Diss. ETH Nr. 13865

The global two-component regulatory system RegSR of *Bradyrhizobium japonicum*: from biochemical characterization to the *in vivo* role

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

presented by
RALF EMMERICH
Dipl. Biochem.
born on March 2, 1971
from the Federal Republic of Germany

Prof. Dr. Hauke Hennecke, examiner
Prof. Dr. Thomas Leisinger, co-examiner
PD Dr. Hans-Martin Fischer, co-examiner

Zürich 2000

Table of contents

Abstract

This work presents the biochemical analysis of the RegSR two-component regulatory system of *Bradyrhizobium japonicum* and the determination of its *in vivo* properties. Furthermore, functional similarities between three homologous response regulators of α-Proteobacteria are documented.

Microaerobic expression of many genes required for nitrogen fixation in *B. japonicum* depends on the redox-responsive transcriptional activator NifA encoded in the *fixR-nifA* operon. Expression of this operon was shown previously to depend on the *regR* gene and an upstream activating site (UAS) located around position –68 in the *fixR-nifA* promoter region. The amino acid sequence deduced from *regR* and *regS*, which is encoded upstream of *regR*, indicated that RegS and RegR are the sensor and regulator proteins, respectively, of a novel two-component regulatory system (Bauer *et al.*, 1998).

To investigate the functional properties of RegR and the interaction with its putative, cognate kinase RegS, histidine-tagged derivatives of the RegR protein and of a truncated, soluble variant of RegS ($RegS_C$) were overproduced and affinity-purified. The autokinase activity of $RegS_C$ was demonstrated by *in vitro* phosphorylation experiments. $RegS_C$~P catalyzed the transfer of the phosphoryl label to RegR. The phosphorylated form of $RegS_C$ exhibited phosphatase activity on RegR-phosphate. The phosphorylated residues H219 in RegS and D63 in RegR were identified by chemical stability tests and site-directed mutagenesis. Competition experiments with isolated domains of RegR demonstrated that only its N-terminal but not the C-terminal domain interacts with $RegS_C$. Gel retardation experiments revealed that phosphorylation enhances substantially the binding activity of RegR to the UAS. The *in vivo* role of RegR phosphorylation was studied by complementation of a *B. japonicum regR* deletion mutant with the *regR** gene encoding the RegR-D63N mutant protein. Expression of a *fixR'-'lacZ* fusion was strongly reduced in the *regR**-complemented mutant as compared with a *regR*-complemented mutant. These data demonstrated that the RegSR proteins exhibit the structural and functional properties typical for a two-component regulatory system.

The RegR binding site was characterized using an *in vitro* binding-site selection assay (SELEX: systematic evolution of ligands by exponential enrichment) and site-

specific mutagenesis of the UAS. Eleven critical nucleotides were identified, which are located within a 17 bp minimal RegR-binding site centered at position –64 upstream of the *fixR-nifA* transcriptional start site. A 5 bp imperfect inverted repeat located within this region is necessary but not sufficient for RegR binding. A RegR box was defined, which comprises the eleven critical nucleotides as well as the imperfect inverted repeat. This RegR box led to the identification of a new RegR-binding site located around position –63 upstream of the *regR* transcription start site. Gel retardation experiments showed binding activity of RegR to this newly identified RegR box. Expression of a *regR'-'lacZ* fusion in a *B. japonicum regR* mutant was decreased to about 60% of the level observed in the wild type suggesting that *regR* is partially autoactivated.

RegR is highly homologous to RegA of *Rhodobacter capsulatus* and ActR of *Sinorhizobium meliloti*. To analyze if these response regulators can functionally replace each other, a *B. japonicum regR* mutant was complemented with constitutively expressed *regA* and *actR*. Both genes were able to complement the *regR* deficiency with respect to the expression of a *fixR'-'lacZ* fusion and the formation of a nitrogen-fixing symbiosis with soybean. *In vitro*, RegA was phosphorylated when $RegS_C$ was added to an *Escherichia coli* extract containing overexpressed RegA. Conversely, RegR activated in *R. capsulatus* the expression of the genuine RegA-dependent *puc* gene and showed *in vitro* DNA-binding activity to two RegA-targets. In conclusion, these results showed that *B. japonicum* RegR, *R. capsulatus* RegA and *Sinorhizobium meliloti* ActR are functionally similar.

