth conditions. The resulting *hrcA* and *grpE* transcripts derived from plasmids pRJ5542 and pRJ5543, respectively, are of the expected lengths (286 and 373 nucleotides, respectively; Fig. 3.3A and B), as judged from a comparison with RNA size markers (not shown) and the *dnaKJ* transcript (358 nucleotides), which was previously shown to be transcribed efficiently by core RNA polymerase reconstituted with $RpoH_2$-H_6 (Fig. 3.3A, and Narberhaus *et al.*, 1998b). The core enzyme alone produced only small amounts of transcript (Fig. 3.3A), most probably due to some residual σ^{32} protein present in the preparation as described elsewhere (Narberhaus *et*

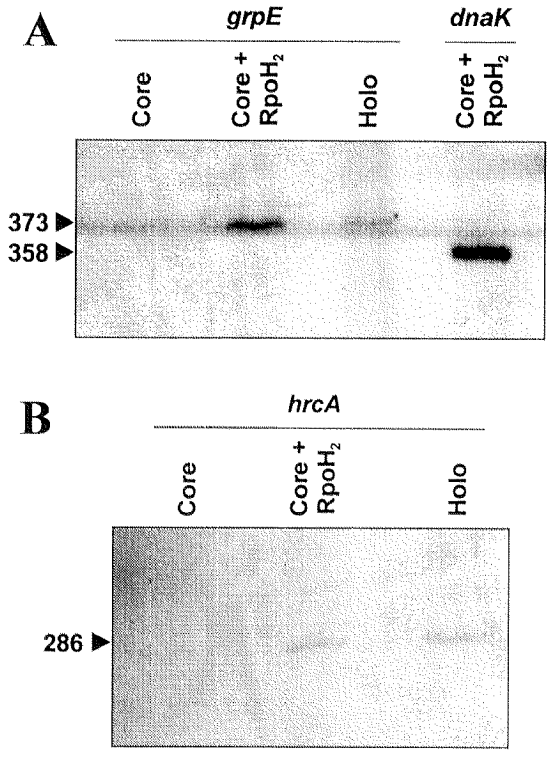


Fig. 3.3 *In vitro* transcription from the *B. japonicum grpE*, *dnaK* (**A**), and *hrcA* (**B**) promoters. The enzymes used are indicated: Core, *B. japonicum* RNA polymerase core enzyme; $RpoH_2$, purified $RpoH_2$-H_6 protein; Holo, *B. japonicum* RNA polymerase holoenzyme. Plasmids pRJ5099, pRJ5542 and pRJ5543 were used as templates to transcribe the 5' ends of *dnaK*, *hrcA* and *grpE*, respectively. Numbers on the left mark transcript lengths (in nucleotides) of the expected transcripts which were affirmed by suitable *in vitro* synthesised RNA size markers (not shown). The signal extending across the entire gel (**A**) was caused by overloaded RNA size markers on the left.

al., 1998b). Similarly, *hrcA* and *grpE* transcripts were obtained with the *B. japonicum* RNA polymerase holoenzyme which contains a significant amount of RpoH protein (Narberhaus *et al.*, 1998b). According to these results we conclude that *hrcA* belongs to the *rpoH* regulon of *B. japonicum* together with the genes coding for the DnaK machinery.

3.4.4 Heat shock gene expression in a *hrcA* deletion mutant

The *B. japonicum hrcA* mutant 5549 was constructed by replacing an internal *hrcA* fragment with a kanamycin resistance cassette (see Fig. 3.1 and Materials and Methods). The effect of the *hrcA* deletion on expression of heat shock genes was determined by both primer extension analysis and β-galactosidase measurements of suitable translational *lacZ* fusions.

The analysis of RNA isolated from the *B. japonicum* wild type and strain 5549 revealed strongly derepressed transcription of the CIRCE-dependent genes $groESL_4$ and $groESL_5$ under non-heat shock conditions (Fig. 3.4). The transcription intensity slightly surpassed even the heat-induced transcription after a 30-min heat shock from 30 to 43°C. By contrast, transcription of σ^{32}-dependent genes (e.g. $groESL_1$, *hrcA*) and heat

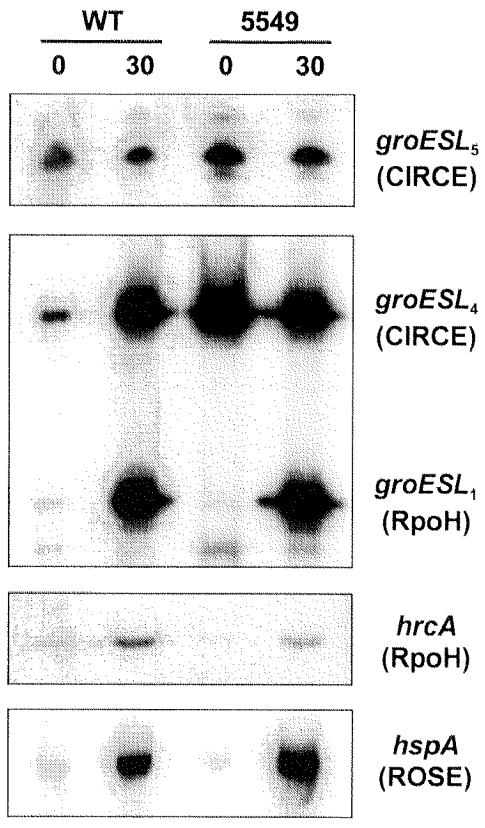


Fig. 3.4 Effect of a *hrcA* mutation on transcription of different heat shock genes in *B. japonicum*. Total RNA was isolated from *B. japonicum* wild-type and 5549 (*hrcA*⁻) cells harvested before (0) and 30 min after a heat shock from 30°C to 43°C (30) and analysed by primer extension experiments. The extension reactions were performed with the primers ES5UP2 (*groESL*$_5$) (Babst *et al.*, 1996), 702 (*groESL*$_4$, *groESL*$_1$) (Babst *et al.*, 1996), HrcA11 (*hrcA*) and Sig107 (*hspA*) (Narberhaus *et al.*, 1998a). The mode of regulation of each gene or operon is indicated.

shock genes regulated by the ROSE element (e.g. *hspA*) was not influenced by the lack of HrcA. In line with the absence of a CIRCE element upstream of *B. japonicum hrcA*, the gene is not subject to autoregulation as it is in *B. subtilis* (Schulz & Schumann, 1996).

Table 3.3 shows the expression of different *lacZ* fusions that were integrated into the chromosome of *B. japonicum* wild type and strain 5549 (see Materials and Methods for the construction of the strains). The two CIRCE-regulated fusions (*groEL*$_4$'-'*lacZ* and *groEL*$_5$'-'*lacZ*) exhibited an increased expression in the absence of HrcA, which supports the primer extension results. However, the calculated induction factors (3.2 for *groEL*$_4$'-'*lacZ* and 2.3 for *groEL*$_5$'-'*lacZ*) appeared to be lower as compared with the strongly increased transcription of the corresponding genes in strain 5549 (Fig. 3.4). The lack of HrcA did not affect the expression of the *lacZ* fusions to *groEL*$_1$, *grpE* and *dnaK* (σ^{32}-regulated), *hspA* (ROSE-regulated), and *groEL*$_2$ (constitutively expressed) (Table 3.3). The latter finding supports an earlier notion that the putative CIRCE-homologous element in the promoter region of *groESL*$_2$ might not be functional (Babst *et al.*, 1996). Although transcription from the *hrcA* promoter was not influenced by a

Table 3.3 Effect of a *hrcA* deletion on the expression of chromosomally integrated *'lacZ* fusions in *B. japonicum*

B. japonicum strains[a]	*'lacZ* fusion	Induction factor[b]	Mode of regulation
H8092 / 8092	*groEL*$_5$*'-'lacZ*	2.3 ± 0.6	CIRCE
H8548 / 8548	*groEL*$_4$*'-'lacZ*	3.2 ± 0.7	CIRCE
H8067 / 8067	*groEL*$_1$*'-'lacZ*	1.2 ± 0.3	σ^{32}
H8174 / 8174	*dnaK'-'lacZ*	0.9 ± 0.1	σ^{32}
H5554 / 5554	*grpE'-'lacZ*	0.9 ± 0.1	σ^{32}
H5559 / 5559	*hrcA'-'lacZ*	2.8 ± 0.6	σ^{32}
H5400 / 5400	*hspA'-'lacZ*	1.1 ± 0.3	ROSE
H7998 / 7998	*groEL*$_2$*'-'lacZ*	1.3 ± 0.7	constitutive

[a] The *'lacZ* fusion indicated in the second column was chromosomally integrated into *B. japonicum* wild type (resulting strains numbered without prefix) and into *hrcA* mutant 5549 (strains numbered with prefix "H").
[b] The induction factor corresponds to the ratio of the β-galactosidase activity measured in the *hrcA*$^-$ mutant and wild-type background. The mean values and deviations are based on at least three independent measurements.

deletion of the corresponding gene (Fig. 3.4), the expression of the *hrcA'-'lacZ* fusion was increased 2.8-fold in strain H5559 (Table 3.3). Posttranscriptional events, not further investigated here, might be responsible for this effect.

Complementary evidence that derepression of the *groESL*$_4$ and *groESL*$_5$ operons in the *hrcA* mutant raised the cellular GroEL pool was obtained by immunoblots performed with anti *E. coli* GroEL serum and by two-dimensional gel analysis (data not shown).

3.4.5 Phenotypic characterisation of a *hrcA* deletion mutant under symbiotic and heat shock conditions

As it was known that GroEL is critical for nitrogen fixation in *B. japonicum* (Fischer *et al.*, 1999) we tested whether the elevated GroESL$_4$ and GroESL$_5$ concentration in the *hrcA* mutant would affect the performance of *B. japonicum* in root-nodule symbiosis and under heat stress conditions. The strain 5549 (*hrcA*$^-$) was indistinguishable from the wild-type strain with respect to the ability to nodulate soybean roots and to fix nitrogen. Strain 5549 was further analysed for its ability to survive a temperature

upshift from 30 to 48°C which is lethal to wild-type *B. japonicum* (Narberhaus *et al.*, 1998c). The survival rate of strain 5549 after exposure to the non-permissive temperature showed no significant deviation from the wild type. Since the elevated GroEL concentration caused by the *hrcA* deletion did not improve the heat tolerance of *B. japonicum*, we conclude that the GroEL pool in *B. japonicum* wild-type cells is not limiting under the conditions tested.

3.4.6 Purification of soluble H_6-HrcA

To obtain *in vitro* evidence for a physical interaction between CIRCE and HrcA we aimed at performing gel retardation assays with purified components. To this end, an amino-terminally histidine-tagged version of *B. japoncium* HrcA (H_6-HrcA) was expressed in *E. coli*. In contrast to overproduced HrcA from other organisms, a large fraction of *B. japonicum* H_6-HrcA remained in the supernatant even after ultracentrifugation. This allowed purification under non-denaturing conditions, yielding approximately 4 mg purified protein per litre *E. coli* culture (Fig. 3.5).

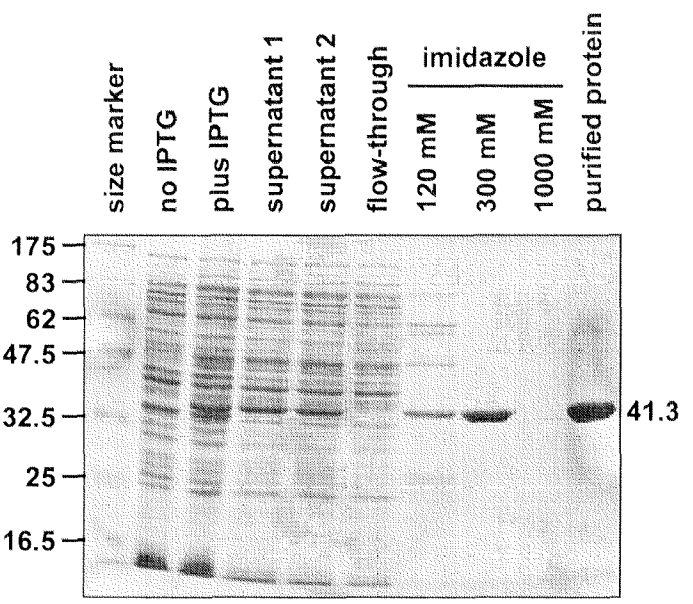


Fig. 3.5 Overexpression and purification of H_6-HrcA. Crude extracts of the H_6-HrcA overproducing *E. coli* strain harvested before and after induction with IPTG, aliquots from each purification step and 2 µl of the dialysed protein fraction, which was subsequently used for *in vitro* experiments, were separated on a 12% SDS polyacrylamide gel. The proteins were stained with Coomassie Blue. Supernatant 1 and 2 mark aliquots of the soluble protein fraction after centrifugation at 30,000 and 116,000 x g, respectively (see Materials and Methods). The apparent molecular masses (in kilodaltons) of H_6-HrcA (41.3) and the marker proteins are indicated.

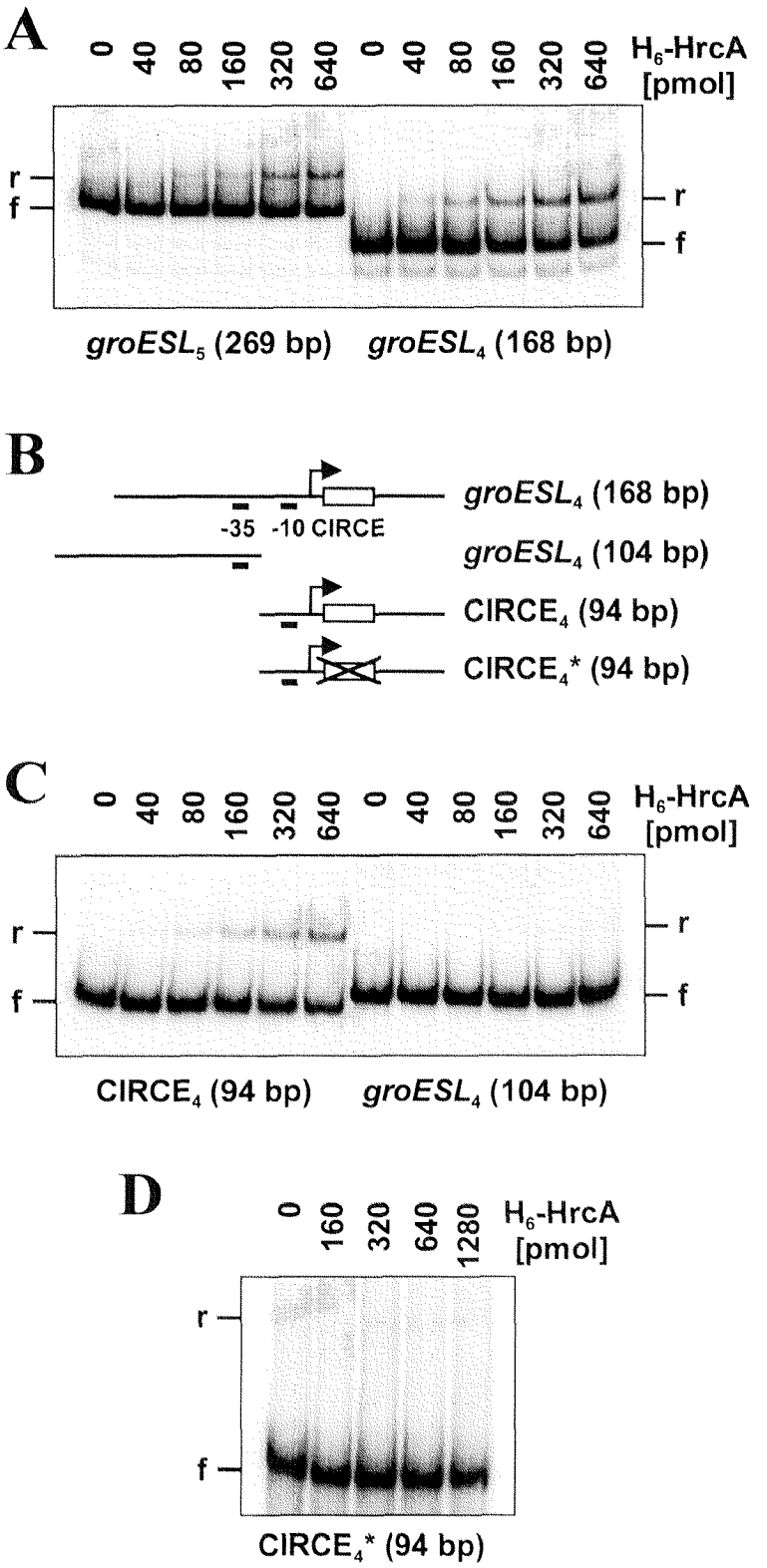


Fig. 3.6 Gel retardation experiments with purified *B. japonicum* HrcA. DNA-binding reactions were performed with DNA fragments derived from the promoter region of *groESL*$_5$ (**A**) and *groESL*$_4$ (**A,C,D**). The *groESL*$_4$ fragments are represented schematically in (**B**). CIRCE$_4$* represents a DNA fragment in which the CIRCE element was mutated (see Materials and Methods). The used amount of H_6-HrcA is indicated above each lane in all panels. The positions of radiolabeled free DNA (f) and retarded bands (r) are marked.

3.4.7 Binding of purified HrcA to CIRCE

Gel retardation experiments with increasing amounts of H_6-HrcA and with radioactively labelled DNA fragments containing CIRCE and the promoter region of *groESL*$_4$ or *groESL*$_5$ showed a protein-dependent DNA retardation (Fig. 3.6A). The *groESL*$_4$ fragment was used to further delineate the DNA region responsible for HrcA binding (Fig. 3.6B). DNA binding of H_6-HrcA was observed only with the 94 bp subfragment that contained the −10 promoter region and CIRCE (Fig. 3.6C) but not with a comparable fragment in which the CIRCE element was replaced by a sequence that had no similarity to CIRCE (Fig. 3.6D; for details see Materials and Methods).

Additional support for the specificity of the HrcA-CIRCE interaction was provided by DNA competition experiments. Gel retardation of the radiolabelled 94 bp *groESL*$_4$ promoter fragment containing CIRCE could be efficiently competed by the addition of an increasing amount of the same unlabeled fragment (Fig. 3.7A). The fragment in which the CIRCE element had been mutated (CIRCE$_4$*) was much less efficient in competing for HrcA binding. A quantitative assessment of the competition efficiency revealed that a 20-fold higher concentration of CIRCE$_4$* was required in comparison with wild-type CIRCE in order to produce a 50% decrease of the amount of retarded fragments (Fig. 3.7B).

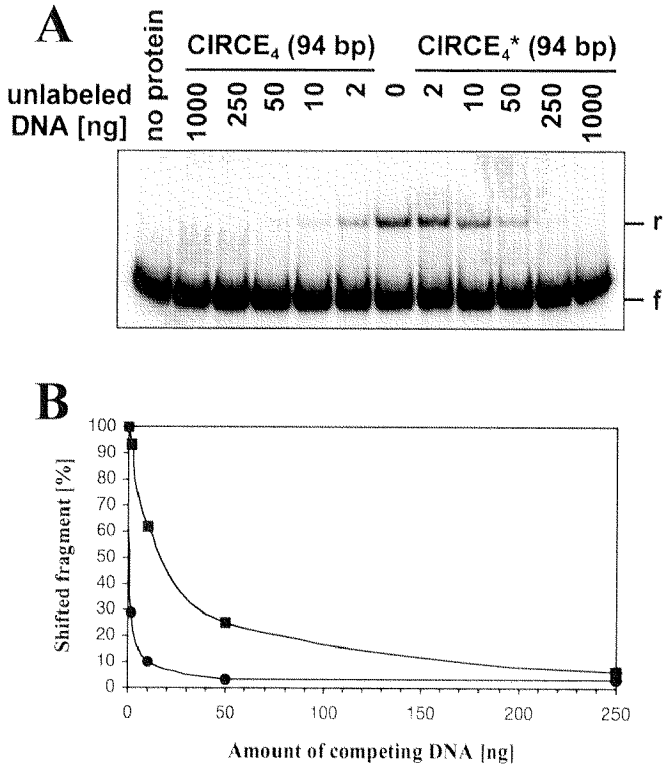


Fig. 3.7 Specificity of the HrcA-CIRCE binding. **A** The interaction between the radiolabelled CIRCE$_4$ fragment and H_6-HrcA (320 pmol) was competed by different amounts of unlabeled CIRCE$_4$ (wild-type CIRCE element) and CIRCE$_4$* (mutated CIRCE element) as indicated. The positions of radiolabeled free DNA (f) and retarded bands (r) are indicated. **B** Graphical representation of the competition efficiency of unlabeled CIRCE$_4$ (●) and CIRCE$_4$* (■).

3.4.8 Chaperones had no stimulating effect on HrcA-CIRCE binding

In an additional set of gel retardation experiments we attempted to lend support to the concept that the HrcA repressor activity might be chaperonin-dependent. H_6-HrcA was preincubated in binding buffer containing either *E. coli* GroEL and GroES (approximate molar ratio of protomers of HrcA:GroEL:GroES = 3:10:1 or 3:40:4) or *E. coli* DnaK, DnaJ and GrpE (approximate molar ratio of protomers of HrcA:DnaK:DnaJ:GrpE = 10:10:1:1 or 10:40:4:4). Each DNA binding assay was started by the addition of radiolabelled 168 bp DNA fragment containing the promoter region and the CIRCE element of *groESL*$_4$. It is evident that the DNA binding activity of our soluble HrcA preparation was unaffected by the presence of the DnaK chaperone machinery, whereas the addition of GroESL led to a strong reduction of HrcA-CIRCE complexes (Fig. 3.8). The apparent decrease of free DNA in the presence of large amounts of GroESL (Fig. 3.8, lane 7) was not accompanied by the appearance of a specific band shift signal. In control experiments we confirmed that the GroE preparation had chaperonin activity in the buffer system we used for the gel retardation experiments. This was done by measuring its ability to reactivate heat denatured malate dehydrogenase (data not shown).

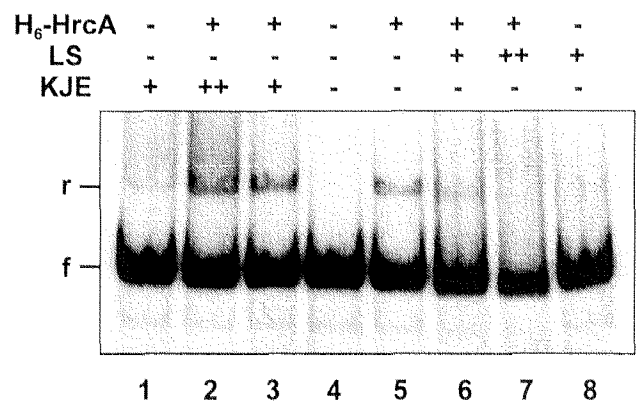


Fig. 3.8 Effect of chaperone addition on HrcA-CIRCE binding. Gel retardation assays were performed with the radiolabeled 168 bp *groESL*$_4$ fragment (*cf.* Fig. 3.6B), 53 pmol H_6-HrcA which had been preincubated for 10 min with either of the two *E. coli* chaperone complexes DnaK-DnaJ-GrpE (KJE) or GroEL-GroES (LS). ATP was added to a final concentration of 1.2 mM together with the chaperone complexes. The positions of radiolabeled free DNA (f) and retarded bands (r) are indicated. Lane 1 and 3: 57 pmol DnaK, 4.9 pmol DnaJ, 4.35 pmol GrpE; lane 2: 228 pmol DnaK, 19.6 pmol DnaJ, 17.4 pmol GrpE; lane 6 and 8: 175 pmol GroEL, 19.3 pmol GroES; lane 7: 700 pmol GroEL, 77 pmol GroES (protein quantities calculated as protomers). Note that the DnaK chaperone machinery alone led to the appearance of a weak signal at the position of retarded bands (lane 1).

3.5 Discussion

This work presents an in-depth *in vivo* and *in vitro* investigation of a representative CIRCE-HrcA system, which holds responsible for a wide-spread heat shock control mechanism in bacteria. It has become evident during the last decade that repressor mechanisms are probably much more abundant among prokaryotes than the *E. coli*-type σ^{32} control of heat shock genes (Narberhaus, 1999). In *B. japonicum*, the CIRCE system is embedded in a complex regulatory network. To elucidate the role of CIRCE in this network, it was imperative to clone the structural gene of HrcA, the putative repressor binding to this DNA element. The amino acid sequences of known HrcA proteins deviate greatly despite the high sequence conservation of the CIRCE elements to which they bind (Roberts *et al.*, 1996; Schulz & Schumann, 1996). This makes the search for a *hrcA* gene with nucleotide probes rather difficult (Grandvalet *et al.*, 1998). Here, we made use of the close genetic linkage that is often observed between *hrcA* and *grpE* and established a novel approach that facilitated the isolation of *B. japonicum hrcA* gene by complementing a temperature-sensitive, *grpE*-deficient *E. coli* strain.

Both *hrcA* and *grpE* are transcribed separately from heat-inducible σ^{32}-dependent promoters. The control of the HrcA repressor by a heat shock sigma factor demonstrates that different regulatory mechanisms involved in the *B. japonicum* heat shock response do not act independently but form a complex network. Evidence for a similar network of positive and negative heat shock regulatory mechanisms was also obtained in *C. crescentus*, in which *hrcA* transcription depends on a heat-inducible σ^{32}-like promoter (Roberts *et al.*, 1996). Moreover, its *groESL* operon is subject to dual control by a heat-inducible σ^{32}-dependent promoter and a CIRCE element which is involved in the cell cycle-regulated gene expression (Baldini *et al.*, 1998). Importantly, basal expression of *B. japonicum hrcA* was observed at normal growth temperatures thus guaranteeing a sufficient amount of HrcA protein to repress the CIRCE regulon. The sigma factor $RpoH_2$ appears to be responsible for this basal transcription, as it is for a certain threshold level of DnaK (Narberhaus *et al.*, 1998b). Although not proven rigorously, it is reasonable to predict that $RpoH_1$, the heat-shock induced RpoH factor of *B. japonicum*, is responsible for temperature induction of *hrcA* expression.

Elevated levels of GroESL in a *hrcA* mutant had no influence on free-living growth and symbiotic nitrogen fixation of *B. japonicum*. Similarly, no obvious effect with

regard to growth under normal and under stress conditions was observed in *C. crescentus* and *Streptomyces albus hrcA* mutants (Roberts *et al.*, 1996; Grandvalet *et al.*, 1998). However, comparable studies in *B. subtilis* showed that the *hrcA* mutant was able to recover from a shift to lethal temperatures whereas the wild type was not (Yuan & Wong, 1995b). A possible reason for this discrepancy between the bacterial species lies in the fact that *B. japonicum*, *C. crescentus* and *S. albus hrcA* mutants enhance only the concentration of GroE chaperonins whereas the lack of HrcA in *B. subtilis* leads to an overexpression of both chaperone complexes, DnaKJ-GrpE and GroESL. Probably, only an increased level of the complete set of major chaperones improves the capacity of bacterial cells to survive temperature upshifts. This interpretation is also supported by the model that DnaK and GroE chaperone complexes interact with each other in order to cope with heat shock situations (Hartl, 1996).

Previous attempts to perform *in vitro* studies with HrcA have been impeded by the insolubility of this protein. Gel retardation experiments could only be performed with cell extracts containing HrcA (Duchêne *et al.*, 1994; Kuroda *et al.*, 1999) or with renatured HrcA that had been purified under denaturing conditions (Mogk *et al.*, 1997). By contrast, we found that a large fraction of *B. japonicum* H_6-HrcA remained in a soluble form upon overproduction and during purification. The reason for the comparatively high solubility of *B. japonicum* HrcA remains unknown at present. Gel mobility experiments performed with this preparation corroborated the *in vivo* results in showing a sequence-specific binding of HrcA to CIRCE. However, a large excess of HrcA over DNA was necessary to obtain a band shift. This might be due to an intrinsic tendency of the repressor to aggregate or to reach a non-functional conformation. It should be noted that our *B. japonicum* HrcA preparation also tended to precipitate from solution over time. Thus, only a small fraction of the purified protein might really be active. Another, physiologically more relevant reason for the poor retardation activity might be an inherently weak or a transient interaction between CIRCE and its repressor. This could explain why both CIRCE-dependent operons in *B. japonicum* are only partially repressed *in vivo* at normal growth temperatures resulting in an appreciable basal transcription of the corresponding genes (see Fig. 3.4, and Babst *et al.*, 1996). Leaky transcription from CIRCE-regulated promoters was also reported for *B. subtilis*, *Clostridium acetobutylicum*, *C. crescentus* and other organisms (Narberhaus & Bahl,

1992; Schön & Schumann, 1992; Roberts *et al.*, 1996) indicating that the CIRCE binding site is not permanently occupied by HrcA. It thus appears as if the CIRCE-HrcA system is not designed for complete repression of heat shock genes under physiological temperatures because major chaperones are also required for folding processes during normal growth.

There is compelling *in vivo* evidence that HrcA activity depends on the presence of GroESL (Babst *et al.*, 1996; Mogk *et al.*, 1997). The interpretation of these findings was an appealing titration model in which the availability of GroE chaperones controls HrcA activity. At a first glance, our *in vitro* results appear incompatible with this model. While gel retardation of a CIRCE fragment with *B. stearothermophilus* HrcA was significantly improved in the presence of GroEL (Mogk *et al.*, 1997) our gel shift experiments showed that the addition of the complete GroE machinery rather impaired the formation of *B. japonicum* HrcA-CIRCE complexes. Since we added *E. coli* chaperones to the *in vitro* system one could argue that these heterologous proteins behave as non-cognate partners of the rhizobial HrcA repressor. This seems unlikely because the *E. coli* GroE chaperone machinery enables the formation of active *B. subtilis* HrcA when the latter is expressed in *E. coli* (Mogk *et al.*, 1998). Moreover, functional analyses with the *B. japonicum* GroEL family revealed that these chaperonins possess no strict substrate specificity and that they can be functionally replaced to some extent by *E. coli* GroEL in the nitrogen fixation process (Fischer *et al.*, 1999). A more reasonable explanation for the apparent contradiction between the *in vitro* results with *B. japonicum* HrcA and those with *B. stearothermophilus* HrcA might be that the latter was tested in the presence of 0.5 M urea after it had been completely denatured during purification. It is thus conceivable that GroEL conferred a rather general chaperone activity on this partially denatured HrcA preparation as was indicated by the prevention of HrcA aggregation in the presence of GroEL. Moreover, it remains unclear why stimulation of gel retardation was observed even in the absence of GroES and ATP (Mogk *et al.*, 1997). The prevention of aggregate formation might enable partially unfolded HrcA protein to reach an active DNA-binding conformation and, hence, a measurable gel retardation activity with a CIRCE fragment. Experiments with *C. acetobutylicum* HrcA (OrfA), which had been purified under denaturing conditions, revealed that HrcA aggregation was prevented by the addition of DnaK,

DnaJ, or the whole DnaK chaperone machinery (Rüngeling *et al.*, 1999), indicating that urea-denatured HrcA is a substrate not only for the GroE but also for the DnaK chaperone machinery. The results of our own gel shift experiments, performed under non-denaturing conditions, suggest that the active fraction of the *B. japonicum* HrcA preparation interacts with GroESL, whereby the amount of HrcA-CIRCE complexes decreases. Apparently, the GroE chaperone machinery was not able to improve the DNA-binding activity of HrcA. It is conceivable that *in vivo* only nascent HrcA protein might be able to interact with the GroE chaperone machinery to gain a DNA binding-competent conformation. We propose that in the absence of free GroESL, e.g. immediately after a heat shock, HrcA adopts an inactive conformation which cannot be refolded into a repressor-competent form by chaperones. This might be the reason why all *hrcA* genes studied so far are under heat shock control either via autoregulation or via RpoH. Assuming that heat-inactivated HrcA cannot be converted into a DNA-binding form, elevated *de novo* synthesis of HrcA might be required to tune down the expression of CIRCE-regulated genes in a rapid and transient heat shock response. In contrast to GroESL, the addition of the DnaK chaperone machinery showed no influence on the DNA-binding activity of HrcA indicating that HrcA is not a substrate for these chaperones. This finding clearly supports the *in vivo* data showing a DnaKJ-independent heat shock response of CIRCE-regulated genes in *B. subtilis* (Mogk *et al.*, 1997).

Circumstantial evidence suggests that HrcA is probably not a direct thermosensor. First of all, HrcA does not only respond to temperature changes but is also responsible for the cell-cycle coordination of *groESL* expression in *C. crescentus* (Baldini *et al.*, 1998). Secondly, *Streptomyces coelicolor* protein extracts isolated from non-heat shocked and heat shocked cell cultures caused a similar gel retardation of a CIRCE-containing DNA fragment (Duchêne *et al.*, 1994). Finally, the ability to shut off CIRCE-dependent heat shock gene transcription during prolonged exposure of cells to high temperatures (Babst *et al.*, 1996; Mogk *et al.*, 1997) is also incompatible with the idea that the repressor is temperature-sensitive *per se*. We addressed this question more directly by performing gel retardation experiments in which purified HrcA protein was preheated to 43°C either before or after addition of the CIRCE fragment. DNA-binding activity was not impaired by this treatment. This could be interpreted to mean that

HrcA is not inherently temperature sensitive, unless it re-gains activity in the course of the electrophoresis process. Regulated activity of the repressor might in fact be mediated solely through the availability of free GroEL, which is the prerequisite for production of functional HrcA *in vivo*.

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CHAPTER 4

Synthesis of chaperones and other heat shock proteins in *Bradyrhizobium japonicum*

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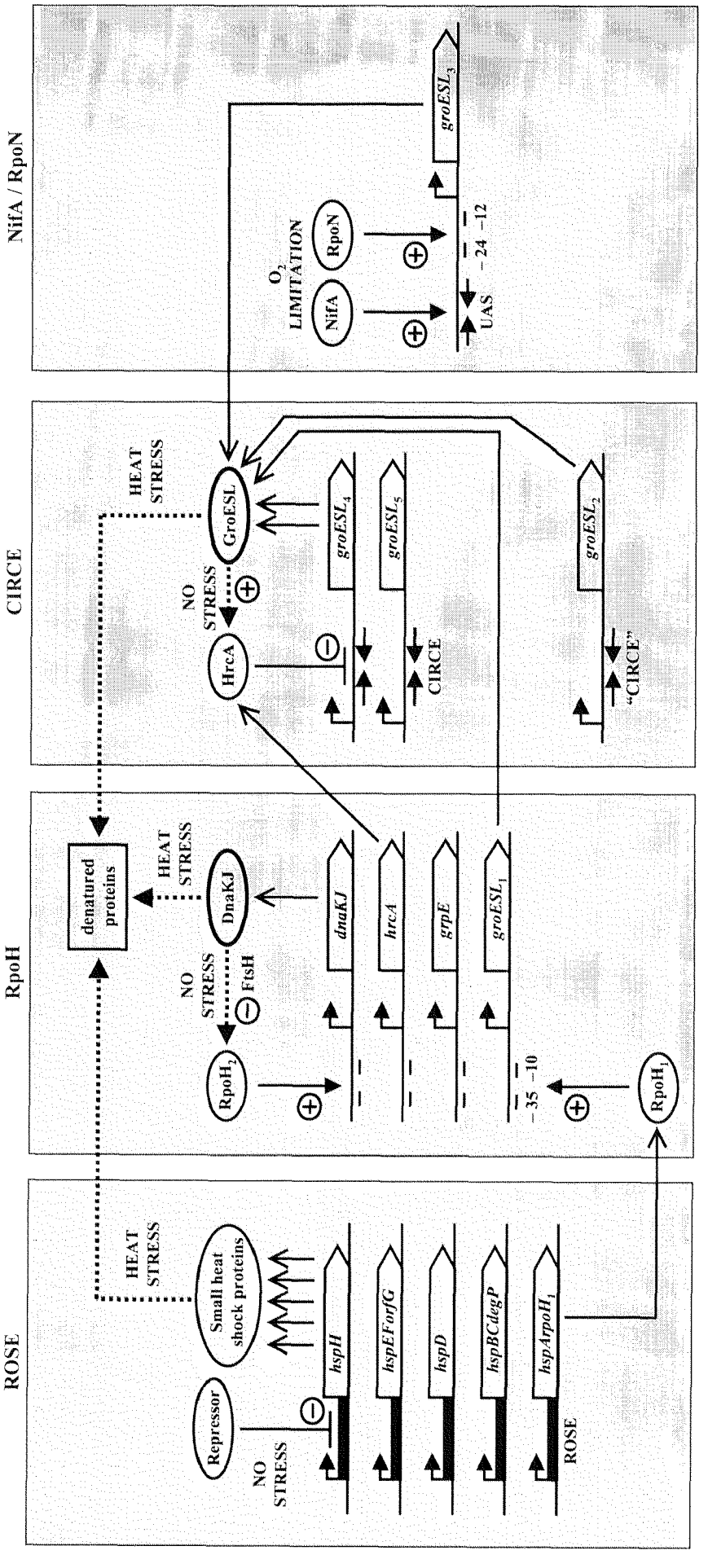
Over the last few years, our knowledge of heat shock regulation in *Bradyrhizobium japonicum*, the nitrogen-fixing root-nodule symbiont of soybean, has constantly increased revealing an unusually complex network of both positive and negative regulatory mechanisms (Fig. 4.1; Narberhaus, 1999). The starting point to investigate the heat shock regulation in this organism was the discovery of a family of five very similar, though not identical, *groESL* operons (Fischer *et al.*, 1993). The recently cloned sixth *groEL* gene (*groEL*$_6$; accession number AF119120) is not further considered in the text, as it was identified as a pseudogene. Transcriptional analysis revealed that the five functional chaperonin operons are differentially regulated depending on the environmental conditions. The expression of *groESL*$_3$ is co-regulated with symbiotic nitrogen fixation genes via the specialised sigma factor RpoN and the oxygen-responsive transcriptional activator protein NifA (Fischer *et al.*, 1993). The *groESL*$_2$ operon is expressed under all growth conditions tested (Babst *et al.*, 1996; Münchbach *et al.*, 1999a) and three chaperonin operons (*groESL*$_1$, *groESL*$_4$ and *groESL*$_5$) show an increased transcription under heat shock conditions (Babst *et al.*, 1996). A detailed regulatory study of these heat-inducible *groESL* operons revealed that their transcriptional control is not uniform. While regulation of *B. japonicum groESL*$_1$ involves σ^{32}-like transcription factors (RpoH proteins), the *groESL*$_4$ and *groESL*$_5$ operons are negatively controlled by the widespread CIRCE-HrcA system (Babst *et al.*, 1996). A second negative regulatory mechanism depending on a highly conserved DNA element called ROSE controls a large regulon coding for small heat shock proteins and the RpoH$_1$ sigma factor (Narberhaus *et al.*, 1998a).

The aim of this chapter is to give an overview of the different mechanisms of heat shock regulation in *B. japonicum* and to present our current model of the heat shock regulatory network (Fig. 4.1).

Fig. 4.1 (following page) Model of the regulatory network controlling the synthesis of chaperones and other heat shock proteins in *Bradyrhizobium japonicum*. Dotted arrows indicate protein–protein interactions; (+) symbolises activation, and (–) repression. The CIRCE element in the promoter region of *groESL*$_2$ is written in quotation as it is not functional as a repressor binding site. The abbreviation UAS (upstream activation site) marks the binding site for the oxygen-responsive transcriptional activator protein NifA. Details are explained in the text.


NifA / RpoN
CIRCE
RpoH
ROSE
groESL3
O2 LIMITATION
RpoN
NifA
UAS
–24 –12
HEAT STRESS
GroESL
NO STRESS
HrcA
groESL4
groESL5
CIRCE
groESL2
"CIRCE"
denatured proteins
DnaKJ
FtsH
RpoH2
dnaKJ
hrcA
grpE
groESL1
–35 –10
RpoH1
Small heat shock proteins
Repressor
hspH
hspEForfG
hspD
hspBCdegP
hspArpoH1
ROSE

4.1 CIRCE / HrcA

CIRCE (controlling inverted repeat of chaperone expression; Zuber & Schumann, 1994) comprises a widespread regulatory *cis* element in the promoter regions of chaperone operons from more than 40 different eubacteria (Hecker *et al.*, 1996; Narberhaus, 1999). DNA sequence analysis of the *B. japonicum groESL* operons revealed a putative CIRCE element in the promoter regions of $groESL_2$, $groESL_4$ and $groESL_5$ (Babst *et al.*, 1996). However, increased transcription after exposure to elevated temperatures was found only in the case of $groESL_4$ and $groESL_5$. Induction of $groESL_4$ transcription progressed transiently with a maximal mRNA level at 35 min after a temperature upshift from 30 to 39°C (Babst *et al.*, 1996). In contrast to $groESL_4$ and $groESL_5$, transcription of $groESL_2$ was found to be unregulated under all growth conditions tested. Consequently, it was concluded that the CIRCE-like DNA element downstream of the $groESL_2$ promoter is not functional (Babst *et al.*, 1996). An inspection of the proteome of *B. japonicum*, however, revealed that the $GroESL_2$ proteins were induced upon heat shock (Münchbach *et al.*, 1999a). Undefined posttranscriptional mechanisms, probably at the level of mRNA stability, might be responsible for this discrepancy between the mRNA and protein data.

First evidence for a negative regulatory function of the *B. japonicum* CIRCE element was provided by the fact that a deletion of 4 bp within the CIRCE element of $groESL_4$ resulted in elevated transcription of the $groESL_4$ operon under non-heat shock conditions (Babst *et al.*, 1996). A similar increase of the $groESL_4$ (and $groESL_5$) transcription was observed in *B. japonicum* mutant strains containing a deletion of the *hrcA* gene coding for the putative CIRCE binding protein HrcA (*cf.* chapter 3). To further investigate the specificity of this putative repressor protein, an amino-terminally histidine-tagged version of *B. japonicum* HrcA was purified under non-denaturing conditions. Noteworthy, this purification yielded a large fraction of soluble protein, which contrasts previous attempts to purify the HrcA protein from other organisms. *In vitro* evidence for a specific interaction between HrcA and the CIRCE element was obtained in gel retardation experiments which demonstrated the ability of HrcA to bind to a DNA fragment that contained the CIRCE element but not to a comparable fragment in which the CIRCE element was replaced by a sequence that had no similarity to CIRCE (*cf.* chapter 3).

A dependence of the HrcA repressor activity on the level of GroESL, as shown for the CIRCE-HrcA system in *B. subtilis* (Mogk *et al.*, 1997), had initially been observed in *B. japonicum* cells (Babst *et al.*, 1996). Deletion of $groEL_4$, which supplies the cell with a basal steady-state level of GroEL protein, led to the increased expression from its own CIRCE-dependent promoter but not from the σ^{32}-dependent $groESL_1$ promoter. Accordingly, it was proposed that the cellular pool of free chaperonins controls the expression of CIRCE-HrcA-regulated genes (Babst *et al.*, 1996). All attempts to simulate this feedback control *in vitro* failed (*cf.* chapter 3). In contrast to experiments performed with *Bacillus stearothermophilus* HrcA (Mogk *et al.*, 1997), our gel shift experiments showed that the addition of the GroE chaperonin machinery did not improve the DNA binding activity of purified HrcA but rather impaired the formation of *B. japonicum* HrcA-CIRCE complexes. The physiological relevance of these data remains to be elucidated.

4.2 ROSE

The second negative regulatory mechanism of heat shock gene transcription in *B. japonicum* depends on a highly conserved DNA element of approximately 100 bp called ROSE (repression of heat shock gene expression; Narberhaus *et al.*, 1998a). This element is positioned between the transcriptional and the translational start sites of the first genes of at least five different heat shock operons (see Fig. 4.1). Although these operons are transcribed from putative housekeeping promoters, the presence of a ROSE element prevents expression of the downstream genes. A temperature upshift results in induced transcription of the ROSE regulon and in a strongly increased steady-state level of the corresponding gene products (Narberhaus *et al.*, 1998a; Münchbach *et al.*, 1999b).