Kurzfassung

Diese Arbeit beschreibt die biochemische Charakterisierung des RegSR Zweikomponenten-Regulationssystems von *Bradyrhizobium japonicum* und die Analyse seiner *in vivo* Eigenschaften. Zusätzlich wurde der Nachweis der funktionellen Gleichwertigkeit von homologen Regulatorproteinen aus drei α-Proteobakterienspezies erbracht.

Die mikroaerobe Expression vieler Stickstoffixierungsgene in *B. japonicum* wird von dem sauerstoff-labilen Transkriptionsaktivator NifA reguliert, welcher im *fixR-nifA* Operon kodiert ist. Die basale Expression dieses Operons ist abhängig von dem *regR* Gen und einer DNA-Region („upstream activating site", UAS) 68 Nukleotide oberhalb der Transkriptionsstartstelle. Die abgeleiteten Aminosäuresequenzen der Gene *regR* und *regS*, welches oberhalb von *regR* kodiert ist, deuteten darauf hin, daß RegS und RegR den Sensor bzw. Regulator eines Zweikomponenten-Regulationssystems darstellen (Bauer *et al.*, 1998).

Zur Analyse der funktionellen Eigenschaften von RegR und seiner Wechselwirkung mit RegS wurden His_6-Derivate von RegR sowie von einer verkürzten, löslichen Variante von RegS ($RegS_C$) überproduziert und mittels Affinitätschromatographie gereinigt. Die Autokinaseaktivität von $RegS_C$ wurde durch Phosphorylierungsexperimente gezeigt. Autophosphoryliertes $RegS_C$~P übertrug den Phosphatrest auf RegR. Die phosphorylierte Form von $RegS_C$ konnte phosphoryliertes RegR dephosphorylieren. Die phosphorylierten Reste H219 in RegS und D63 in RegR wurden mittels chemischen Stabilitätsuntersuchungen und gezielter Mutagenese identifiziert. Kompetitionsexperimente mit isolierten Domänen von RegR wiesen darauf hin, daß alleine die N-terminale Domäne und nicht die C-terminale Domäne mit $RegS_C$ interagieren. Gelretardationsstudien zeigten, daß die Phosphorylierung von RegR dessen DNA-Bindeaktivität stark erhöht. Die *in vivo* Rolle der Phosphorylierung von RegR wurde mittels Komplementation einer *B. japonicum regR* Deletionsmutante mit dem *regR**-Gen, welches das RegR-D63N Mutantenprotein kodiert, untersucht. Die Expression einer *fixR'-'lacZ* Fusion war in der *regR**-komplementierten Mutante stark reduziert verglichen mit einer *regR*-komplementierten Mutante. Diese Daten zeigten, daß die RegSR Proteine die

strukturellen und funktionellen Eigenschaften eines typischen Zweikomponenten-Regulationssystems aufweisen.

Die UAS, an welche RegR bindet wurde durch ein SELEX Verfahren ("systematic evolution of ligands by exponential enrichment") und gezielte Mutagenese charakterisiert. Elf kritische Nukleotide wurden innerhalb einer 17 Nukleotide langen minimalen RegR-Bindestelle identifiziert, welche 64 Nukleotide oberhalb der *fixR-nifA* Transkriptionsstartstelle liegt. Ein fünf Basenpaar langes imperfektes "inverted repeat" in dieser Region ist notwendig, aber nicht ausreichend für die RegR Bindung. Eine "RegR Box" wurde definiert, welche die elf kritischen Nukleotide und das imperfekte "inverted repeat" umfasst. Mit Hilfe dieser RegR-Box wurde eine neue RegR-Bindestelle identifiziert, welche sich 63 Nukleotide oberhalb des *regR*-Promotors befindet. Gelretardationsexperimente zeigten, daß RegR an diese neue RegR Box bindet. Die Expression einer *regR'-'lacZ* Fusion in einer *B. japonicum regR* Mutante war auf 60% des Wildtypniveaus reduziert, was darauf hindeutet, daß *regR* einer partiellen Autoaktivierung unterliegt.

RegR besitzt grosse Ähnlichkeit zu RegA aus *Rhodobacter capsulatus* und ActR aus *Sinorhizobium meliloti*. Zur Analyse, ob diese Regulatoren sich funktionell ersetzen können, wurde eine *B. japonicum regR* Mutante mit den konstitutiv exprimierten Genen *regA* oder *actR* komplementiert. Beide Gene konnten den *regR*-Defekt bezüglich der Expression einer *regR'-'lacZ* Fusion und der Bildung einer funktionellen Symbiose mit Sojabohnen komplementieren. *In vitro* ließ sich RegA phosphorylieren, wenn $RegS_C$ zu einem Extrakt von *Escherichia coli* Zellen zugegeben wurde, welcher überproduziertes RegA enthielt. Andererseits aktivierte RegR die Expression des RegA-abhängigen *puc* Gens in *R. capsulatus* und zeigte *in vitro* DNA-Bindeaktivität bezüglich zweier natürlicher, RegA-kontrollierter Promotoren. Somit konnte gezeigt werden, daß *B. japonicum* RegR, *R. capsulatus* RegA und *Sinorhizobium meliloti* ActR funktionell ähnlich sind.