The current model assigns two different regulatory functions to the ROSE element. First, it might act as a binding site for a putative repressor protein under normal growth conditions (Narberhaus *et al.*, 1998a). This assumption was supported by gel shift experiments, which demonstrated that extracts from unstressed cells but not from heat-shocked cells retarded the full-length $ROSE_1$ element. Moreover, this retardation was shown to depend on the presence of the $ROSE_1$ core region (positions +50 to +74) which is part of a highly conserved inverted repeat. Deletion of this core region in the *B. japonicum* wild-type strain resulted in derepression of the downstream genes at low temperature. A recent mutational analysis further supported the importance of the core region for ROSE-mediated repression (Hausherr, 1999). However, the fact that derepressed $ROSE_1$ mutants still responded weakly to a thermal shock by further increasing the transcription of the downstream genes (Narberhaus *et al.*, 1998a) and the finding that the transcription of $ROSE_1$-dependent genes was also repressed in *E. coli*, which most likely does not contain a ROSE-binding protein (Hausherr, 1999), pointed to an intrinsic regulatory function of the ROSE element. According to the nucleotide sequence, ROSE-containing mRNA might form a distinct secondary structure which consists of four consecutive inverted repeats (Hausherr, 1999). Assuming that this secondary structure is formed under normal growth conditions, it might either terminate transcription or cause rapid degradation of the corresponding mRNA as was described for the CIRCE element in *B. subtilis* (Homuth *et al.*, 1999). Hence, the formation of a

secondary structure by the ROSE-containing mRNA might provide a second level of control for the repression of ROSE-regulated genes.

The identification of critical residues in ROSE is currently being attempted by an extensive mutational analysis and by the search for new ROSE elements in other bacterial species. Southern blot hybridisations using *B. japonicum* $ROSE_1$ as a probe demonstrated that this element might be restricted to the genus of *Bradyrhizobium* (F. Narberhaus, personal communication). Recently, four new ROSE elements with significant homology to the already known ROSEs were identified in *Bradyrhizobium* sp. Parasponia (A. Nocker, personal communication).

Another area of investigations concerns the characterisation of ROSE-dependent *B. japonicum* genes (see Fig. 4.1). Seven out of ten identified ROSE-dependent genes code for small heat shock proteins (sHsps). These proteins can be further divided into two distinct classes on the basis of sequence similarities (Münchbach *et al.*, 1999b). A recent model suggests that sHsps bind to denatured proteins accumulated under stress conditions and maintain them in a folding-competent state (Ehrnsperger *et al.*, 1997; Lee *et al.*, 1997). In order to elucidate the function of the multiple sHsps of *B. japonicum*, two proteins of each class of sHsps were purified and are currently being analysed for their effect on heat-induced aggregation of citrate synthetase (S. Studer, personal communication). Apart from the genes that encode sHsps, three other ROSE-dependent *B. japonicum* genes were found (*orfG*, *degP* and $rpoH_1$; Fig. 4.1). The putative gene product of *orfG* showed no significant similarity to any known protein sequence. Moreover, a deletion of *orfG* had no effect on the expression of $hspArpoH_1$, which makes it unlikely that the deduced protein is involved in the ROSE-dependent heat shock regulation (Narberhaus *et al.*, 1998a). The *degP* gene product displays significant amino acid sequence similarity to the heat-inducible, periplasmic serine endoprotease HtrA (DegP) of *E. coli* (Lipinska *et al.*, 1988). While the *E. coli* protein was shown to be essential for survival at high temperature and the degradation of abnormal periplasmic proteins (Strauch & Beckwith, 1988; Lipinska *et al.*, 1989; Strauch *et al.*, 1989), no growth condition was found under which the DegP homologue of *B. japonicum* is required (Narberhaus *et al.*, 1998c). The $rpoH_1$ gene, which encodes a σ^{32}-type heat shock sigma factor, will be presented in the following section.

4.3 RpoH

The identification of a functional σ^{32}-dependent promoter upstream of *groESL*$_1$ (Babst *et al.*, 1996) initiated the search for a σ^{32}-like factor in *B. japonicum*, which resulted in the identification of three different *rpoH*-homologous genes (Fig. 4.2 and Narberhaus *et al.*, 1996; Narberhaus *et al.*, 1997). The designation of the deduced proteins as RpoH sigma factors was based on the presence of a highly conserved RpoH box within their amino acid sequences and their ability to initiate transcription from the σ^{32}-dependent *groE* promoter in *E. coli* (Narberhaus *et al.*, 1997). At present, *B. japonicum* is the only organism known to possess three RpoH factors. As outlined below, the three *rpoH* genes are disparately regulated, their products have different promoter specificities and they are differentially resistant to degradation by the FtsH protease.

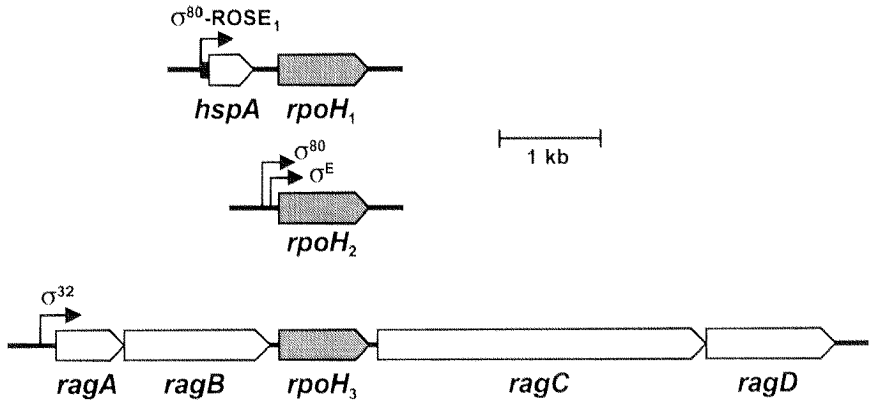


Fig. 4.2 Physical map of the *B. japonicum rpoH* gene regions. Arrows mark the identified transcription start sites. σ^{80} and σ^{32} indicate promoters that are recognised by the *B. japonicum* housekeeping sigma factor SigA (Beck *et al.*, 1997) and the heat shock sigma factors RpoH, respectively. σ^{E} denotes a promoter that resembles the *E. coli* extreme heat shock promoter.

RpoH$_1$. Heat-inducible transcription of the *B. japonicum hspArpoH*$_1$ operon is controlled by the ROSE mechanism (Fig. 4.2 and Narberhaus *et al.*, 1996). Mutants containing a deletion in *rpoH*$_1$ showed no apparent growth defect and still produced RpoH protein indicating the presence of a *rpoH* gene family. After a temperature upshift, *B. japonicum* induces the synthesis of a RpoH protein, which remains at an elevated level for several hours. A comparison of RpoH protein levels in the wild type, an *rpoH*$_1$-deficient mutant and an *rpoH*$_{1+3}$ double mutant demonstrated that the increased steady-state level of RpoH at elevated temperatures was primarily caused by

the accumulation of $RpoH_1$ protein (Narberhaus *et al.*, 1997). This accumulation is consistent with the heat shock-induced transcription of *B. japonicum* $groESL_1$, whose promoter is efficiently transcribed by $RpoH_1$-containing RNA polymerase (Babst *et al.*, 1996; Narberhaus *et al.*, 1998b). By contrast, the *dnaKJ* promoter was only weakly transcribed *in vitro* by RNA polymerase reconstituted with $RpoH_1$ (Narberhaus *et al.*, 1998b). We thus conclude that $RpoH_1$ is mainly responsible for the heat-induction of $groESL_1$ in *B. japonicum* (Fig. 4.3).

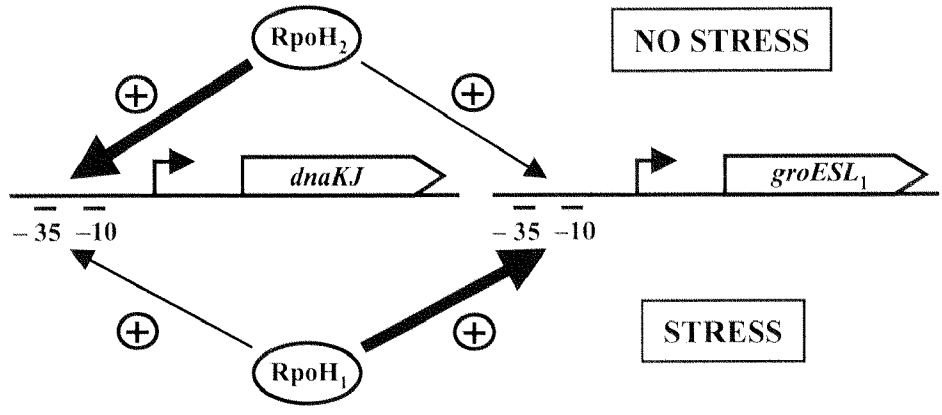


Fig. 4.3 Model of the proposed regulatory function of the *B. japonicum* heat shock sigma factors $RpoH_1$ and $RpoH_2$ in the regulation of *dnaKJ* and $groESL_1$. (+) symbolises activation. The model is adapted from (Narberhaus *et al.*, 1998b). Details are explained in the text.

$RpoH_2$. Two transcription start sites were identified upstream of *B. japonicum* $rpoH_2$ (Narberhaus *et al.*, 1997). Under normal growth conditions, $rpoH_2$ is transcribed from a σ^{70}-type housekeeping promoter whereas a second promoter, which resembles the *E. coli* σ^E-type promoter (Raina *et al.*, 1995; Rouvière *et al.*, 1995), allows $rpoH_2$ transcription at extreme temperatures (48°C) (Fig. 4.2). The first promoter provides a basal $RpoH_2$ level at normal growth temperatures that might guarantee the basal expression of essential σ^{32}-regulated genes (e.g. genes of the DnaK machinery; *cf.* chapter 2 and 3). In favour of this assumption, $RpoH_2$ efficiently promoted transcription of the *B. japonicum dnaKJ* operon *in vitro* (Narberhaus *et al.*, 1998b) and two-dimensional gel analysis revealed that DnaK is indeed significantly expressed under normal growth conditions (Münchbach *et al.*, 1999b). The importance of $rpoH_2$ and *dnaK* for the viability of the cell at normal growth temperatures was supported by the fact that neither gene could be deleted (*cf.* chapter 2 and Narberhaus *et al.*, 1997). We thus conclude that $RpoH_2$ represents the 'housekeeping' RpoH sigma factor of *B.*

japonicum. An important function of $RpoH_2$ is to provide a steady-state level of DnaKJ chaperones to the cell under normal growth conditions (Fig. 4.3). GrpE, the third component of the DnaK machinery, the HrcA repressor and the $GroESL_1$ chaperonins are also supplied via $RpoH_2$ as we have shown that $RpoH_2$ initiated transcription of the *B. japonicum grpE*, *hrcA* and *groESL*$_1$ promoters (*cf.* chapter 3 and Narberhaus *et al.*, 1998b).

$RpoH_3$. In comparison with the amount of data concerning *B. japonicum rpoH*$_1$ and *rpoH*$_2$, only little is known about the function of *rpoH*$_3$ in *B. japonicum*. It is located in an operon flanked by two genes encoding a classical two-component system with unknown function (*ragA* and *ragB*; Narberhaus *et al.*, 1997) and two genes whose deduced proteins display high sequence similarity to heavy metal or multidrug efflux pumps (*ragC* and *ragD*; Krummenacher & Narberhaus, 1999). The transcription of this putative pentacistronic operon is initiated from a σ^{32}-type promoter (Narberhaus *et al.*, 1997). Although a significant amount of transcript is produced even under normal growth conditions, $RpoH_3$ protein could not be detected by Western blot analysis in cell extracts indicating that this heat shock sigma factor does not significantly contribute to the RpoH pool in *B. japonicum* (F. Narberhaus, personal communication). Mutants carrying a deletion in the *rpoH*$_3$ gene region exhibited neither an apparent growth defect nor a deficiency in the symbiotic interaction with soybean roots. Moreover, a direct involvement of $RpoH_3$ in *rag* gene expression was excluded because transcription from the promoter upstream of *ragA* was normal in the *rpoH*$_3$ deletion strain (Krummenacher & Narberhaus, 1999). Complementation experiments in *E. coli* showed that *rpoH*$_3$ encodes a functional sigma factor capable of interacting with *E. coli* RNA polymerase and transcribing the *E. coli groE* promoter (Narberhaus *et al.*, 1997). Obviously, the function of $RpoH_3$ in *B. japonicum* remains to be elucidated.

RpoH stability. In addition to their different modes of regulation and their distinct promoter preferences, the *B. japonicum* RpoH sigma factors are also distinguished by their susceptibility towards proteolytic degradation. In *E. coli*, the degradation of RpoH depends on the presence of the DnaK chaperone machinery and is mainly performed by the membrane-anchored ATP-dependent metalloprotease FtsH (Herman *et al.*, 1995; Tomoyasu *et al.*, 1995). Whether the *B. japonicum* heat shock sigma factors are subject to a similar proteolytic degradation is currently under investigation. Recently, the *B.*

japonicum ftsH gene has been cloned and the ability of the corresponding gene product to degrade *E. coli* RpoH was demonstrated (Narberhaus *et al.*, 1999). As the *ftsH* gene could not be deleted in *B. japonicum*, we investigated the degradation of $RpoH_1$, $RpoH_2$ and $RpoH_3$ in a heterologous *E. coli* background. This study showed that only $RpoH_2$ was degraded by *E. coli* FtsH and that this proteolysis depended on the presence of DnaK and DnaJ as it is the case for *E. coli* RpoH (Urech *et al.*, 1999). $RpoH_3$ was also rapidly degraded in *E. coli* but this degradation was found to be performed by an alternative, *ftsH*-independent pathway. Most interestingly, $RpoH_1$ was not degraded by *E. coli* FtsH. This sigma factor thus represents a useful tool to study the substrate selectivity of the *E. coli* FtsH protease.

4.4 Heat shock regulatory network

The current view of the complex heat shock regulatory network of *B. japonicum* is outlined in Fig. 4.1. Although not all of the predictions have been confirmed yet, it is already evident that the three regulatory mechanisms do not coexist independently of each other. There is important cross-talk between the systems as shown by the RpoH-dependent synthesis of the CIRCE binding protein HrcA and the ROSE-dependent expression of $rpoH_1$. Interestingly, two different strategies for the production of the major chaperone systems DnaKJ and GroESL have evolved. DnaK, DnaJ and GrpE, the components of the DnaK chaperone machinery, are produced by the RpoH mechanism only. Accordingly, several elements of the DnaK system or factors involved in their biosynthesis were found to be essential (DnaK (*cf.* chapter 2), GrpE (*cf.* chapter 3), $RpoH_2$ (Narberhaus *et al.*, 1997), FtsH (Narberhaus *et al.*, 1999)). By contrast, the cellular GroE chaperonin pool is provided from five *groESL* operons which are differentially regulated and whose products are able to replace each other (Fischer *et al.*, 1999). While one *groESL* operon ($groESL_3$) is specifically transcribed under nitrogen-fixing conditions (Fischer *et al.*, 1993), other *groESL* operons are classical heat shock operons. The CIRCE-controlled $groESL_4$ operon is transiently expressed after heat shock whereas transcription of the RpoH-controlled $groESL_1$ operon proceeds at an elevated level for several hours (Babst *et al.*, 1996). This maximal flexibility in adjusting the cellular chaperonin level to the given environmental condition might be advantageous for survival both in the soil and in the host plant cell. In summary, the amazing complexity of regulatory systems controlling the synthesis of chaperones and other heat shock proteins in *B. japonicum* makes it an interesting model system for studying large regulatory networks in bacteria.

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PART B

Genes involved in the phosphate metabolism

The following chapters introduce two genes whose corresponding proteins are involved in the complex phosphate metabolism of *Bradyrhizobium japonicum*.

Chapter 5 presents the characterisation of the *B. japonicum phoB* gene encoding the central phosphate regulatory protein PhoB which was fortuitously cloned in the course of our attempts to identify alternative σ factors (see also Addendum). Studies mainly performed in *Escherichia coli* showed that under conditions of phosphate limitation, phosphorylated PhoB acts as a transcriptional activator necessary for a suitable starvation response (reviewed in Torriani-Gorini *et al.*, 1994; Wanner, 1996). In accordance with these data, our results demonstrated an impaired growth of the *phoB*-deficient *B. japonicum* mutants in media containing little phosphate.

Chapter 6 presents results concerning the *B. japonicum* phospholipid *N*-methyltransferase encoded by *pmtA*. This project was initiated by Otto Geiger (Institute of Biotechnology, Technical University of Berlin, Germany) whose laboratory recently identified the *pmtA* gene of *Sinorhizobium meliloti* encoding an enzyme which catalyses the three successive methylations of phosphatidylethanolamine to obtain the major membrane-forming phospholipid phosphatidylcholine. Our collaboration, was aimed at the phenotypical characterisation of *B. japonicum pmtA* which turned out to be necessary for the symbiotic interaction of the bacteria with its soybean host plant.

Interestingly, a recent study in *S. meliloti* revealed a connection between phospholipid synthesis and the phosphate regulatory protein PhoB (Geiger *et al.*, 1999). Under phosphate-limiting conditions, *S. meliloti* replaces its membrane phospholipids by lipids not containing phosphorus, e.g. sulpholipids, ornithine lipids, and by high amounts of diacylglyceryl-*N,N,N*-trimethylhomoserine (DGTS). The regulation of this phosphate-starvation induced change in the membrane lipid composition, and specifically in the DGTS biosynthesis, was shown to be mediated by PhoB.

CHAPTER 5

The *Bradyrhizobium japonicum phoB* gene is required for phosphate-limited growth but not for symbiotic nitrogen fixation

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5.1 Abstract

We identified by cloning and DNA sequence analysis the phosphate regulatory gene *phoB* of *Bradyrhizobium japonicum*. The deduced gene product displayed pronounced similarity to the PhoB protein of *Sinorhizobium meliloti* (71.4 % identical amino acids), *Escherichia coli* (50.2 %) and other bacterial species. Insertion of a kanamycin resistance cassette into *phoB* led to impaired growth of the *B. japonicum* mutant in media containing approximately 25 µM phosphate, or less. A standard plant infection test using wild-type and *phoB*-defective *B. japonicum* strains showed that the *phoB* mutation had no effect on the symbiotic properties of *B. japonicum* with its soybean host plant.

5.2 Introduction

Phosphorus in the environment is one of several important nutritional factors for the growth of bacteria. Symbiotically living bacteria such as *Bradyrhizobium japonicum*, the nitrogen-fixing root-nodule symbiont of soybean, are particularly affected because the symbiotic dinitrogen fixation process is highly phosphorus-dependent (Israel, 1987). Israel (1987) showed that the environmental phosphorus concentration specifically influences both nodule size and nodule number in addition to being critical for supporting host plant growth.

The role of phosphorus in bacterial growth has been investigated mainly with *Escherichia coli* (for reviews see Torriani-Gorini *et al.*, 1994; Wanner, 1996). The genetic control system is based on the phosphate regulatory protein PhoB. Its activity is modulated by specific phosphorylation and dephosphorylation, mediated by the transmembrane sensor protein PhoR in response to the environmental phosphate concentration. Under conditions of phosphate limitation, phosphorylated PhoB acts as a transcriptional activator by binding to a specific 18-bp sequence (*pho* box) in the promoter region of genes belonging to the so-called *pho* regulon. The *pho* regulon in *E. coli* consists of at least 31 genes arranged in eight transcriptional units, whose gene products are involved in the transport (e.g. the PstSCAB phosphate transport system) or mobilisation (e.g. the PhoA phosphatase) of phosphates and phosphorus compounds.

Comparatively little is known about the phosphate regulatory system in symbiotically living bacteria. Only recently, Bardin *et al.* (1996) described an ABC-type phosphate uptake system, PhoCDET, of *Sinorhizobium meliloti*, whose transcription is induced in response to phosphate starvation. An analysis of the corresponding promoter region revealed two elements that are similar to the previously described *pho* boxes present in phosphate-regulated *E. coli* promoters. Mutants having mutations in the *S. meliloti phoCDET* operon grew poorly at a relatively high phosphate concentration of 2 mM and failed to form nitrogen-fixing root nodules. By analogy with the situation in *E. coli* it seems reasonable to propose that the product of the *phoB* gene, which has been identified in *S. meliloti*, regulates the cellular response to environmental phosphate limitation.

We report here that the sequence analysis of a fortuitously cloned *B. japonicum* DNA region revealed an open reading frame with similarity to the phosphate regulatory

gene *phoB*. The results from a mutational analysis of the *S. meliloti* phosphate uptake system PhoCDET (Bardin *et al.*, 1996) prompted us to investigate the significance of *phoB* for cell growth under phosphate limitation and for the symbiotic interaction of *B. japonicum* with its soybean host plant.

5.3 Materials and methods

5.3.1 Bacterial strains, plasmids, and growth conditions

E. coli cells were grown in Luria-Bertani (LB) medium (Miller, 1972) supplemented with ampicillin (200 µg/ml), kanamycin (30 µg/ml), or tetracycline (10 µg/ml), if required. The growth temperature for *E. coli* strains was 37°C. *B. japonicum* was routinely grown aerobically at 28°C in PSY medium (Regensburger & Hennecke, 1983). For phosphate limitation experiments *B. japonicum* strains were propagated aerobically at 28°C in HM minimal medium (Cole & Elkan, 1973) containing different concentrations of phosphate. Both *B. japonicum* media were supplemented with 0.1 % (w/v) L-arabinose. If appropriate, antibiotics were added at the following concentrations µg/ml): chloramphenicol, 20 (for counterselection against *E. coli* donor strains); kanamycin, 100; spectinomycin, 100; and tetracycline, 50. The spectinomycin-resistant *B. japonicum* strain 110*spc*4 (Regensburger & Hennecke, 1983) was used as parental strain. Strains 5518 and 5519 are derivatives of strain 110*spc*4 in which a neomycinphosphotransferase II cassette (Km^r) was inserted into the *Nru*I site located in the *phoB* gene. The orientation of the cassette relative to *phoB* in the individual mutants is shown in Fig. 5.1. Plasmid pRJ5510 is a pUC18 derivative, which carries the *B. japonicum phoB* gene region on a 1.8 kb insert as shown in Fig. 5.1.

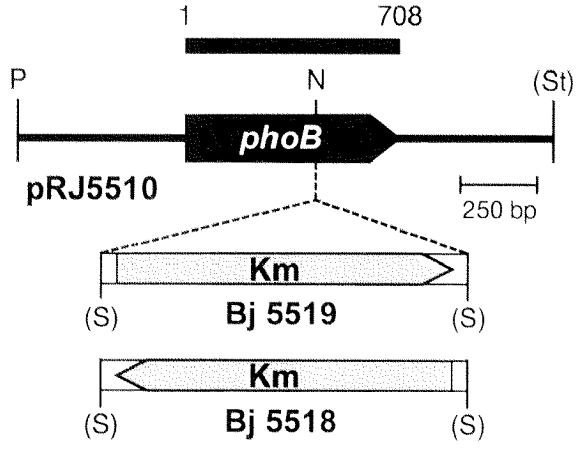

Fig. 5.1 Physical map of the *B. japonicum phoB* gene. The genetic organisation of the 1.8 kb insert of plasmid pRJ5510 and the *B. japonicum phoB* insertion mutations in strains 5518 and 5519 are shown. Abbreviations: Km, neomycinphosphotransferase II gene; N, *Nru*I; P, *Pst*I; S, *Sma*I; St, *Stu*I. Restriction sites in *parentheses* were destroyed during the cloning procedures. The nucleotide sequence, which has been deposited in the GenEMBL database under accession number AJ223073, and the corresponding numbers respresenting the first and last nucleotide of the start and stop codon of *phoB*, respectively, are indicated above the physical map.

5.3.2 DNA manipulations

Recombinant DNA techniques were used according to standard protocols (Sambrook *et al.*, 1989). DNA was sequenced by the chain termination method (Sanger *et al.*, 1977) with a model 373 DNA sequencer and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The DNA region sequenced and the deduced amino acid sequence were analysed using the software package of the UWGCG (Genetics Computer Group of the University of Wisconsin, Madison, USA; version 8.0) or the NCBI (National Center for Biotechnology Information) BLAST network server. The 'CODONPREFERENCE' program was applied using a previously established *B. japonicum* codon usage table (Ramseier & Göttfert, 1991).

5.3.3 Construction of *B. japonicum phoB* mutant strains

The 1.2 kb *Sma*I fragment of pBSL15 (Alexeyev, 1995) containing the neomycinphosphotransferase II cassette (Km^r) was inserted into the *Nru*I site located in the *phoB* gene present on plasmid pRJ5510 (Fig. 5.1). Both orientations of the resistance cassette relative to the *phoB* gene were obtained. The inserts of the resulting plasmids were subcloned into pSUP202pol3 (H. M. Fischer, unpublished) and mobilised from *E. coli* S17-1 into *B. japonicum* 110*spc*4 for marker replacement mutagenesis as described previously (Hahn & Hennecke, 1984). The correct genomic structure of these mutant strains was confirmed by Southern blot hybridisation of genomic DNA digested with appropriate restriction enzymes, using a *B. japonicum phoB* probe.

5.3.4 Plant infection test

The symbiotic phenotype of the *B. japonicum phoB* mutants was determined in a soybean plant infection test as described previously (Hahn & Hennecke, 1984; Göttfert *et al.*, 1990).

5.4 Results

5.4.1 Sequence analysis of the *B. japonicum phoB* gene

In the course of our attempts to identify alternative σ factors in *B. japonicum* we had cloned a 1.8 kb chromosomal fragment that hybridised to an *E. coli rpoS* probe (data not shown). DNA sequence analysis of the insert of the resulting plasmid pRJ5510 revealed an open reading frame of 708 nucleotides. The corresponding gene product consists of 235 amino acids with a predicted molecular weight of 26,895. A search in the GenEMBL database using the program 'TFASTA' showed that the deduced amino acid sequence exhibited significant similarity to the phosphate regulatory protein PhoB of *S. meliloti* (71.4% identical amino acids; accession number M96261), *E. coli* (50.2%; P08402) and other bacterial species (Fig. 5.2). Moreover, the highly conserved phosphate-accepting residue Asp-53 as well as residue Thr-83, which plays an important role in the phosphate transfer as described for *E. coli* PhoB (Makino *et al.*, 1994), are also present in the *B. japonicum* protein. On the basis of these similarities the predicted open reading frame found on pRJ5510 was designated *phoB*. As the cloned DNA fragment did not contain *rpoS*-like sequences, the reason for the initially observed cross-hybridisation with *E. coli rpoS* remains obscure.

```
          1                                                          80
Bj PhoB MGARIMVVEDEEALTELLRYNLEGDGYDVETVMRGDDADTRLKEHIPDLIVLDWMLPGLSGIELCRRLRTRPETKQLPII
Sm PhoB MLPKIAVVEDEEALSVLLRYNLEAEGFEVDTILRGDEAEIRLQERLPDLLILDWMLPGVSGIELCRRLRQRPETERLPII
Ec PhoB MARRILVVEDEAPIREMVCFVLEQNGFQPVEAEDYDSAVNQLNEPWPDLILLDWMLPGGSGIQFIKHLKRESMTRDIPVV
          *   * *****          **  *         * *   * *  ***  ******* ***     *     *   *
         81                                                         160
Bj PhoB MLTARGEESERVRGLATGADDYIVKPFSVPELLARVKGLLRRASPERLATVLAYGDIELDRDKRRVARSGRPIDLGPTEY
Sm PhoB MLTARGEESERVRGLATGADDYVVKPFSTPELMARVKAMLRRAKPEVLSTLLRCGDIELDRETHRVHRRSREVRLGPTEF
Ec PhoB MLTARGEEEDRVRGLETGADDYITKPFSPKELVARIKAVMRRISPMAVEEVIEMQGLSLDPTSHRVMAGEEPLEMGPTEF
          ********  ***** ******  ****  ** ** *   **  *            **    **        ****
        161                                                        235
Bj PhoB RLLEFFLEHPGRVFSREQLLDSVWGRDIYIDERTVDVHIGRLRKLLNLGREQDPIRTVRGAGYALDDRFAKAEQT
Sm PhoB RLLEFLMSSPGRVFSRSQLLDGVWGHDIYVDERTVDVHVGRLRKALNFSNMPDVIRTVRGAGYSLES.....
Ec PhoB KLLHFFMTHPERVYSREQLLNHVWGTNVYVEDRTVDVHIRRLRKALEPGGHDRMVQTVRGTGYRFSTRF.....
           ** *    * ** ** ***  ***   *   ******  **** *          **** **
```

Fig. 5.2 Alignment of deduced amino acid sequences of the PhoB protein of *B. japonicum* (*Bj*, accession number AJ223073), *S. meliloti* (*Sm*, M96261), and *E. coli* (*Ec*, Makino *et al.*, 1986). Amino acids are given in the single-letter code. The *asterisks* indicate residues that are identical in all three sequences. The putative phosphate-accepting residue (D), and a second residue (T) that plays an important role in the phosphate transfer reaction of the *E. coli* protein, are boxed.

5.4.2 Construction and phenotypic analysis of *phoB* mutants

B. japonicum phoB mutants 5518 and 5519 were constructed by insertion of a kanamycin resistance cassette into the *Nru*I site located in the *phoB* gene (see Fig. 5.1

and Materials and methods). The symbiotic properties of these mutants were tested in a plant infection test. The mutant strains did not differ from the wild type with respect to the ability to nodulate soybean roots and to fix nitrogen under symbiotic conditions. Nitrogenase activity was determined as 108% ± 9% and 111% ± 11% of wild-type activity for *B. japonicum* 5518 and 5519, respectively. This indicates that the *phoB* gene product is not essential for symbiosis under the conditions tested.

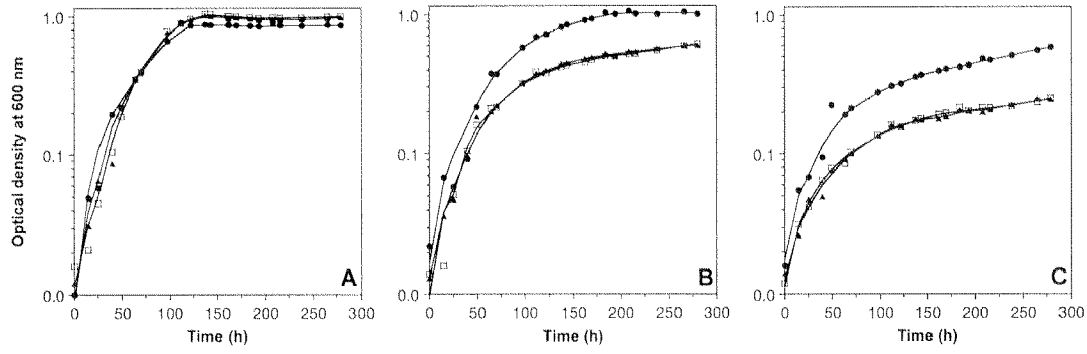

Fig. 5.3 Effect of phosphate-limited conditions on the growth of the *B. japonicum* wild type and of *phoB* mutants. Cultures of *B. japonicum* wild type (●) and *phoB* mutants 5518 (□) and 5519 (▲) were grown in minimal medium (HM) containing 88.1 µM (**A**), 26.4 µM (**B**), and 8.8 µM phosphate (**C**) under aerobic conditions at 28°C. The growth curves are based on mean values from two parallel cultures. Deviations between parallel cultures were less than 10%.

The mutants were further analysed for their growth behaviour in media containing different phosphate concentrations. Aerobic growth of the mutants in rich medium (PSY medium) or minimal medium (HM medium) was not affected as compared with the wild type (data not shown). Only when the phosphate concentration in the minimal medium was strongly reduced, growth of the mutants was slowed down. Fig. 5.3 shows the behaviour of the wild type and the mutants growing aerobically over a period of 280 h in minimal medium containing 88.1 µM, 26.4 µM, and 8.8 µM phosphate, which corresponds to 10%, 3%, and 1% of the phosphate in standard HM medium, respectively. At a phosphate concentration of 88.1 µM, the doubling times of the wild type and the mutants were similar (19 h), and comparable final cell densities were reached (Fig. 5.3A), whereas at 26.4 µM phosphate the respective doubling times were calculated as 21 h for the wild type and 26 h for the mutants (Fig. 5.3B). After 280 h, the optical density of mutant cultures had reached only about half of the one observed in wild-type cultures. This is due to a rather short exponential growth phase of the

mutant cultures, as growth slowed down after approximately 80 h. In the presence of 8.8 μM phosphate, limited growth was observed for both the wild-type and the mutant strains (Fig. 5.3C). Again the *phoB* mutants reached a lower final optical density after 280 h than the wild type (0.25 and 0.58, respectively). Notably, no difference was observed between mutants 5518 and 5519 under all conditions tested.

5.5 Discussion

The identification of the *B. japonicum phoB* gene was based on the pronounced similarity of the deduced *phoB* gene product to PhoB proteins from other bacteria. Furthermore, a functional role of the *B. japonicum phoB* gene was documented by the impaired growth of *phoB* mutants under phosphate-limited conditions.

As shown for *E. coli*, a group of genes belonging to the *pho* regulon is activated by the regulatory protein PhoB during phosphorus limitation. The objective of this regulation is to achieve a higher phosphorus concentration in the cell by increasing the number of phosphate, organophosphate and phosphonate uptake and mobilisation systems (Torriani-Gorini *et al.*, 1994; Wanner, 1996). The deletion of a phosphate uptake system can cause severe effects on cell growth as demonstrated by the mutant analysis of the *S. meliloti* PhoCDET system (Bardin *et al.*, 1996). Mutants of *phoCDET* grew poorly at a relatively high phosphate concentration of 2 mM. Assuming that *B. japonicum*, a rather close relative of *S. meliloti*, possesses a phosphate transport system functionally equivalent to PhoCDET, one may ask why the *B. japonicum phoB* mutants described here showed a growth defect only at a phosphate concentration lower than 25 µM. One possible explanation is that the phosphate transport system is synthesised at a basal level independently of PhoB. In fact, a basal expression of the high-affinity PhoB-regulated phosphate transport system PstSCAB (K_m 0.4 µM) was observed in *E. coli* under conditions of phosphate excess where PhoB is not active. However, under these conditions the majority of phosphate is taken up by the so-called Pit transporter, a low-affinity phosphate uptake system (K_m 38.2 µM) that appears to be synthesised constitutively (see Wanner, 1996; and references therein). A similar, alternative phosphate uptake system might also be present in *B. japonicum* which would enable the *phoB* mutants to grow normally at phosphate concentrations as low as approximately 100 µM. The presence of several phosphate uptake systems with different substrate affinities seems to be a common feature in bacteria because a *pit*-like gene has recently been identified also in *S. meliloti* (Bardin *et al.*, 1996; Voegele *et al.*, 1997).

Shortly before our work was submitted, Voegele *et al.* (1997) published a thorough study on the PhoCDET high-affinity phosphate uptake system (K_m 0.2 µM) and the OrfA-Pit low-affinity system (K_m 1–2 µM) in *S. meliloti*. Under phosphate-limited conditions the PhoB regulatory protein activated the expression of the *phoCDET*

operon and repressed the OrfA-Pit system. The failure of *S. meliloti phoCDET* mutants to grow under conditions of excess phosphate (2 mM) was apparently due to a reduced activity or expression of the OrfA-Pit system in these mutants via an unknown mechanism. By contrast, the normal growth of *B. japonicum phoB* mutants under high phosphate conditions would suggest that the function of an OrfA-Pit-like system, if it exists in this species, is not affected by the *phoB* mutation.

The growth phenotype of *B. japonicum phoB* mutants might explain why they showed no obvious defect in the plant infection test. The Jensen medium used to grow soybeans contains approximately 2.5 mM phosphate. Thus, it seems likely that bacteroids living in the root nodules are provided with non-limiting amounts of phosphate and, therefore, a defective PhoB protein might cause no disadvantage for the cell in this assay. We considered it unrealistic to perform plant infection tests under phosphate limitation, because this condition does not only affect *B. japonicum* but also soybean growth *per se* (Drevon & Hartwig, 1997) which makes it difficult, if not impossible, to interpret *in planta* N_2 fixation data.

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CHAPTER 6

The *Bradyrhizobium japonicum pmtA* gene encoding a phospholipid *N*-methyltransferase is required for an efficient symbiosis with the soybean host plant

Parts of this chapter will be published in collaboration with O. Geiger (TU Berlin, Germany).

6.1 Abstract

Phosphatidycholine, the major membrane component in most eukaryotes, is found only in few bacteria including the family of *Rhizobiaceae*. Bacterial phosphatidylcholine is mainly synthesised by *N*-methylation of phosphatidylethanolamine. Here we report on the isolation of the *Bradyrhizobium japonicum pmtA* gene whose deduced 22 kDa gene product displays significant homology to the recently found phospholipid *N*-methyltransferase PmtA of *Sinorhizobium meliloti*. *In vivo* transcript mapping identified a transcription start site 46 nucleotides upstream of *pmtA* and revealed a putative *pmtA* promoter that resembles typical housekeeping promoters of *B. japonicum*. Insertion of a kanamycin resistance cassette into *pmtA* led to slightly reduced growth rates of the mutants in rich media under aerobic and microaerobic conditions. Interestingly, this growth defect was alleviated by the addition of 1 mM choline to the medium suggesting the presence of an alternative, choline-dependent pathway of phosphatidylcholine biosynthesis. Soybean root nodules of plants infected with *B. japonicum pmtA* mutants showed a nitrogen fixation activity of only 18% and their interior colour was beige suggesting reduced amounts of leghemoglobin. Moreover, ultrastructure analysis of these nodules demonstrated a strongly reduced number of bacteroids within infected plant cells. According to these data we assume that the *pmtA* gene product is necessary for an efficient symbiotic interaction of *B. japonicum* with its soybean host plant. The findings of this study are discussed in relation to the results of the phospholipid analysis of *B. japonicum* wild type and *pmtA* mutants performed by the group of Otto Geiger (Institute of Biotechnology, Technical University of Berlin, Germany).

6.2 Introduction

Phosphatidylcholine (PC) is the major membrane-forming phospholipid in eukaryotes. Beside its essential function in the formation of the eukaryotic membrane bilayer, PC was found to be involved in a wide variety of systems, e.g. as a main component of pulmonary surfactant (reviewed in Veldhuizen *et al.*, 1998), or as a precursor of diacylglycerol which is implicated in the cellular signal transduction (reviewed in Exton, 1994; Exton, 1997).

Eukaryotes have evolved two principal mechanisms for the biosynthesis of PC. In all nucleated eukaryotic cells, PC is derived predominantly from the CDP-choline pathway (Kennedy & Weiss, 1956). Following its uptake into the cell, choline is activated to choline phosphate and subsequently to CDP-choline, which finally condenses with diaclyglycerol to obtain PC (reviewed in Kent, 1995). The second possibility of PC biosynthesis involves three successive *N*-methylations of phosphatidylethanolamine (PE) using *S*-adenosyl-L-methionine as methyl donor (reviewed in Vance & Ridgway, 1988). This PE *N*-methylation pathway has an important function in mammalian liver cells when dietary choline is insufficient (Walkey *et al.*, 1998) and it is used as an alternative pathway of PC formation in the eukaryotic microorganisms *Neurospora crassa* (Crocken & Nyc, 1964), *Saccharomyces cerevisiae* (reviewed in Henry & Patton-Vogt, 1998) and *Schizosaccharomyces pombe* (Kanipes *et al.*, 1998). In contrast to the PE *N*-methylation in liver cells where only a single enzyme is necessary for the complete conversion of PE to PC (Ridgway & Vance, 1987), there is genetic evidence that in these microorganisms two distinct enzymes are involved in this conversion (Scarborough & Nyc, 1967; Kodaki & Yamashita, 1987). One enzyme, the so-called PE methyltransferase, catalyses the first methylation step from PE to monomethlyphosphatidylethanolamine (MMPE) whereas the second enzyme, the phospholipid methyltransferase, converts MMPE to dimethylphosphatidylethanolamine (DMPE) and PC.

Among bacteria, only few species contain PC within their membranes (Goldfine, 1982). Until recently, the formation of this bacterial PC was thought to derive exclusively from the PE *N*-methylation pathway. This assumption was supported by the fact that *Zymomonas mobilis* and *Rhodobacter sphaeroides* mutants deficient in phospholipid *N*-methyltransferase (Pmt) had lost their ability to synthesise PC (Arondel

et al., 1993; Tahara *et al.*, 1994). Moreover, a single methyltransferase able to catalyse all three methylation reactions along the PE *N*-methylation pathway was found in both *Z. mobilis* (Tahara *et al.*, 1987) and *R. sphaeroides* (Arondel *et al.*, 1993). Interestingly, *R. sphaeroides pmt* mutants were fully functional in their vegetative properties. In contrast, in *Agrobacterium tumefaciens* two methyltransferase activities were described. While one enzyme activity is capable to perform all three methylation reactions, a second methyltransferase catalyses only the first methylation of PE to MMPE (Kaneshiro & Law, 1964). Moreover, an additional pathway to produce PC in this organism might involve direct incorporation of choline into PC. However, an enzymatic activity indicative of a functional CDP-choline pathway was not detected in cell extracts (Sherr & Law, 1965).

New insights into the bacterial PC biosynthesis were obtained by two consecutive studies in *Sinorhizobium meliloti*. The observation that mutants with a defective PE *N*-methylation pathway were still able to form PC in wild-type amounts when propagated on complex medium indicated the existence of an alternative pathway of PC biosynthesis (de Rudder *et al.*, 1997). The mechanism was recently identified as a direct condensation of choline with CDP-diacylglycerol yielding PC in a one step reaction (de Rudder *et al.*, 1999). The corresponding enzymatic activity identified in cell extracts was named phosphatidylcholine synthase (Pcs). As the legume host plants of *S. meliloti* exude choline into their rhizosphere it was proposed that this alternative pathway enables the bacterium to utilise this choline source for its PC biosynthesis (de Rudder *et al.*, 1999).

Here, we report the cloning of the *Bradyrhizobium japonicum pmtA* gene encoding a phospholipid *N*-methyltransferase which turned out to be necessary for the symbiotic interaction of the bacterium with its soybean host plant.

6.3 Materials and Methods

6.3.1 Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 6.1. *Escherichia coli* cells were grown at 37°C in Luria-Bertani (LB) medium (Miller, 1972) supplemented with ampicillin (200 µg/ml), kanamycin (30 µg/ml), or tetracycline (10 µg/ml) if required. *B. japonicum* strains were propagated aerobically at 30°C in PSY complex medium (Regensburger & Hennecke, 1983) or HM minimal medium (Cole & Elkan, 1973), both supplemented with 0.1 % (w/v) L-arabinose. Microaerobic conditions were reached by aerating closed cell cultures twice a day with a mixture of 0.1% O_2 and 99.9% N_2. If appropriate, antibiotics were added at the following concentrations (µg/ml): chloramphenicol, 20 (for counterselection against *E. coli* donor strains); kanamycin, 100; spectinomycin, 100; tetracycline, 50.

Table 6.1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype	Reference or origin
E. coli		
DH5α	*supE44* Δ*lacU169* (ψ80*lacZ*ΔM15) *hsdR17* *recA1 gyrA96 thi-1 relA1*	Gibco-BRL, Gaithersburg, MD, USA
S17-1	Sm^r Sp^r *hdsR* RP4-2 *kan*::Tn*7* *tet*::Mu, integrated in the chromosome	(Simon *et al.*, 1983)
B. japonicum		
110*spc*4	Sp^r (wild type)	(Regensburger & Hennecke, 1983)
5569	Sp^r Km^r *pmtA::kan*; *pmtA* and *kan* oriented in the same direction	This study
5570	Sp^r Km^r *pmtA::kan*; *pmtA* and *kan* oriented in opposite directions	This study
Plasmids		
pBluescript II KS+	Ap^r	Stratagene, La Jolla, CA, USA
pBSL86	Ap^r Km^r	(Alexeyev, 1995)
pUC19	Ap^r	(Norrander *et al.*, 1983)

(continued)

(Table 6.1 continued)

pSUP202	Ap^r Cm^r Tc^r *oriT* from RP4	(Simon *et al.*, 1983)
pSUP202pol4	Tc^r (pSUP202) part of polylinker from pBluescript II KS+ between *Eco*RI and *Pst*I	(Fischer *et al.*, 1993)
pRK290	Tc^r	(Ditta *et al.*, 1980)
pRK290*X*	Tc^r (pRK290) containing an additional *Xho*I site	(Alvarez-Morales *et al.*, 1986)
pTB2054	Ap^r (pUC19) 2.9 kb *Pst*I fragment containing *S. meliloti pmtA*	O. Geiger, unpublished
pRJ5565 [a]	Ap^r (pBSL86) 1.12 kb *Eco*RI fragment from pRJ8162 containing *B. japonicum pmtA*	This study
pRJ5569	Tc^r Km^r (pSUP202pol4) *pmtA::kan*; *pmtA* and *kan* oriented in the same direction	This study
pRJ5570	Tc^r Km^r (pSUP202pol4) *pmtA::kan*; *pmtA* and *kan* oriented in opposite directions	This study
pRJ5575	Tc^r (pRK290*X*) 1.12 kb *Eco*RI fragment from pRJ8162 containing *B. japonicum pmtA*	This study
pRM5576	Tc^r (pRK290*X*) 1.46 kb *Sal*I fragment from pTB2054 containing *S. meliloti pmtA*	This study
pRJ8162 [a]	Ap^r (pBluescript II KS+) 3.3 kb *Xho*I fragment containing *B. japonicum 'dnaJ* and *pmtA* in opposite orientation to α*lacZ*	This study

[a] The insert of this plasmid is depicted in Fig. 6.1.