Chapter 1

Introduction

1.1. Two-component regulatory systems

Bacteria react to changes in environmental conditions by using a large number of regulatory systems with which they adapt transiently to new conditions, for example the availability of nutrients (sugars, amino acids), different physical conditions (temperature, osmolarity) or the presence of chemical compounds (e.g. oxygen). The two-component regulatory systems represent widespread and common signaling cascades responding to environmental changes (reviewed by Hoch & Silhavy, 1995). In prokaryotes, an increasing number of different two-component regulatory systems are identified which are involved in such diverse actions like quorum sensing (Bassler & Silverman, 1995), resistance to antibiotics (Arthur *et al.*, 1992) or citrate sensing (Kaspar *et al.*, 1999). For example, 62 open reading frames were identified as putative members of the two-component signal transducers in the genome of *Escherichia coli* (Mizuno, 1999). In the past few years, these systems were discovered even in eukaryotic organisms playing a role in yeast osmoregulation, fungi hyphal development and plant ethylene response (reviewed by Loomis *et al.*, 1997).

Two-component regulatory systems usually consist of two proteins: a membrane-bound or cytoplasmic histidine protein kinase which detects the signal, and a response regulator which normally regulates gene expression. The sensor responds to the signal by autophosphorylation at a conserved histidine using ATP (Fig. 1.1.). The phosphoryl group is then transferred to a conserved aspartate of the response regulator. The addition of the phosphoryl group can either activate or repress the activity of the regulator. The response regulator usually binds to DNA to activate or repress the transcription of target genes. These target genes encode proteins whose synthesis is regulated in response to the original signal. For example, the presence of citrate induces under anaerobic conditions target genes which encode a citrate carrier and a citrate-degrading enzyme (Bott *et al.*, 1995). Similarly, when oxygen concentration drops below a threshold level, the sensor FixL in *Sinorhizobium meliloti* detects the "low-oxygen" signal and autophosphorylates (reviewed by Agron & Helinski, 1995). Its phosphate group is transferred to the regulator FixJ, which then activates the expression of the so-called *nif* and *fix* genes. The newly synthesized proteins enable the bacterium to fix nitrogen during symbiosis with alfalfa. Because oxygen is detrimental to some of these proteins, they are synthesized only under low-oxygen conditions. To switch off the response, the phosphatase activity of many

sensor proteins or another phosphatase removes the phosphoryl group from the response regulator.