6.3.2 DNA manipulations

Recombinant DNA techniques were performed according to standard protocols (Sambrook *et al.*, 1989). The isolation of chromosomal DNA from *B. japonicum* was done as described previously (Hahn & Hennecke, 1984). DNA was sequenced by the chain termination method (Sanger *et al.*, 1977) with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The DNA region sequenced and the deduced proteins were analysed using the software package of the UWGCG (Genetics Computer Group of the University of Wisconsin, Madison, USA; version 8.1) or the NCBI (National Center for Biotechnology Information) BLAST network server. The 'CODONPREFERENCE' program was applied using the *B. japonicum* codon usage table of group III genes (Ramseier & Göttfert, 1991).

6.3.3 Construction of strains and plasmids

For the construction of *pmtA* deletion mutants, the 1.12 kb *Eco*RI fragment (position 463 to 1579 in Fig. 6.1) of pRJ8162 was ligated into the 2.36 kb *Eco*RI vector fragment of pBSL86. In the resulting plasmid pRJ5565 the 81 bp *Sal*I (985 to 1066) internal fragment of *pmtA* was replaced by the 1.18 kb *Sal*I fragment of pBSL86 containing the neomycin phosphotransferase II cassette (Km^r). Both orientations of the resistance cassette relative to the *pmtA* gene were obtained. The 2.3 kb *Eco*RI inserts of the resulting plasmids were ligated into the 6.7 kb *Eco*RI vector fragment of pSUP202pol4, yielding plasmids pRJ5569 and pRJ5570. These plasmids were mobilised from *E. coli* S17-1 into *B. japonicum* 110*spc*4 for marker replacement mutagenesis as described previously (Hahn & Hennecke, 1984). The correct genomic structures of the resulting mutant strains 5569 and 5570 (see Fig. 6.1) were confirmed by Southern blot hybridisation of genomic DNA digested with appropriate restriction enzymes, using a DIG (digoxigenin-11-dUTP)-labelled *B. japonicum pmtA* probe according to the manufacturer's instructions (Boehringer Mannheim).

In order to complement the *pmtA*-defective *B. japonicum* strain 5569, the wild-type *pmtA* gene and the corresponding gene of *S. meliloti* were cloned into the broad host range vector pRK290*X*. The resulting plasmids pRJ5575 and pRM5576 and, for control purposes, the vector pRK290*X* alone were mobilised from *E. coli* S17-1 into *B. japonicum* 5569.

6.3.4 Transcript mapping

RNA isolation and primer extension analysis was performed as described (Babst *et al.*, 1996). The following oligonucleotides were used to determine the transcription start site upstream of *pmtA*:

DnaK35 (907) 5'-ATCTGAGAAAGCGCACCTCGTCATC-3'

DnaK36 (927) 5'-GAGCGGCTTTTCGATCCACGATCTG-3'

The numbers in parentheses indicate the position of the 5' end based on the numbering used in the physical map shown in Fig. 6.1.

6.3.5 Plant infection test

The symbiotic phenotypes of the *B. japonicum pmtA* mutants were determined in a soybean plant infection test as described previously (Hahn & Hennecke, 1984; Göttfert *et al.*, 1990). Pictures of nodule cross-sections were taken with a Color Chilled 3CCD Camera (Hamamatsu Photonics, Herrsching, Germany) connected to a Stemi 2000-C binocular (Carl Zeiss, Jena, Germany). The nodule ultrastructure was analysed by transmission electron microscopy (E. Wehrli, Institute for Cell Biology, Eidgenössische Technische Hochschule, Zürich) according to the protocol described elsewhere (Studer *et al.*, 1987).

6.3.6 Nucleotide sequence accession number

The nucleotide sequence of the *B. japonicum pmtA* gene region corresponding to the insert of plasmid pRJ8162 (*cf.* Fig. 6.1) has been deposited in the GenEMBL database under accession number AF193038.

6.4 Results

6.4.1 Cloning of the *B. japonicum pmtA* gene

Database searches with the deduced amino acid sequences of the recently found *S. meliloti* phospholipid *N*-methyltransferase gene *pmtA* revealed a significant similarity (38% identical amino acids) to the *B. japonicum orf>65* product encoded 87 bp downstream of *dnaJ* (O. Geiger, personal communication; *cf.* chapter 2). On the basis of this finding the complete DNA sequence of *orf>65* was expected to be located on plasmid pRJ8162 which had been constructed in the course of characterising the *dnaKJ* gene region (Fig. 6.1). DNA sequence analysis of a 1.1 kb *Eco*RI subfragment (insert of

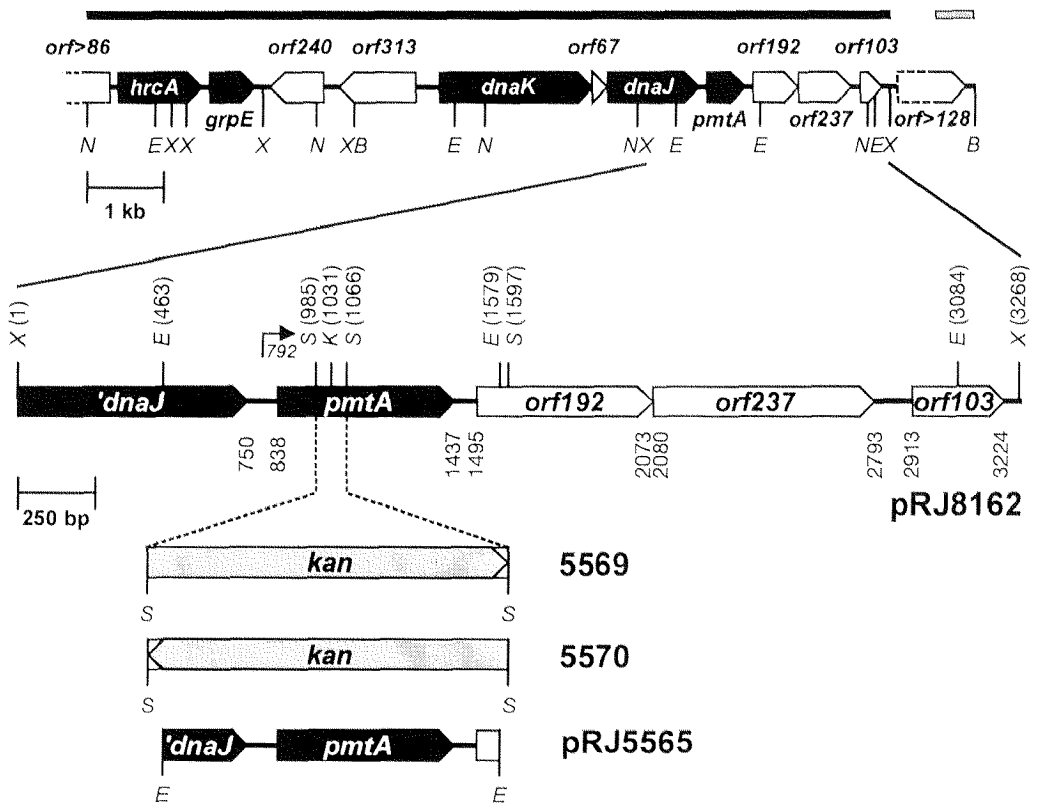


Fig. 6.1 Physical map of the *B. japonicum* gene region containing the *pmtA* gene and the heat shock genes *hrcA*, *grpE*, *dnaK*, and *dnaJ*. The black bar above the physical map indicates the DNA region that was sequences on both strands. The region represented by the grey bar was sequenced on a single strand only. The blow-up below the physical map shows the insert of plasmids pRJ8162 and pRJ5565. Numbers indicate start and stop codon positions of open reading frames, the transcription start site (horizontal arrow), and recognition sites of the following restriction enzymes: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; N, *Not*I; S, *Sal*I; X, *Xho*I. The strategy to construct the *B. japonicum pmtA* deletion strains 5569 and 5570 is indicated.

plasmid pRJ5565) revealed an open reading frame of 600 nucleotides (position no. 838 to 1437 in Fig. 6.1). The corresponding gene product consists of 199 amino acids (M_r 21,955) which indeed showed significant similarity to the *S. meliloti* PmtA protein (34.2% identical amino acids). According to this similarity and the below described enzymatic activity (see Discussion) the predicted *B. japonicum* open reading frame was designated *pmtA*.

To address the question of whether there is more than one copy of the *pmtA* gene in the *B. japonicum* chromosome, as it is the case for *groESL* (Fischer *et al.*, 1993) and *rpoH* (Narberhaus *et al.*, 1996), a suitable *pmtA* probe was hybridised under low stringency conditions (5 x SSC, 57°C) against *B. japonicum* chromosomal DNA digested with several different restriction enzymes. No evidence for a *pmtA* gene family was obtained.

6.4.2 Nucleotide sequence of the *pmtA* gene region

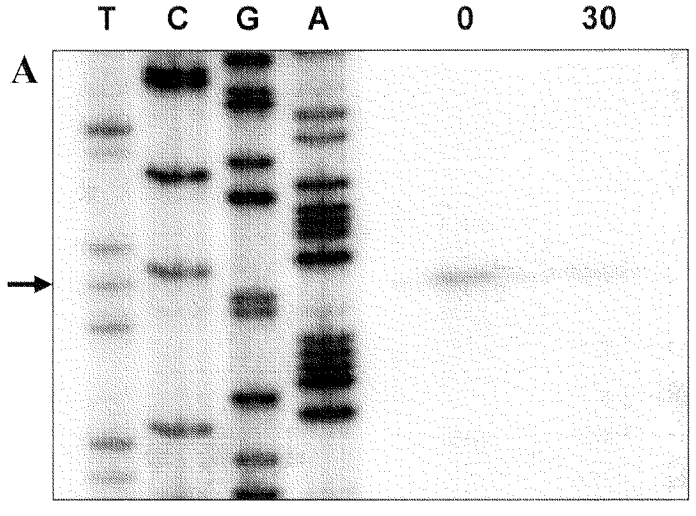
In order to get an overview of the genetic structure of the *B. japonicum pmtA* gene region, the DNA sequence of the 3.3 kb *Xho*I insert of plasmid pRJ8162 was established continuing the previously described sequence of the *dnaKJ* gene region (Fig. 6.1; *cf.* chapter 2). The investigation of this DNA region revealed three additional open reading frames, all oriented as *pmtA*. The first open reading frame (*orf192*) starts 58 bp downstream of *pmtA* and codes for a putative protein of 192 amino acids (M_r 20,669) which shows a significant similarity to a hypothetical 20.4 kDa protein of *E. coli* (33.9% identical amino acids; accession number P31465) encoded by the open reading frame *yieF*. Only 7 bp downstream of *orf192* starts a second open reading frame (*orf237*). Its gene product consists of 237 amino acids (M_r 24,673) and exhibits a striking sequence homology to orotidine-5'-monophosphate decarboxylases (e.g. 42.3% identical amino acids as compared to the *pyrF* gene product of *Pseudomonas aeruginosa* (Strych *et al.*, 1994)). The gene product of the third *B. japonicum* open reading frame (*orf103*; M_r 11,350) showed no significant similarity to any known protein sequence in the database. Interestingly, a similar chromosomal organisation of *pmtA* with regard to an adjacent *orf* of unknown function and a *pyrF*-homologous gene was also found in *S. meliloti* (O. Geiger, personal communication). However, the

deduced gene products of *B. japonicum orf192* and the corresponding *orf* of *S. meliloti* display no significant similarity.

Sequence information resulting from a single sequencing reaction in the downstream region of *orf103* revealed the 3'-end of an additional open reading frame (*orf>128*; see physical map in Fig. 6.1). The deduced fragment of a polypeptide of 128 amino acids shows high sequence homology to the carboxy-terminal end of dihydrodipicolinate reductases (e.g. 45.3% identical amino acids compared to the *dapB* gene product of *E. coli* (Bouvier *et al.*, 1984)).

6.4.3 Transcriptional analysis of the *pmtA* gene

Primer extension analysis with oligonucleotides complementary to the 5' end of *pmtA* revealed a single transcription start site 46 nucleotides upstream of the proposed



B

Promoter	−35 Region		−10 Region	+1
B. japonicum pmtA	ACTCGTC **TTGAC**C	GTATCGCCTTCGGCC	**TATA**CG	TCTTTATG**A**
B. japonicum rrn	ATTGGCG **TTGACA**	GCCCGGAAGGTGGGGCC	**TATAA**C	CCCAACC**A**
E. coli σ^{70} consensus	**TTGACA**	17 bp	**TATAAT**	

Fig. 6.2 (**A**) Determination of the transcription start sites of *pmtA* by primer extension mapping. Total RNA was isolated from *B. japonicum* 110*spc*4 cells harvested before (0) and 30 min after a heat shock from 30°C to 43°C (30). The extension and sequencing reactions (TCGA) were performed with the primer DnaK36. The transcription start site is marked with an arrow. (**B**) Comparison of the deduced *B. japonicum pmtA* promoter sequence with the promoter of *B. japonicum rrn* (Kündig *et al.*, 1995). Nucleotides matching the *E. coli* σ^{70} consensus promoter (Lisser & Margalit, 1993) and transcriptional start sites are emphasised in bold letters.

translational start site of *pmtA* (Fig. 6.2A). Since the amount of *pmtA* transcript was reduced under heat shock conditions (3.7-fold decrease of reverse products; Fig. 6.2A) and the putative *pmtA* promoter region shows significant homology to the constitutive *B. japonicum rrn* –10/–35-type promoter (Fig. 6.2B and Beck *et al.*, 1997) we conclude that *pmtA* belongs to the class of housekeeping genes.

6.4.4 Effect of a *pmtA* deletion on the cellular growth rate

As it is known that PC is a major phospholipid within species of *Bradyrhizobium* (Miller *et al.*, 1990) we tested whether a deletion of the *pmtA* gene, which is expected to cause a disruption of PC biosynthesis via the PE *N*-methylation pathway, would affect the growth rate of *B. japonicum*. Moreover, the influence of choline addition on the cellular growth rate was investigated with regard to the potential existence of a second, choline-dependent pathway of PC biosynthesis.

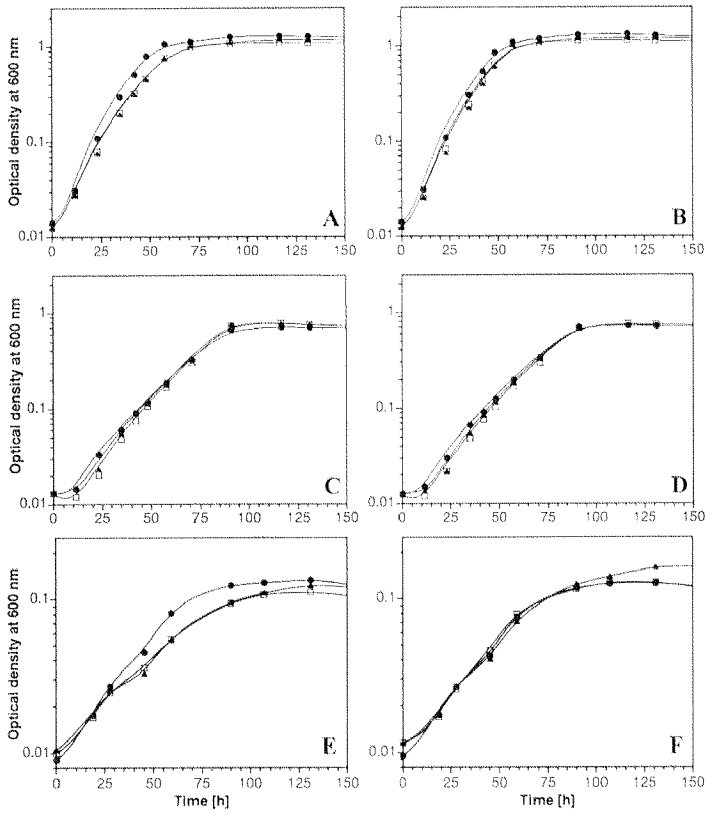


Fig. 6.3 Aerobic and microaerobic growth of *B. japonicum* wild type and *pmtA* mutants. Cultures of *B. japonicum* wild type (●) and *pmtA* mutants 5569 (□) and 5570 (▲) were grown in PSY (**A,B,E,F**) or HM minimal medium (**C,D**) under aerobic (**A,B,C,D**) or microaerobic (0.1% O_2, 99.9% N_2; **E,F**) conditions at 30°C in the absence (**A,C,E**) or presence (**B,D,F**) of 1 mM choline. The growth curves are based on mean values from two parallel cultures. Deviations between parallel cultures were less than 10%.

Under aerobic growth conditions in rich medium (PSY medium) the generation time of the *pmtA* mutant strains 5569 and 5570 (about 8.7 h) were slightly elevated as compared with the wild type (about 7.5 h) (Fig. 6.3A). This minor growth defect disappeared when 1 mM choline was added to the medium as indicated by similar

generation time of all three strains (about 7.5 h) (Fig. 6.3B). In contrast to these findings, the generation time of mutants and the wild type were indistinguishable (approximately 14 h) when cells were grown in minimal medium (HM medium) either in the presence or absence of choline (Fig. 6.3C and D). Possibly, growth in this medium is limited by processes other than PC biosynthesis.

In its symbiotic state *B. japonicum* is confronted with low-oxygen concentrations. To simulate such microaerobic growth conditions, closed bacterial cells cultures in PSY medium were aerated twice a day with a mixture of 0.1% oxygen and 99.9% nitrogen. As had been observed with aerobic cultures in PSY medium the absence of choline resulted in a slightly extended generation time of the mutant strains (about 24 h) as compared to the wild-type strain (about 19 h) (Fig. 6.3E). The addition of choline resulted in identical growth rates of all three strains (generation times of approximately 19 h) (Fig. 6.3F).

6.4.5 Symbiotic phenotype of *pmtA* deletion mutants

The ability of the *pmtA* mutant strains 5569 and 5570 to nodulate soybean roots and to fix nitrogen symbiotically was examined in a plant infection test (Table 6.2; since both mutants were phenotypically identical only the data for strain 5569 is shown).

Table 6.2 Symbiotic phenotype of *B. japonicum pmtA* mutants

Strain	Plasmid	Relevant genotype [c]	Characteristics (mean ± SE) [a]		
			Nodule number	Dry weight /nodule [mg]	Fix activity [b] [% of wild type]
110*spc*4		wild type	32 ± 11	0.9 ± 0.2	100 ± 32
5569		*pmtA*⁻	33 ± 10	0.5 ± 0.1	18 ± 5
5569	pRJ5575	*pmtA*⁻, *Bj pmtA*	37 ± 13	0.7 ± 0.3	92 ± 30
5569	pRM5576	*pmtA*⁻, *Sm pmtA*	38 ± 8	0.6 ± 0.1	64 ± 17
5569	pRK290*X*	*pmtA*⁻	39 ± 11	0.4 ± 0.1	22 ± 8

[a] Values are the means ± standard errors of at least 13 individual plants.
[b] Fixation (Fix) activity was measured as the amount of C_2H_2 reduced per minute per milligram of nodule weight (dry weight).
[c] Abbreviations: *Bj*, *B. japonicum*; *Sm*, *S. meliloti*.

Soybean plants infected with the *pmtA* mutant strain 5569 produced about the same number of nodules as compared with the wild-type situation. However, the nodules were significantly smaller. Moreover, cross-sections of these mutant-elicited nodules showed a beige colour (Fig. 6.4B), in contrast to the reddish colour of nodule cross-sections from plants infected with *B. japonicum* wild-type cells (Fig. 6.4A). Thus, nodules hosting the *pmtA* mutant seem to contain lower amounts of leghemoglobin. The analysis of the ultrastructure of these nodules revealed a strongly reduced number of normally shaped bacteroids within the plant cells accompanied by an increased amount of starch granules (Fig. 6.5). Accordingly, the specific symbiotic nitrogen fixation activity of the *pmtA* mutant strain 5569 was reduced to only 18% of that of the wild type (Table 6.2).

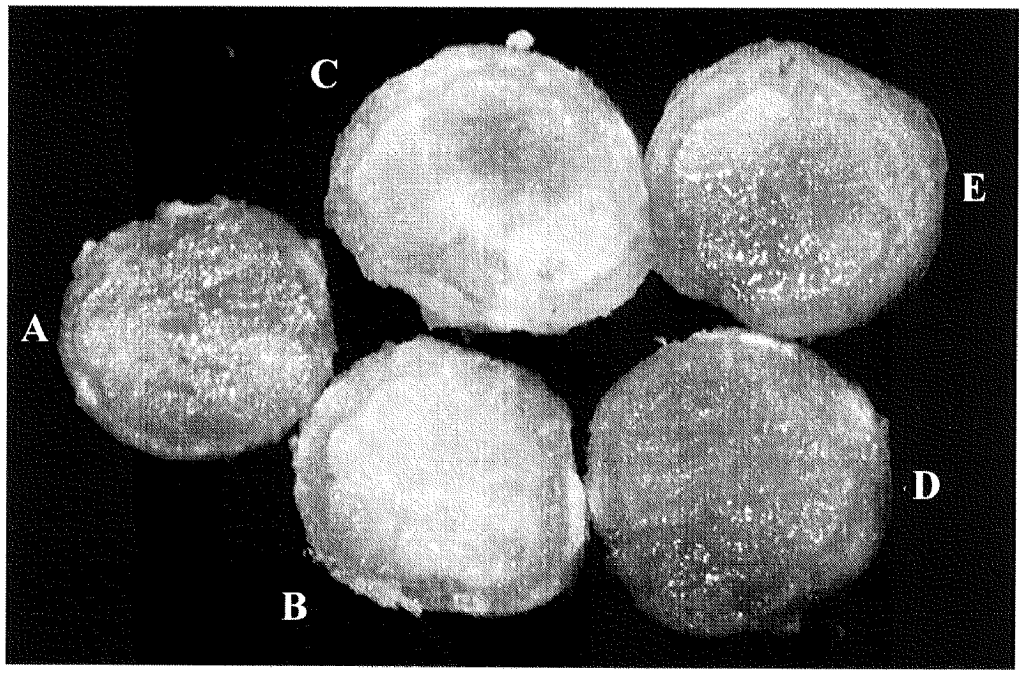


Fig. 6.4 Cross-sections of soybean root nodules infected by the *B. japonicum* wild type (**A**), the *pmtA* mutant 5569 (**B**), and strain 5569 containing either vector DNA (pRK290*X*; **C**) or the plasmid-encoded *pmtA* gene of *B. japonicum* (pRJ5575; **D**) or *S. meliloti* (pRM5576; **E**).