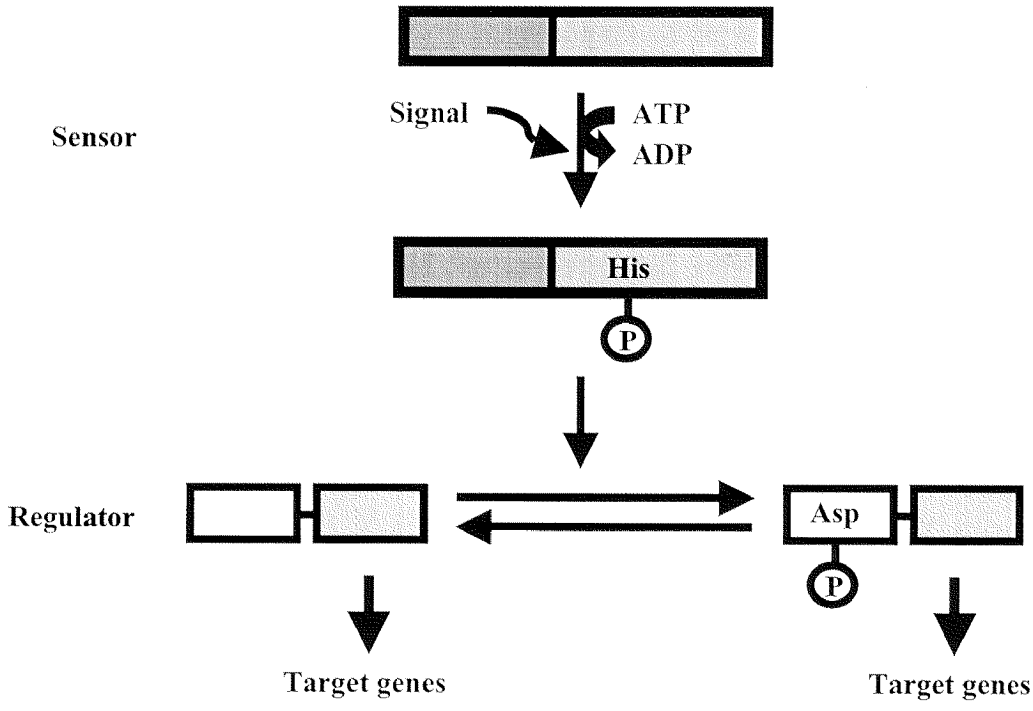


Fig. 1.1. Schematic representation of a two-component regulatory system. In response to a signal, the sensor autophosphorylates at a conserved histidine. The phosphoryl group is then transferred to a conserved aspartate of the response regulator. The response regulator can regulate target genes in the unphosphorylated or phosphorylated state. Often, the histidine kinase also can dephosphorylate the phosphorylated response regulator. The domain structure of the two proteins is indicated by the differently shaded boxes. Many sensors contain two domains: a N-terminal membrane-bound domain and a C-terminal cytoplasmic domain, containing the phosphorylated His. Note that also soluble sensors exist which do not have a membrane-spanning domain. Many regulators consist of an N-terminal domain comprising the phosphorylated Asp and a C-terminal domain containing the DNA-binding motif.

1.1.1. Histidine protein kinases: structures and signaling mechanisms

Signal reception and transmission is performed by the histidine kinases. They are identified by characteristic sequence motifs, called H, N, G1, F and G2 boxes (Parkinson & Kofoid, 1992; Swanson *et al.*, 1994) and divided in two classes by their domain organization (Bilwes *et al.*, 1999; Fig. 1.2.). The difference between the two classes relates to the position of the conserved histidine. In class I kinases, like Spo0B, the H box is located in the same domain as the other four boxes, whereas in class II kinases, like CheA, other domains separate the H box from the other four boxes. The so-called hybrid kinases contain two additional domains: one domain which is normally present in response regulators and a second His-containing domain.

For example, the hybrid kinase ArcB is first phosphorylated at the conserved histidine in the H box. The phosphoryl group is then transferred to an aspartate and then to a second histidine from which it is transferred to the response regulator ArcA (Iuchi & Lin, 1990; Iuchi & Lin, 1995). Many histidine kinases are membrane anchored, e.g. the sensor EnvZ of *E. coli* possesses two membrane-spanning parts, placing its N-terminal domain in the periplasm (Forst *et al.*, 1987). This periplasmic domain is thought to sense the environmental osmolarity which represents the stimulus for EnvZ. Another general feature of histidine kinases is that the autophosphorylation takes place *in trans*, i.e. the kinase is a dimer and the catalytic domain of one monomer phosphorylates the conserved histidine of the other monomer.

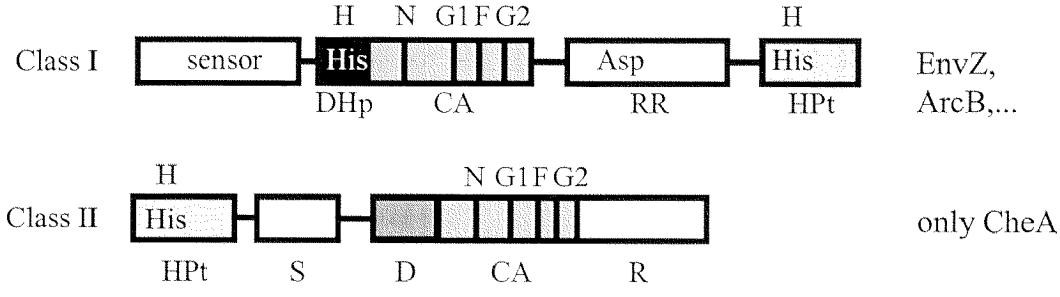


Fig. 1.2. Schematic diagram of the two classes of histidine kinases. In class I histidine kinases, the conserved His residue in the H box of the DHp domain is the primary autophosphorylation site. The two distal domains RR and HPt are only found in hybrid kinases, where the phosphoryl group is sequentially transferred from the conserved His in the DHp domain to the conserved aspartate (Asp) in the RR domain and eventually to the conserved His residue in the H box of the HPt domain. In class II histidine kinases, the conserved residue is located in the H box of the HPt domain. Sensor, domain needed for detection of the signal; DHp, dimerization and histidine phosphotransfer domain; CA, catalytic and ATP-binding domain; RR, response regulator domain; HPt, histidine-containing phosphotransfer domain; S, substrate binding domain which interacts with the response regulators CheY and CheB; D, dimerization domain; R, regulatory domain which binds to CheW (Dutta *et al.*, 1999).