Further plant infection tests were performed to investigate whether *B. japonicum pmtA* (plasmid pRJ5575) or *S. meliloti pmtA* (pRM5576) would be able to complement the *pmtA* deficiency of strain 5569. Soybean plants infected with either one of these complemented *B. japonicum* strains produced larger nodules than plants infected with strain 5569 (Table 6.2). Moreover, the reddish colour of nodule cross-sections indicated

the presence of normal levels of leghemoglobin (Fig. 6.4D and E). The ability of *B. japonicum pmtA* to completely correct the *pmtA* mutation of strain 5569 was further demonstrated by the symbiotic nitrogen fixation activity of 92% of the complemented mutant. By contrast, the nitrogen fixation activity of strain 5569 was only partially restored by *S. meliloti pmtA* (64%, Table 6.2). The control strain harbouring the vector pRK290*X* showed similar properties as the non-complemented mutant 5569 (Table 6.2; Fig. 6.4C).

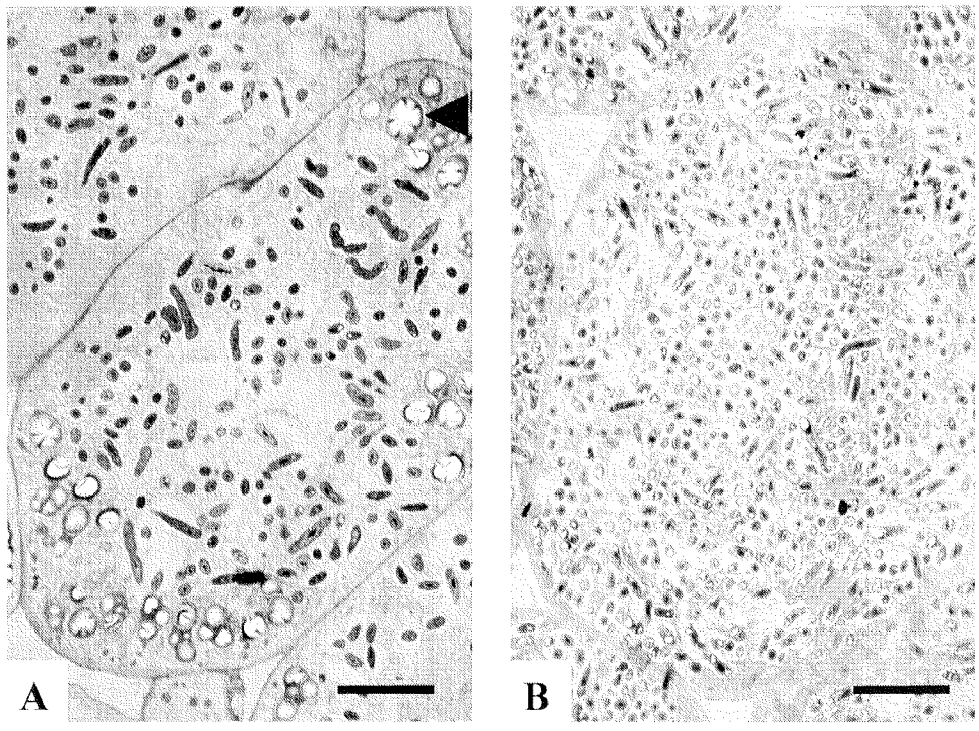


Fig. 6.5 Electron micrographs showing the structure of soybean nodule cells infected by the *B. japonicum pmtA* mutant 5569 (**A**) and the wild-type strain (**B**). The black arrowhead in panel A marks starch granules. Black bars correspond to 5 µm.

Soybean plants suffering from nitrogen starvation are recognisable by pale green leaves. This property was used to further characterise the *pmtA* mutant 5569 and its complemented derivatives (Fig. 6.6). Leaves of plants infected with strain 5569 or strain 5569 containing pRK290*X* were pale green (Fig. 6.6B and C), which corresponds well with the reduced nitrogen fixation activity of these strains. By contrast, leaves of plants inoculated with *B. japonicum* wild type or strain 5569 containing pRJ5575 were indistinguishably green (Fig. 6.6A and D) whereas infection with strain 5569 harbouring pRM5576 resulted in plants which had leaves of intermediate green colour (Fig. 6.6E).

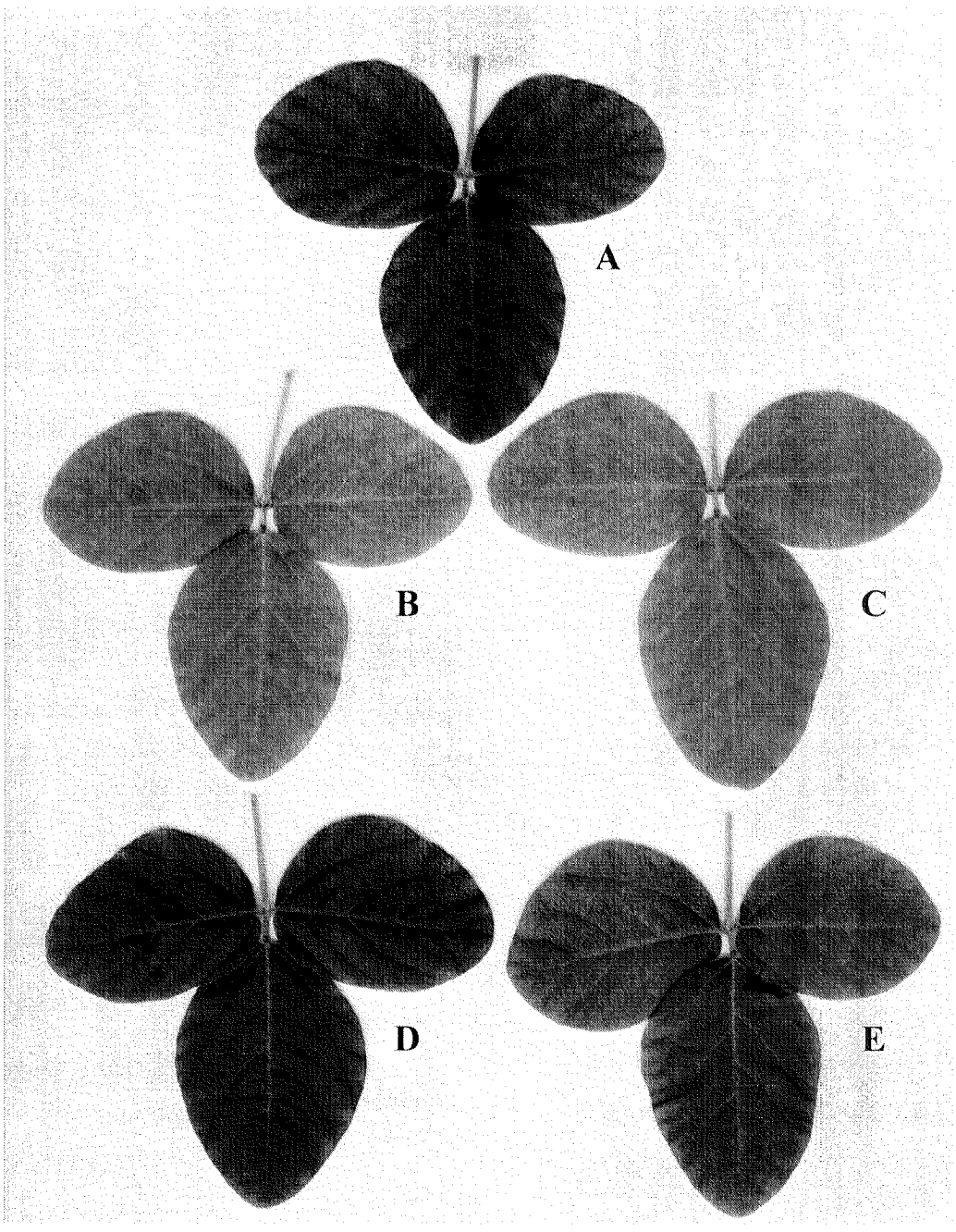


Fig. 6.6 Leaves of 33 days old soybean plants infected by the *B. japonicum* wild type (**A**), the *pmtA* mutant 5569 (**B**), and strain 5569 containing either vector DNA (pRK290*X*; **C**) or the plasmid-encoded *pmtA* gene of *B. japonicum* (pRJ5575; **D**) or *S. meliloti* (pRM5576; **E**).

6.5 Discussion

This work represents our contribution to a collaboration with the laboratory of O. Geiger (Institute of Biotechnology, Technical University of Berlin, Germany) on the *B. japonicum* phospholipid *N*-methyltransferase encoded by *pmtA*. Within this collaboration we focussed mainly on the symbiotic phenotype of *pmtA* mutants. The collaborating laboratory is particularly interested in the significance of the *pmtA* gene product in the phospholipid biosynthesis and especially in the transformation of PE into PC by transmethylation. Two results obtained in these parallel studies are relevant in the context of this discussion. First, the analysis of the phospholipid composition in *B. japonicum pmtA* mutants revealed a strong, but not complete depletion of PC combined with a comparable increase of PE. Second, studies with a *S. meliloti pmtA* mutant and with *E. coli*, which usually does not produce PC, demonstrated that the introduction of *B. japonicum pmtA* elicited the production of large amounts of MMPE, whereas DMPE and PC were not accumulated. Accordingly, the *B. japonicum pmtA* gene product seems to be involved in the PC biosynthesis by catalysing the first step of the PE *N*-methylation pathway only. This suggests the existence of further phospholipid methyltransferases, which are able to perform the second and third methylation step (see Fig. 6.7). However, a preliminary search revealed no *pmtA*-homologous gene in *B. japonicum* neither by analysing the vicinity of the *pmtA* gene nor by low-stringency Southern blot hybridisations of chromosomal DNA using a *pmtA* probe.

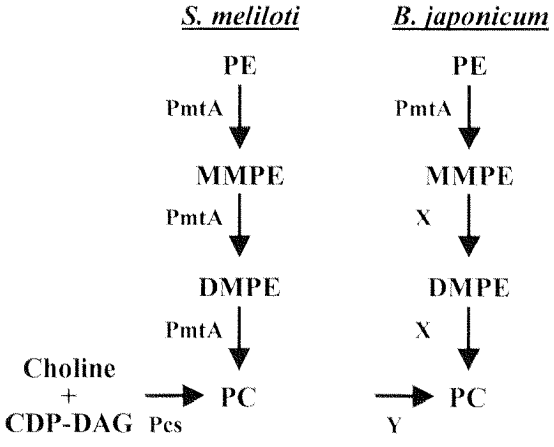


Fig. 6.7 Comparison between the current model of phosphatidylcholine biosynthesis in *B. japonicum* and the identified pathways of phosphatidylcholine formation in *S. meliloti*. Abbreviations: PE, phosphatidylethanolamine; MMPE, monomethylphosphatidylethanolamine; DMPE, dimethylphosphatidylethanolamine; PC, phosphatidylcholine; CDP-DAG, CDP-diacylglycerol; PmtA, phospholipid *N*-methyltransferase; Pcs, phosphatidylcholine synthase. On the basis of our results and in analogy to the identified PC biosynthesis pathways in *S. meliloti*, PC might be synthesised in *B. japonicum* either via the PE *N*-methylation pathway including at least two distinct phospholipid *N*-methyltransferases (PmtA and X) or via an alternative choline-dependent pathway including the hypothetical enzyme Y.

The existence of at least two distinct enzymatic activities in the *B. japonicum* PE *N*-methylation pathway supports a new concept of PC biosynthesis in bacteria. Previous studies on this subject in *Z. mobilis* and *R. sphaeroides* revealed the existence of a single enzyme which is able to perform all three methylation steps from PE to PC (Arondel *et al.*, 1993; Tahara *et al.*, 1994). A similar enzymatic activity was also demonstrated for the *S. meliloti pmtA* gene product, which shows a significant homology to the *B. japonicum* PmtA protein (see Fig. 6.7; O. Geiger, personal communication). In addition to this type of methyltransferases, a second methyltransferase activity was described in *A. tumefaciens*, which catalyses primarily the first methylation step from PE to MMPE (Kaneshiro & Law, 1964). Interestingly, the involvement of two distinct methyltransferases in the PE *N*-methylation pathway as we propose it for *B. japonicum* is well established in eukaryotic systems, e.g. *N. crassa*, *S. cerevisiae* and *S. pombe* (Scarborough & Nyc, 1967; Kodaki & Yamashita, 1987; Kanipes *et al.*, 1998).

The analysis of the lipid composition demonstrated that the *B. japonicum pmtA* mutants still contained a significant amount of PC. The formation of this phospholipid might either be catalysed by a second phospholipid methyltransferase which is able to perform to a certain extent the methylation of PE to MMPE besides its function of catalysing the subsequent methylation steps or PC is produced along a second, hypothetical biosynthetic pathway (see Fig. 6.7). In order to provide evidence for an alternative PC biosynthetic pathway dependent on choline, we compared the growth rates of the *B. japonicum* wild type and *pmtA* mutants in the absence or presence of choline. The results showed that the slightly reduced generation time of the mutant strains propagated in rich medium without choline were reverted to wild-type values by the addition of choline. Whether choline is directly incorporated into PC remains to be elucidated. First evidence for a direct choline incorporation into bacterial PC was demonstrated in *A. tumefaciens* (Sherr & Law, 1965), but only the recently demonstrated phosphatidylcholine synthase activity (Pcs) of *S. meliloti* proved the existence of a bacterial PC biosynthesis pathway starting from choline and CDP-diacylglycerol (see Fig. 6.7; de Rudder *et al.*, 1999). Assuming that a similar choline-dependent pathway exists in *B. japonicum*, its contribution to the PC biosynthesis would be small as choline is taken up poorly by *B. japonicum* cells compared to the

high constitutive choline uptake activity of *A. tumefaciens* and *S. meliloti* (Boncompagni *et al.*, 1999).

The presence of PC within bacterial membranes is rather unusual and limited to few species including the family of *Rhizobiaceae* (Goldfine, 1982). The appearance of PC in rhizobia as a major membrane component synthesised by two separate pathways prompted several authors to suggest that it might play an important role during plant infection (Goldfine, 1982; Miller *et al.*, 1990; Geiger, 1998). Accordingly, PC should be detectable in bacteroids, the symbiotic state of rhizobia within root nodules. While PC was indeed found to represent the major phospholipid in bacteroids of a *Bradyrhizobium* ssp. infecting *Lotus pedunculatus* (Gerson & Patel, 1975), a recent study with free-living *B. japonicum* showed that the amount of this phospholipid was greatly reduced in cells grown under low-oxygen conditions as compared with aerobically grown cells (Tang & Hollingsworth, 1998). As these microaerobic conditions are comparable to those in the symbiotic state, the small amount of PC seems to question its significance in symbiosis. The defective symbiotic phenotype of the *pmtA* mutants, however, proves the contrary. This defect is most likely related to the strongly reduced number of bacteroids in root nodule cells of plants infected with the *pmtA*-deficient *B. japonicum* strain. However, the exact reason for the weak performance of *pmtA* mutants in the endosymbiotic state remains to be determined. Slower growth of the mutants as compared to the wild type can probably be excluded as the sole reason because the differences between the growth rates were only marginal in free-living cells. Furthermore, no improvement of the symbiotic properties was observed in root nodules grown for an additional week, during which the retarded mutant strains had the chance to further multiply (data not shown). Unlike *B. japonicum pmtA*, the *pmtA* gene of *S. meliloti* corrected the symbiotic defect of mutant strain 5569 only partially. A possible explanation for this finding could be weak expression of the heterologous *S. meliloti pmtA* gene in the *B. japonicum* background. Also, it cannot be excluded that pRM5576 was lost more frequently than pRJ5575 under the non-selective conditions during the plant infection test. Unfortunately, the results of the bacteroid reisolation experiment could not be evaluated statistically due to the low number of colonies that grew on those plates.

On the basis of our results, we propose that the *pmtA* gene product is necessary for a functional symbiosis between *B. japonicum* and soybean, presumably by providing the major membrane component PC or other biomolecules derived from PC. A candidate could be phosphocholine-substituted β-1,3;1,6 cyclic glucan (PCCG) (Rolin *et al.*, 1992), an unusual cyclic oligosaccharide, which seems to be involved in the adaptation of *B. japonicum* cells to osmotic stress conditions (Pfeffer *et al.*, 1994). As *B. japonicum* is exposed to a change of osmotic conditions on its way from the free-living state in the soil to the cytosol of the host plant cell, a potentially reduced amount of PCCG might interfere with the adaptation to the new environment.

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APPENDIX

A.1 In the search of new *Bradyrhizobium japonicum* sigma factors

A.2 In the search of *Bradyrhizobium japonicum hrcA*

The following sections present DNA sequence data which were established in the course of our attempts to identify alternative *Bradyrhizobium japonicum* σ factors as well as the *B. japonicum hrcA* gene (*cf.* chapter 3).

A.1 In the search of new *Bradyrhizobium japonicum* sigma factors

Bacterial gene expression is mainly regulated by the different availability of sigma factors in response to altered environmental or developmental conditions. As the subunit of RNA polymerase which recognises a specific promoter sequences, each sigma factor is responsible for the transcription of a specific set of genes and therefore provides the bacteria with the ability to adapt rapidly to the new growth condition (recently reviewed in Wösten, 1998).

Based on amino acid sequence similarity, sigma factors are grouped into two distinct families. The majority of the sigma factors belong to the σ^{70} family, which is named after the 70-kDa primary sigma factor from *Escherichia coli*. Some members of this family are required to maintain basal gene expression ('housekeeping' sigma factors, RpoD) whereas others guarantee the survival of cells under stress conditions, e.g. during stationary growth phase (RpoS) or under heat shock conditions (RpoH). The σ^{54} family consists of sigma factors (RpoN) which are not only structurally but also functionally distinct from the σ^{70} family, as they require the presence of an activator protein and ATP hydrolysis to initiate transcription. Several of these activators have been characterised in different bacteria, e.g. the NtrC protein of *E. coli* and *Salmonella typhimurium* and the NifA protein of *Klebsiella pneumoniae*. The name of the σ^{54} family originates from the 54-kDa sigma factor RpoN (NtrA, GlnF) of *E. coli* which was found to be responsible for gene transcription under nitrogen-limiting conditions (reviewed in Merrick, 1993; Morett & Segovia, 1993; Shingler, 1996).

Symbiotically living bacteria such as *B. japonicum* are confronted with a wide range of different growth conditions. Accordingly, rather complex regulatory networks have evolved within these bacteria. In order to elucidate these networks we were interested in identifying the genes encoding so far unknown sigma factors. The successful purification of *B. japonicum* RNA polymerase core enzyme (Narberhaus *et al.*, 1998)

offered the attractive possibility to investigate the promoter recognition of new sigma factors by *in vitro* transcription assays.

By the time this project was started, two *rpoN* genes encoding members of the σ^{54}-family (Kullik *et al.*, 1991), *sigA* coding for the 'housekeeping' σ^{80} factor (Beck *et al.*, 1997), and three *rpoH* genes of heat-shock sigma factors (Narberhaus *et al.*, 1996; Narberhaus *et al.*, 1997) had been identified. However, hybridisation experiments indicated the existence of additional sigma-factor genes. In these experiments *B. japonicum* chromosomal DNA was hybridised with radioactively labelled oligonucleotides designed according to the amino acid sequence in the highly conserved region 2 of the σ^{70} family (Wösten, 1998). In addition to already known fragments, several unassigned signals were obtained under low stringency hybridisation conditions. These bands were interpreted as DNA fragments containing previously unknown sigma-factor genes (F. Narberhaus, personal communication). Furthermore, a putative *rpoE*-dependent promoter, which was only induced at extreme temperatures (48°C), was found upstream of *B. japonicum* $rpoH_2$ (Narberhaus *et al.*, 1997). This suggested that *B. japonicum* might possess an RpoE-like sigma factor whose homologue in *E. coli* and other bacteria belongs to the ECF class of sigma factors (extracytoplasmic function; Lonetto *et al.*, 1994).

The search for new *B. japonicum* sigma factors was performed by using three different approaches in parallel.

A.1.1 Hybridisation approach

This approach was aimed at the identification of new *B. japonicum* sigma-factor genes which exhibit sequence homology to already sequenced *B. japonicum* and *E. coli rpo* genes. Southern blot hybridisations of *B. japonicum* chromosomal DNA were performed using different DIG (digoxigenin-11-dUTP)-labelled probes under low stringency conditions (5 x SSC, 56°C).

A.1.1.1 Construction of DNA probes

The ***E. coli rpoS* probe** (1012 bp) was PCR-amplified from isolated *E. coli* genomic DNA or from plasmid pRJ40.1 (kindly provided by R. Hengge-Aronis; Lange & Hengge-Aronis, 1994a) using primers SigS1 (5'-GGAATTCCATATGAGTCAGAATA CGCTG-3', the *E. coli rpoS* sequence is underlined) and SigS2 (5'-GGAATTCTCGAG CTCGCGGAACAGCGCTTCG-3').

The ***E. coli rpoE* probe** (595 bp) was obtained by amplifying *E. coli* genomic DNA using primers SigE1 (5'-GGAATTCCATATGAGCGAGCAGTTAACGG-3', the *E. coli rpoE* sequence is underlined) and SigE2 (5'-GGAATTCTCGAGACGCCTGATA AGCGGTTGAAC-3'). All four primers contain an *Eco*RI site at their 5'-ends to facilitate the cloning of amplified DNA fragments.

To generate the ***B. japonicum sigA* (*rpoD*) probe**, a portion of the *sigA* gene (1634 bp) was amplified from plasmid pRJ9507 (Beck, 1998) using primers J10 (5'-CGTGCG CATGTATCTGC-3') and E1 (5'-CCAGCGTGTGGTCGGTG-3').

The ***B. japonicum rpoH*$_3$ probe** representing the entire *rpoH*$_3$ gene was kindly provided by F. Narberhaus.

A '**general *B. japonicum rpo* probe**' was designed on the basis of homologous regions from *B. japonicum* SigA and RpoH factors (Fig. A.1). For PCR amplifications with degenerated oligonucleotides Allg1 and Allg2 genomic DNA of *B. japonicum* strain 09-32 (*rpoH*$_{1+3}^{-}$; Narberhaus *et al.*, 1997) was used in order to prevent amplification of *rpoH*$_1$ or *rpoH*$_3$.

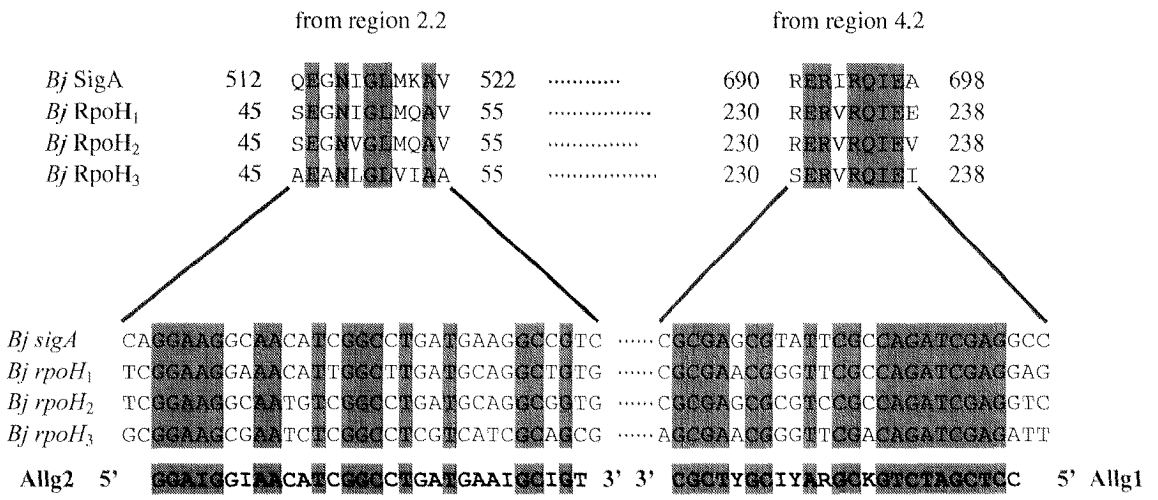
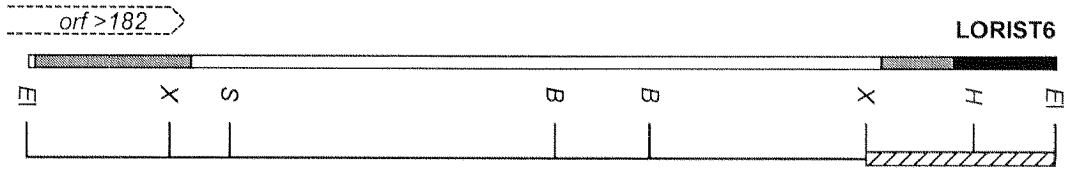


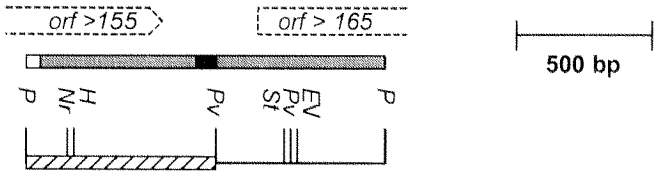
Fig. A.1 Design of degenerated oligonucleotides Allg1 and Allg2. The amino acid sequence alignments show conserved internal regions within known sigma factors of the *B. japonicum* σ^{70}-family (SigA, accession number CAA67902 (Beck *et al.*, 1997); $RpoH_1$, AAC44755 (Narberhaus *et al.*, 1996); $RpoH_2$/$RpoH_3$, CAA70697/CAA70861 (Narberhaus *et al.*, 1997)). The degenerated oligonucleotides Allg1 and Allg2 were deduced from the nucleotide sequences of the corresponding gene regions. Code for mixed nucleotides: K = G or T; R = A or G; Y = C or T; I = inosine.

A.1.1.2 Identification of hybridising DNA fragments

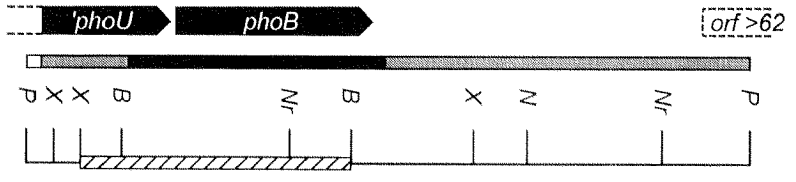
Chromosomal DNA isolated from *B. japonicum* wild-type strain 110*spc*4 was digested with the restriction enzymes *Bam*HI, *Eco*RI, *Pst*I and *Xho*I, electrophoretically separated and transferred to nylon membranes. The *sigA* probe hybridised only with DNA fragments containing the known *sigA* gene and the *rpoE* probe produced no hybridisation signal at all. The *rpoS*, *rpoH*$_3$, and the 'general *rpo*' probe yielded several signals which could not be attributed to DNA fragments of already identified sigma-factor genes. In order to clone these putative sigma-factor fragments, partial gene banks were constructed and pools containing the plasmid DNA isolated from 10 clones were hybridised with the *rpoS*/*rpoH*$_3$ (*Bam*HI gene bank) or the *rpoS* probe (*Pst*I gene bank). The analysis of 468 plasmids containing *Bam*HI inserts of either 3.8 to 5.2 kb (168 plasmids) or 7 to 9 kb (300) and 587 plasmids containing an *Pst*I insert of either 1.0 to 1.4 kb (344) or 2.3 to 4.2 kb (243) yielded two hybridising DNA fragments, the inserts of plasmids pRJ5506 and pRJ5507 (Fig. A.2).



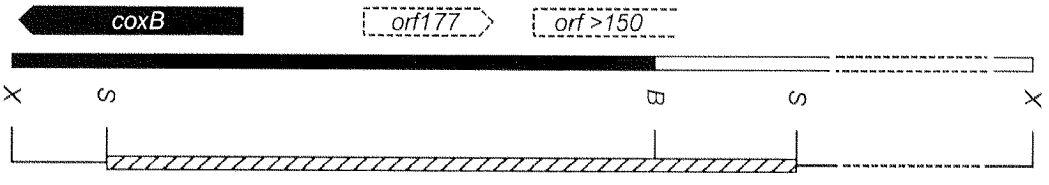
pRJ5503 ~3.8 kb *Eco*RI fragment of cosmid 18/B8 in pUC18
pRJ5504 inverted insert



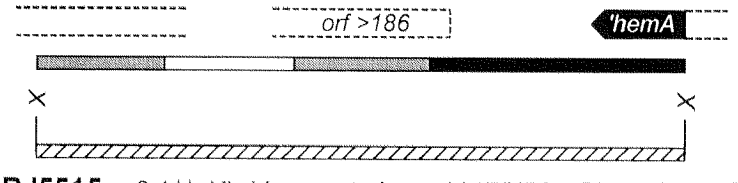
pRJ5506 ~1.3 kb *Pst*I fragment of *B. japonicum* total DNA in pUC18



pRJ5507 ~2.7 kb *Pst*I fragment of *B. japonicum* total DNA in pUC18



pRJ5513 ~5.4 kb *Xho*I fragment of cosmid 17/H7 in pBluescript II KS+
pRJ5514 inverted insert



pRJ5515 ~2.4 kb *Xho*I fragment of cosmid 17/H7 in pBluescript II KS+
pRJ5515r inverted insert

Fig. A.2 Physical maps of *B. japonicum* DNA fragments which were isolated due to their hybridisation with sigma-factor-encoding DNA probes. Regions whose DNA sequences were established on both DNA strands are indicated with black bars above the physical maps. Grey bars mark regions whose sequences were determined in single sequencing reactions on one DNA strand. DNA regions causing the observed hybridisation signals are represented by hatched bars. Abbreviations for restriction enzymes: *B*, *Bam*HI; *E*I, *Eco*RI; *E*V, *Eco*RV; *H*, *Hin*dIII; *N*, *Not*I; *Nr*, *Nru*I; *P*, *Pst*I; *Pv*, *Pvu*I; *S*, *Sal*I; *St*, *Stu*I; *X*, *Xho*I. (see also Table A.1)

In a second hybridisation approach, a *B. japonicum* **cosmid library** based on vector LORIST6 (Kündig, 1994) was hybridised with different sigma-factor probes. Ten cosmids were found to hybridise with the following probes: $rpoH_3$ (cosmid 8/H3, 9/H6, 12/E5); $rpoH_1$ (18/B8; F. Narberhaus, unpublished result); $rpoH_2$ (7/A11, 10/F4, 17/H7; F. Narberhaus, unpublished result) or *sigA* (13/D5, 14/B6, 15/A6; Kläusli, 1994). In this work only cosmids 17/H7 and 18/B8 were chosen for further analysis. Cosmid 17/H7 yielded several hybridising DNA fragments with the *rpoS* probe. A 5.4 and a 2.4 kb *Xho*I fragment were subcloned resulting in plasmids pRJ5513 (pRJ5514) and pRJ5515 (pRJ5515r), respectively (Fig. A.2). Plasmid pRJ5503 (pRJ5504) (Fig. A.2) was constructed by subcloning a 3.8 kb *Eco*RI fragment of cosmid 18/B8 which hybridised with the *rpoS*/$rpoH_3$ probe.

A.1.1.3 Sequence analysis

The DNA fragments hybridising with sigma probes were partially sequenced by the chain termination method (Sanger *et al.*, 1977) with a Model 373 DNA sequencer (Applied Biosystems, Forster City, CA, USA) (*cf.* Fig. A.2). The sequenced DNA regions and the deduced amino acid sequences were analysed using the software package of the UWGCG (Genetics Computer Group of the University of Wisconsin, Madison, USA; version 8.1) or the NCBI (National Center for Biotechnology Information) BLAST network server. The identification of putative open reading frames was performed with the 'CODONPREFERENCE' program using the *B. japonicum* group III codon usage table (based on 19 *B. japonicum* genes which belong neither to the group of nodulation genes nor to the group of NifA-regulated genes; Ramseier & Göttfert, 1991).

Table A.1 Identification of putative open reading frames (ORF) within the DNA fragments depicted in Fig. A.2

Plasmid	ORF	Analysis of deduced amino acid sequence
pRJ5503	*orf>182*	no significant similarity to any known protein sequence
pRJ5506	*orf>155*	minor sequence homology to the exopolysaccharide synthesis protein ExoF of *Sinorhizobium meliloti* (29% identity within 88 amino acids; accession number Q02728 (Müller *et al.*, 1993))

(continued)

(Table A.1 continued)

pRJ5506	*orf>165*	sequence homology to UDP-glucose 4-epimerases (e.g. 49% identity within 161 amino acids compared to the *galE* gene product of *Escherichia coli*; accession number P09147 (Lemaire & Müller-Hill, 1986))
pRJ5507	*'phoU*	sequence homology to the phosphate regulatory protein PhoU (e.g. 63% identity within 110 amino acids compared to the C-terminus of *S. meliloti* PhoU; accession number AAB42025). The complete *B. japonicum phoU* and *phoB* genes are probably present on an approximately 5.0 kb *Not*I fragment
pRJ5507	*phoB*	*B. japonicum* phosphate regulatory protein PhoB (*cf.* chapter 5)
pRJ5507	*orf>62*	sequence homology to the succinylornithine aminotransferase CstC and the acetylornithine aminotransferase ArgD of *E. coli* (50% and 45% identity within 51 amino acids, respectively; accession number of *cstC* P77581 (Fraley *et al.*, 1998); accession number of *argD* P18335 (Heimberg *et al.*, 1990))
pRJ5513	*coxB*	*B. japonicum* cytochrome *c* oxidase subunit II (R. Rossmann, unpublished)
pRJ5513	*orf177*	no significant similarity to any known protein sequence
pRJ5513	*orf>150*	minor sequence homology to the *E. coli* TldD protein which suppresses the inhibitory activity of the carbon storage regulator CsrA (27% identity within 150 amino acids; accession number P46473 (Murayama *et al.*, 1996))
pRJ5515	*orf>186*	no significant similarity to any known protein sequence
pRJ5515	*hemA*	*B. japonicum* 5-aminolevulinic acid synthase HemA (McClung *et al.*, 1987)

Results of the sequence analyses are summarised in Table A.1. Neither plasmid contained an *rpoH*-like gene. The positive hybridisation signal of plasmid pRJ5503 was most probably caused by traces of labelled vector DNA (pBR322) within the probes, which cross-hybridised with the fragment containing a part of vector LORIST6. The reason for the observed hybridisation signals of the other plasmids shown in Fig. A.2 was not further investigated.

A.1.2 PCR approach

The aim of this approach was the direct amplification of DNA fragments of new sigma-factor genes from *B. japonicum* total DNA by using different primer combinations. The PCR reactions were performed as follows: 1 min at 95°C / 1 min at 55°C / 2 min at 72°C / 30 cycles. Resulting amplification products were isolated from agarose gels and directly sequenced.

The DNA fragments E4 and S5 resulted from amplification of *B. japonicum* total DNA using primers SigE1/SigE2 and SigS1/SigS2, respectively (*cf.* chapter A.1.1.1). The SigS1/SigS2 amplification product was also used as a template for nested PCR reactions using primer SigS1 and the internal sigma-factor primer Sig4 (complementary to primer Allg2 described in chapter A.1.1.1; kindly provided by F. Narberhaus). A definite DNA sequence could only be established for two of the resulting DNA fragments (T3 and T4). To avoid the generation of DNA fragments containing an internal region of *B. japonicum* $rpoH_1$ and $rpoH_3$, PCR reactions with primers Allg1 and Allg2 (*cf.* chapter A.1.1.1) were performed with chromosomal DNA of the *B. japonicum* strain 09-32 ($rpoH_{1+3}^-$; Narberhaus *et al.*, 1997). Moreover, amplification products containing parts of $rpoH_2$ or *sigA* were eliminated by digestion with *Ear*I, which cuts in these genes. The remaining DNA fragments of appropriate length were isolated and used as template for another PCR reaction with primers Allg1 and Allg2. The resulting product was again digested with *Ear*I and two unaffected DNA fragments were isolated and designated A1 and A2.

The results of the sequence analysis of all amplification products mentioned above are summarised in Table A.2. Unexpectedly, DNA fragment A1 was identified as part of the *B. japonicum* $rpoH_1$ gene containing an intact internal *Hin*dIII restriction site although this fragment was amplified from chromosomal DNA of the *B. japonicum* $rpoH_{1+3}$ mutant 09-32 in which a kanamycin resistance cassette had been inserted into that *Hin*dIII site of $rpoH_1$. The reason for the appearance of this fragment was not further investigated. Apparently, all other PCR products do not contain a gene coding for a sigma factor.

Table A.2 Analysis of amino acid sequences deduced from sequencing amplified DNA fragments

Fragment	Nucl.[a]	Analysis of deduced amino acid sequence
A1	361	*B. japonicum* heat shock sigma factor $RpoH_1$ (Narberhaus *et al.*, 1996)
A2	443	*B. japonicum* *N*-acetylglucosaminyltransferase NodC (M. Göttfert, unpublished)
E4	317	no significant similarity to any known protein sequence
S5	150	no significant similarity to any known protein sequence
T3	230	sequence homology to the methionine synthase MetE (e.g. 44% identity within 74 amino acids compared to the *metE* gene product of *E. coli*; accession number P25665 (Gonzalez *et al.*, 1992))
T4	186	sequence homology to the cyclolysin secretion protein CyaB of *Bordetella pertussis* (42% identity within 59 amino acids; accession number P18770 (Glaser *et al.*, 1988))

[a] Number of nucleotides of the sequenced DNA region.

A.1.3 Complementation approach

Western blot analysis of *B. japonicum* crude extracts using an antibody against *E. coli* RpoS (kindly provided by R. Hengge-Aronis) revealed a cross-reacting signal whose intensity increased in protein extracts from cells reaching the stationary growth phase (F. Narberhaus, personal communication). However, the apparent molecular mass of the corresponding protein (25 to 28 kDa) was much smaller than expected for an RpoS-homologous protein (38 kDa). To provide evidence for the existence of a functional RpoS-like protein in *B. japonicum* the broad host range plasmids pGM112 and pGM115 (kindly provided by U. Jenal; Miksch & Dobrowolski, 1995) containing the RpoS-dependent *E. coli* reporter gene fusions $P1_{bolA}$::*lacZ* and P_{fic}::*lacZ*, respectively, should be transferred into the *B. japonicum* wild-type strain. Unfortunately, several attempts to perform these conjugations failed.

To isolate a putative *rpoS* gene of *B. japonicum*, gene banks containing DNA fragments of this organism were transformed into two different *rpoS*-deficient *E. coli*

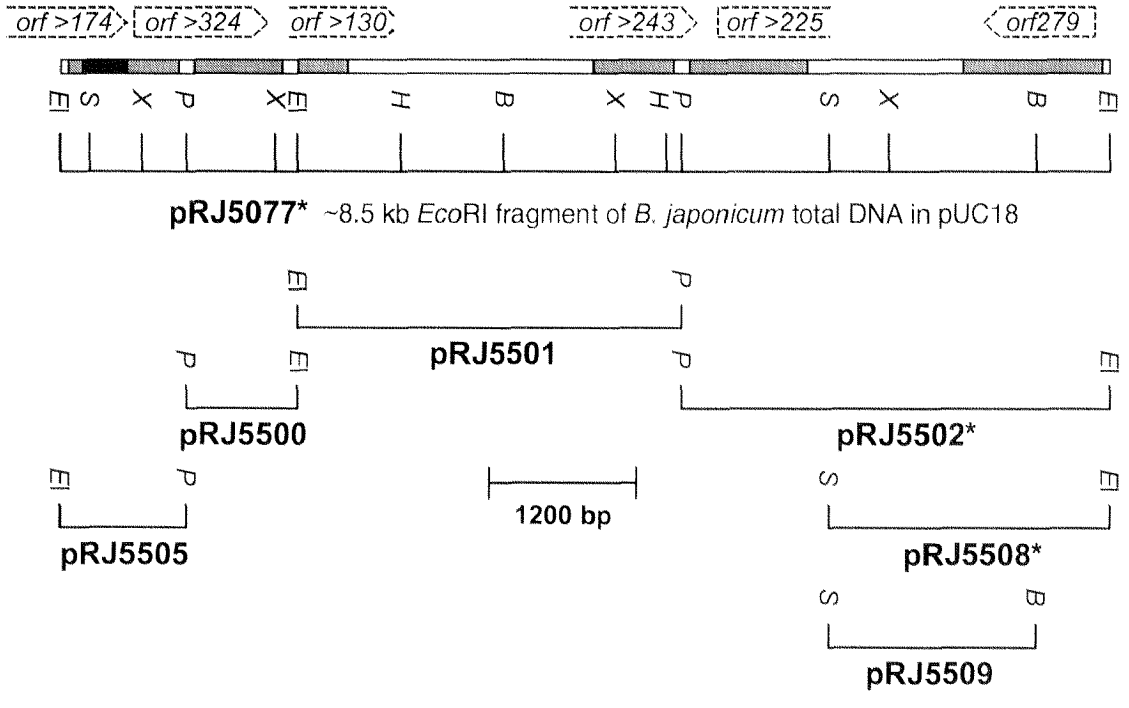


Fig. A.3 (following page) Physical map of the 8.5 kb *Eco*RI insert of plasmid pRJ5077. The region corresponding to the DNA sequence established on both DNA strands is indicated with a black bar above the physical map. Grey bars mark regions whose sequences were determined in single sequencing reactions on one DNA strand. The position of subfragments and the corresponding plasmid numbers are indicated below the physical map. Plasmids causing the blue colouring of the *E. coli* strain RH99 are marked with an asterisk. Abbreviations for restriction enzymes: *B*, *Bam*HI; *E*I, *Eco*RI; *H*, *Hin*dIII; *P*, *Pst*I; *S*, *Sal*I; *X*, *Xho*I. (see also Table A.3)

strains, RH99 and RO45 (kindly provided by R. Hengge-Aronis; Lange & Hengge-Aronis, 1994b). Both strains contain an RpoS-dependent *csi-5::lacZ* reporter gene fusion integrated into the chromosome. Assuming that an RpoS-like *B. japonicum* protein would be functional in *E. coli*, we isolated plasmid pRJ5077 from a blue RH99 colony. The formation of blue colonies by inserting plasmid pRJ5077 or pRL40.1 (*E. coli rpoS*) into RH99 was only detectable in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG). Unexpectedly, however, strain RH99 alone showed blue colonies even in the absence of IPTG. Neither the sequence analysis of the 8.5 kb *Eco*RI insert of plasmid pRJ5077 (Fig. A.3 and Table A.3) nor the Southern blot hybridisation of pRJ5077 fragments using the *E. coli rpoS* probe (*cf.* chapter A.1.1.1) revealed any evidence for an *rpoS*-like *B. japonicum* gene. The DNA fragment causing the blue colour of the *E. coli* strain RH99 was identified to be *orf279*. The reason for this activity remains mysterious.

Table A.3 Identification of putative open reading frames (ORF) within the insert of plasmid pRJ5077 depicted in Fig. A.3

ORF	Analysis of deduced amino acid sequence
orf>174	sequence homology to phosphoenolpyruvate phosphotransferases (e.g. 38% identity within 159 amino acids compared to the *ptsP* gene product of *E. coli*; accession number P37177 (Reizer *et al.*, 1996))
orf>324	sequence homology to the peptide chain release factor 1 (e.g. 46% identical amino acids compared to *E. coli* RF-1; accession number P07011 (Lee *et al.*, 1988))
orf>130	no significant similarity to any known protein sequence
orf>243	sequence homology to the hypothetical protein Rv1751 of *Mycobacterium tuberculosis*, a possible hydroxylase (47% identity within 233 amino acids; accession number CAB09317 (Cole *et al.*, 1998))
orf>225[a]	sequence homology to precursors of outer-membrane proteins (e.g. 30% identity within 216 amino acids compared to the *omp31* gene product of *Brucella melitensis*; accession number Q45322 (Vizcaino *et al.*, 1996))
orf279[a]	sequence homology to the precursor of the 31 kDa outer-membrane protein of *Brucella melitensis* (32% identical amino acids; accession number Q45322 (Vizcaino *et al.*, 1996))

[a] The deduced protein sequences of *orf>225* and *orf279* possess 36% identical amino acids and both N-terminal sequences contain a putative leader peptide.

A.2 In the search of *Bradyrhizobium japonicum hrcA*

Chapter 3 presents the characterisation of the *B. japonicum hrcA* gene encoding the heat shock regulatory protein HrcA which specifically represses the transcription of $groESL_4$ and $groESL_5$ by binding to the CIRCE-element located downstream of their promoter regions. During the search for the *hrcA* gene, several DNA fragments were isolated as putative *hrcA* candidates. The analysis of these fragments and the different approaches, which led to their finding, are described in the following sections.

A.2.1 Hybridisation approach

Although the amino acid sequences of different HrcA proteins deviate greatly (Roberts *et al.*, 1996; Schulz & Schumann, 1996), one of the approaches to identify the *B. japonicum hrcA* gene was based on Southern blot hybridisations of *B. japonicum* chromosomal DNA using different *hrcA* probes. Another hybridisation was performed with a DIG-labelled probe of *C. crescentus grpE* making use of the observation that *hrcA* and *grpE* are often genetically linked (*cf.* chapter 3).

A.2.1.1 Construction of DNA probes

Several attempts to amplify the *C. crescentus hrcA* gene from isolated genomic DNA or plasmid pRR316-3 (kindly provided by L. Shapiro; Roberts *et al.*, 1996) using primers HrcA1 (5'-TTCACGCACCATTTCCAAG-3') and HrcA2 (5'-TATCCATCCA TCATCCGCC-3') failed. (Later it was found that the addition of 10% DMSO to the PCR reactions led to a proper amplification of *hrcA*-containing DNA fragments.) Due to the lack of a DIG-labelled **C. *crescentus hrcA* probe**, a 1.55 kb *Xho*I and a 0.95 kb *Xho*I-*Sma*I fragment of pRR316-3 were radioactively labelled by nick translation. Degenerated oligonucleotide HrcA8 was radioactively labelled and used as a '**general *hrcA* probe**'. This oligonucleotide was designed on the basis of an alignment of DNA sequences encoding a highly conserved amino acid region in the amino-terminal region of all HrcA proteins known by that time (Fig. A.4). A DIG-labelled ***C. crescentus grpE*** **probe** (419 bp) was amplified from isolated *C. crescentus* genomic DNA using primers GrpE1 (5'-TGGAAGTGGCGCAACTGAAG-3') and GrpE3 (5'-CATGGCGGGACGA ACCAG-3').

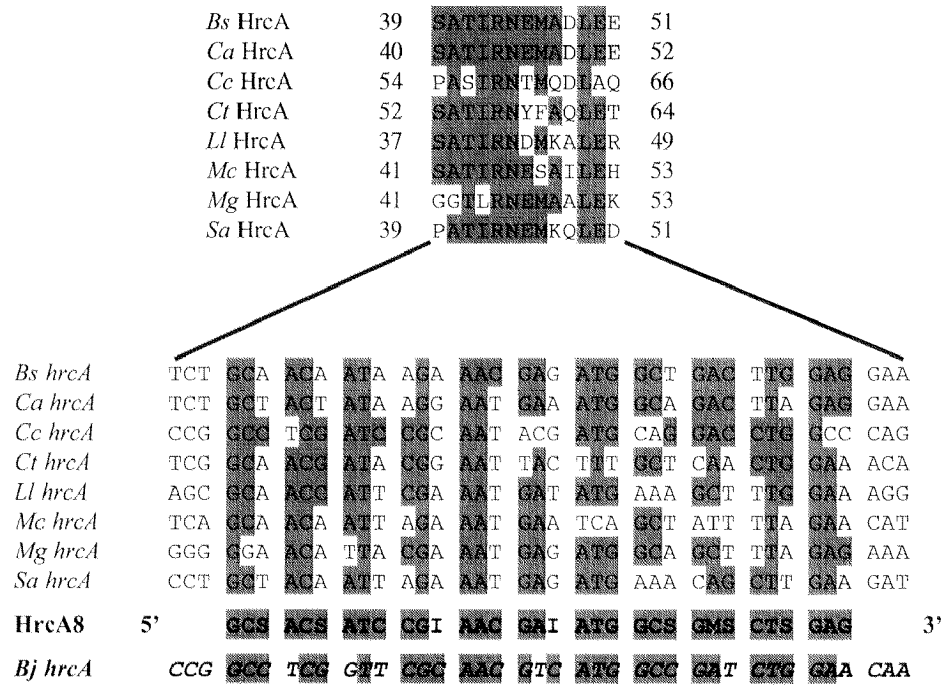


Fig. A.4 Design of degenerated oligonucleotide HrcA8. The amino acid sequence alignment shows a highly conserved internal fragment within HrcA proteins. Abbreviations and protein accession numbers: *Bs*, *Bacillus subtilis*, P25499; *Ca*, *Clostridium acetobutylicum*, P30727; *Cc*, *Caulobacter crescentus*, P54305; *Ct*, *Chlamydia trachomatis*, P36426; *Ll*, *Lactococcus lactis*, P42370; *Mc*, *Mycoplasma capricolum*, P71498; *Mg*, *Mycoplasma genitalium*, P47447; *Sa*, *Staphylococcus aureus*, P45556. Degenerated oligonucleotide HrcA8 was designed according to the nucleotide sequence alignment of the corresponding gene regions. Code for mixed nucleotides: M = A or C; S = C or G; I = inosine. The true nucleotide sequence of the corresponding region of the *B. japonicum hrcA* gene (*Bj hrcA*) is provided in italic letters below HrcA8.

A.2.1.2 Identification and analysis of hybridising DNA fragments

No distinct hybridisation signal was observed in Southern blot hybridisations of *B. japonicum* chromosomal DNA using either one of the *C. crescentus hrcA* probes, although the experiments were performed under low stringency conditions (5 x SSC, 56°C). In contrast, hybridisations of *B. japonicum* total DNA using the radioactively labelled oligonucleotide HrcA8 showed several clear bands even under higher stringency (2 x SSC, 57°C; data not shown). However, the comparison of these signals with the later established restriction map of the *B. japonicum hrcA* gene region demonstrated that the observed hybridisation signals were not caused by a *hrcA*-containing DNA fragment even though 27 of the 33 nucleotides of HrcA8 match the corresponding *hrcA* sequence (Fig. A.4).

The DIG-labelled ***C. crescentus grpE* probe** revealed a distinct hybridisation signal under low stringency conditions (5 x SSC, 56°C) caused by an approximately 1.6 kb *Pst*I fragment of *B. japonicum* total DNA. In order to clone this hybridising DNA fragment, a partial *Pst*I gene bank was constructed. The analysis of 437 plasmids from this gene bank using the *grpE* probe led to the identification of three different DNA fragments, the inserts of plasmids pRJ5516 (pRJ5517), pRJ5520 and pRJ5521 (Fig. A.5). The results of their sequence analyses are summarised in Table A.4 showing that neither of the fragments contained the *B. japonicum hrcA* gene.

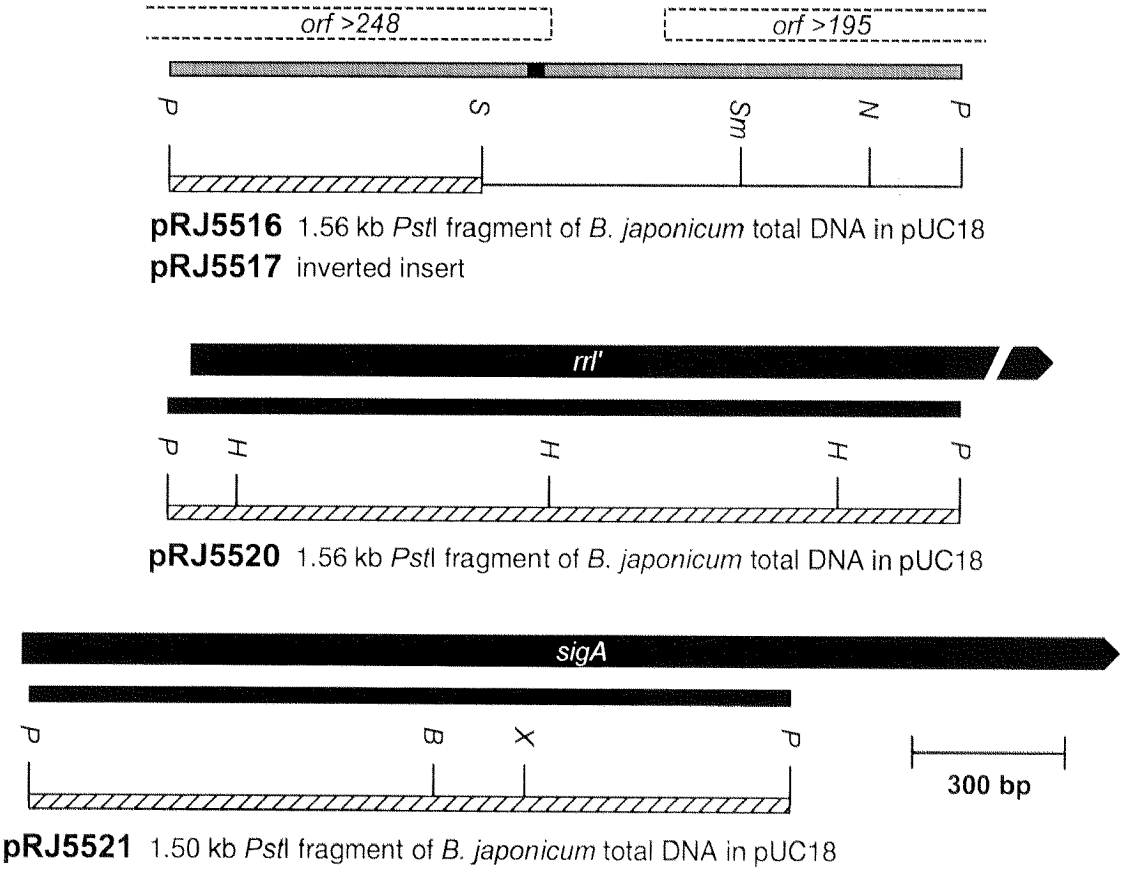


Fig. A.5 Physical maps of *B. japonicum* DNA fragments which were isolated due to their hybridisation with the *C. crescentus grpE* probe. Regions whose DNA sequences were established on both DNA strands are indicated with black bars above the physical maps. Grey bars mark regions whose sequences were determined in single sequencing reactions on one DNA strand. DNA fragments causing the observed hybridisation signals are represented by hatched bars. Abbreviations for restriction enzymes: *B*, *Bam*HI; *H*, *Hin*dIII; *N*, *Not*I; *P*, *Pst*I; *S*, *Sal*I; *Sm*, *Sma*I; *X*, *Xho*I. (see also Table A.4)

Table A.4 Identification of putative open reading frames (ORF) within the DNA fragments depicted in Fig. A.5

Plasmid	ORF	Analysis of deduced amino acid sequence
pRJ5516	*orf>248*	sequence homology to a putative integral membrane protein of *S. meliloti* (35% identity within 248 amino acids; accession number CAB38104)
pRJ5516	*orf>195*	sequence homology to a putative Na^+/H^+ antiporter of *Streptomyces coelicolor* (41% identity within 195 amino acids; accession number CAB45470 (Redenbach *et al.*, 1996))
pRJ5520	*rrl*	*B. japonicum* 23S ribosomal RNA (accession number Z35330 (Kündig *et al.*, 1995))
pRJ5521	*sigA*	*B. japonicum* 'housekeeping' sigma factor (accession number X99588 (Beck *et al.*, 1997))

A subsequent hybridisation of digested *B. japonicum* chromosomal DNA with the radioactively labelled insert of plasmid pRJ5520 showed a similar pattern of hybridising fragments as found with the *grpE* probe. This observation suggests that the *rrl* fragment represents the *Pst*I fragment of *B. japonicum* that interacted with the *C. crescentus grpE* probe.

A.2.2 PCR approach

The primer combinations HrcA1/HrcA2 and GrpE1/GrpE3, initially designed to amplify the *C. crescentus hrcA* and *grpE* probes, respectively (*cf.* chapter A.2.1.1), were also used for an amplification attempt with *B. japonicum* chromosomal DNA. The PCR reaction was performed as follows: 1 min at 95°C / 1 min at 55°C / 2 min at 72°C / 30 cycles. Resulting amplification products were electrophoretically separated, gel isolated and directly sequenced. The DNA sequence of two fragments (8E1 and 10E1), which were generated with the *grpE* primers, could be established. The results of their sequence analyses are shown in Table A.5. Again, the *B. japonicum hrcA* or *grpE* gene was not found.

Table A.5 Analysis of amino acid sequences deduced from amplified DNA fragments

Fragment	Nucl.[a]	Analysis of deduced amino acid sequence
8E1	201	sequence homology to cellulases (e.g. 30% identity within 80 amino acids compared to the precursor protein of the endo-1,4-β-glucanase S (CelS) of *Erwinia carotovora*; accession number P16630 (Saarilahti *et al.*, 1990))
10E1	524	N-terminus of the *B. japonicum* NifS protein (M. Göttfert, unpublished)

[a] Number of nucleotides of the sequenced DNA region.

A.2.3 The DNA region upstream of *hrcA*

To analyse the DNA region upstream of *B. japonicum hrcA*, several fragments of cosmid 15/H1, which contains the region upstream of the *dnaK* gene (Minder, 1995), were subcloned as depicted in Fig. A.6. In contrast to the 4.3 kb *Eco*RI fragment of plasmid pRJ5537, which was identified using a *B. japonicum hrcA* probe, the insert of plasmid pRJ5539 was fortuitously cloned. It is located in an undefined distance and orientation upstream of *hrcA*. The results of the sequence analysis of both DNA fragments are summarised in Table A.6.

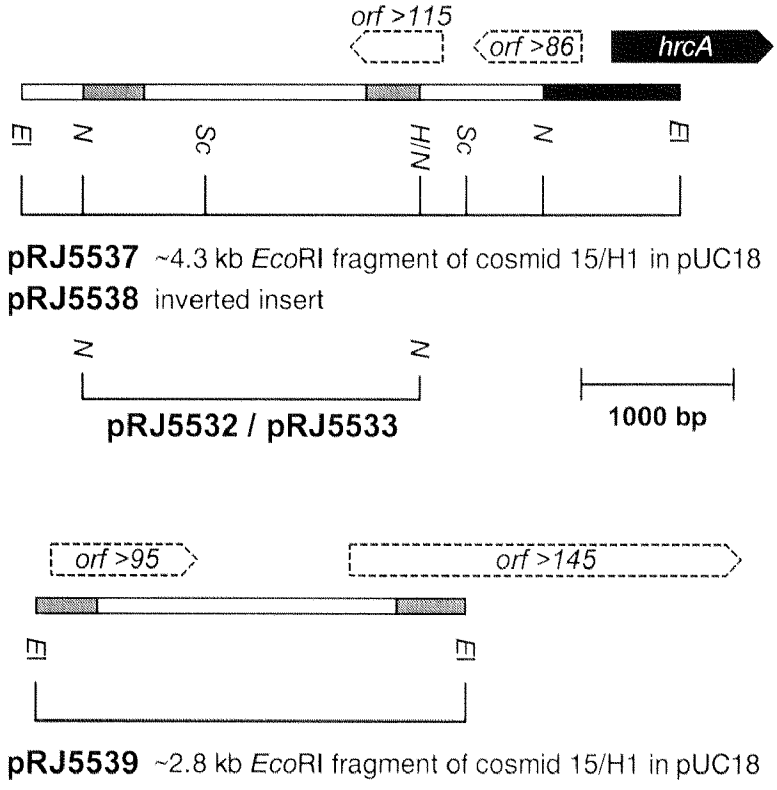


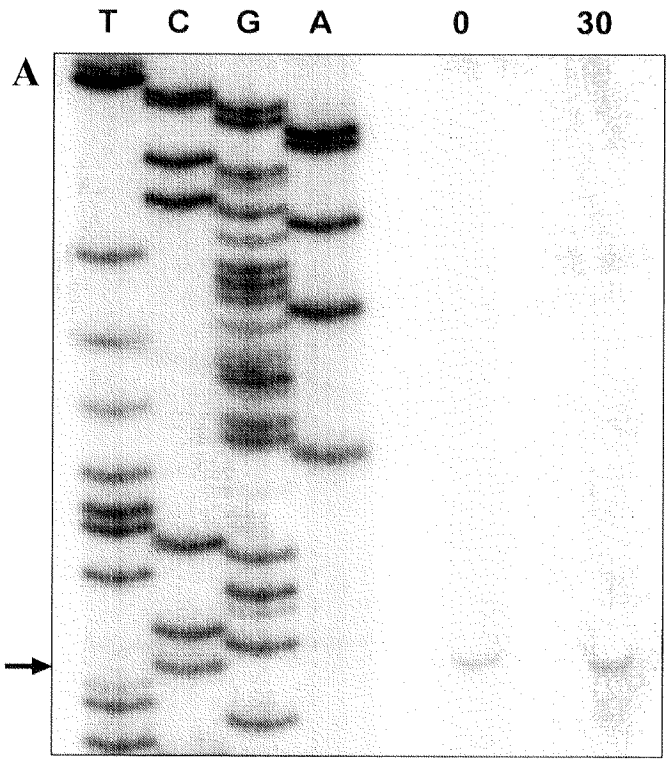
Fig. A.6 Physical maps of DNA fragments from the upstream region of *B. japonicum hrcA*. The region whose DNA sequence was established on both DNA strands is indicated with a black bar above the physical map. Grey bars mark regions whose sequences were determined in single sequencing reactions on one DNA strand. The location of the insert of plasmids pRJ5532 and pRJ5533 is indicated below the physical map of pRJ5537. Abbreviations for restriction enzymes: *E*I, *Eco*RI; *H*, *Hin*dIII; *N*, *Not*I; *Sc*, *Sac*I. (see also Table A.6)

Table A.6 Identification of putative open reading frames (ORF) within the DNA fragments depicted in Fig. A.6.

Plasmid	ORF	Analysis of deduced amino acid sequence
pRJ5537	*orf>115*	sequence homology to a hypothetical protein of *S. coelicolor* (49% identity within 115 amino acids; accession number CAB50983 (Redenbach *et al.*, 1996))
pRJ5537	*orf>86*	sequence homology to the putative tRNA nucleotidyltransferase RnpH of *Caulobacter crescentus* (67% identity within 86 amino acids; accession number P48196 (Roberts *et al.*, 1996); *cf.* chapter 3)
pRJ5537	*hrcA*	*B. japonicum* heat shock regulatory protein HrcA (*cf.* chapter 3)
pRJ5539	*orf>95*	sequence homology to glutathione synthetases (e.g. 38% identity within 95 amino acids compared to *E. coli* Gsh-II; accession number P04425 (Gushima *et al.*, 1984))
pRJ5539	*orf>145*	sequence homology to the hypothetical protein Y4XJ of the *S. meliloti* secretion system (30% identity within 145 amino acids; accession number P55702 (Freiberg *et al.*, 1997))

In order to test whether *orf>86* is a heat shock gene, the transcription start site was determined by primer extension analysis with oligonucleotide RnpH1 (5'-GGCAGGAGCCTTCCGCATATTTGACCAC-3'). The single start site, which was detected 36 nucleotides upstream of the proposed translational start site of *orf>86* (*cf.* chapter 3), corresponds to a promoter with significant homology to the 'housekeeping' promoter of the *B. japonicum rrn* operon and the consensus sequence of *E. coli* σ^{70} promoters (Fig. A.7). Moreover, transcription of *orf>86* appeared to be unaffected by heat shock treatment.

Acknowledgements: We are indebted to Regine Hengge-Aronis for providing the *E. coli* strains RH99 and RO45, plasmid pRL40.1 and the *E. coli* RpoS antiserum, to Urs Jenal for plasmids pGM112 and pGM115, to Lucy Shapiro for plasmid pRR316-3, and to Christoph Kündig for preparation of the cosmid library used in this study. We thank Sandra Röthlisberger for help in automatic DNA sequencing and Michael Spring for technical assistance. This work was supported by grants from the Swiss National Science Foundation and the Federal Institute of Technology, Zürich.



B

Promoter	−35 Region		−10 Region	+1
B. japonicum orf>86	AAAGGCC	**TTG**CC**G** CTCACCCTCACCCCACC	**TA**C**AA**G	CACCGC**G**
B. japonicum rrn	ATTGGCG	**TTGACA** GCCCGGAAGGTGGGGCC	**TATAA**C	CCCAACC**A**
E. coli σ^{70} consensus		**TTGACA** 17 bp	**TATAAT**	

Fig. A.7 (following page) (**A**) Determination of the transcription start site of *orf>86* by primer extension mapping. Total RNA was isolated from *B. japonicum* 110*spc*4 cells harvested before (0) and 30 min after a heat shock from 30°C to 43°C (30). The extension and sequencing reactions (TCGA) were performed with the primer RnpH1. The transcription start site is marked with an arrow. (**B**) Comparison of the deduced *B. japonicum orf>86* promoter sequence with the promoter of the *B. japonicum rrn* operon (Kündig *et al.*, 1995). Nucleotides matching the *E. coli* σ^{70} consensus promoter (Lisser & Margalit, 1993) and transcriptional start sites are emphasised in bold letters.

References (Appendix)

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Publications

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