In recent years, the structures from full-length proteins or individual domains of both classes of kinases were solved. EnvZ, the osmosensor of *E. coli*, ArcB, the anaerobic sensor of *E. coli*, and Spo0B, a histidine kinase involved in sporulation of *Bacillus subtilis* are examples of structurally characterized class I histidine kinases whereas CheA is the only class II member whose structure is known. These structures will be discussed in the following part.

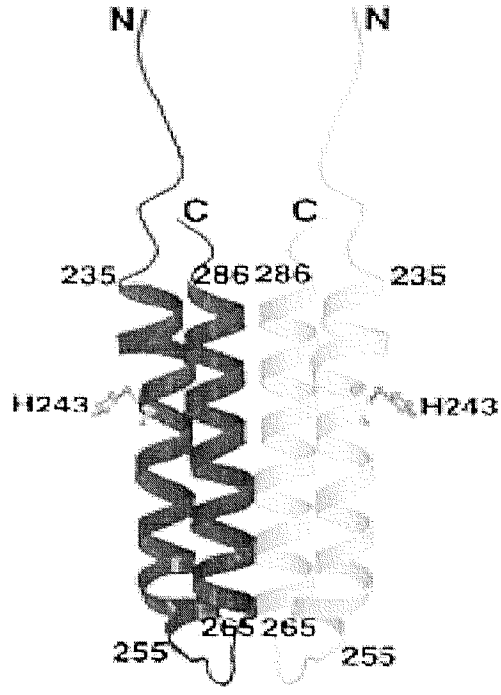


Fig. 1.3. Ribbon diagram of the homodimeric core domain (DHp domain) of *E. coli* EnvZ. The conserved His243 is shown as a ball-and-stick model. The residues at the end of the helices, as well as the N- and C-termini of the protein are labeled (Tomomori *et al.*, 1999).

The **DHp domain** of EnvZ, containing the conserved histidine is shown in Fig. 1.3. (Tomomori *et al.*, 1999). The structure comprises a four-helix bundle formed by two identical helix-turn-helix subunits forming two active sites within the dimeric kinase. The four amphipatic helices pack against each other to form a hydrophobic core. The conserved His243 situated in the middle of the helices is solvent exposed, concordant with its potential to become phosphorylated. NMR titration experiments performed with the isolated DHp domain and the regulatory domain of OmpR, which is phosphorylated by EnvZ, suggested that OmpR binds towards the base of the four-helix bundle of the DHp domain (Tomomori *et al.*, 1999). A monomeric mutant histidine kinase, which is functional with respect to autophosphorylation and phosphotransfer activity, but lacks phosphatase actvity, was constructed by fusing a second DHp domain in front of a DHp and a CA domain (Qin *et al.*, 2000). As a consequence, the four-helix bundle was formed intramolecular. The DHp domain of Spo0B also dimerizes by parallel association of helical hairpins to form a four-helix bundle and the phosphorylated histidine is again solvent exposed (Varughese *et al.*, 1998). The DHp domain seems to be involved also in phosphatase activity. A fragment of *E. coli* NtrB, comprising the DHp domain, has recently been implicated

in harboring the phosphatase activity (Kramer & Weiss, 1999). CheA which lacks a DHp domain does not possess phosphatase activity.

The structure of the individual catalytic domain of EnvZ was determined by Tanaka *et al.* (1998). The **CA domain** assumes an α/β sandwich fold: one layer consists of a five-stranded β-sheet and the other layer comprises three α-helices. The nonhydrolysable ATP analogue, AMP-PNP, is surrounded by an α-helix and a long loop that extends away from the rest of the molecule. The conserved amino acids of the N, G1, F and G2 boxes border this ATP-binding pocket. The triphosphate chain is exposed to the surface, consistent with the potential for transferring the γ-phosphate to the conserved histidine. The structure is highly similar to the CA domains of CheA (Fig. 1.5.) and three ATPases, Hsp90 (Prodromou *et al.,* 1997), DNA gyrase B (Wigley *et al.,* 1991) and MutL (Ban & Yang, 1998). However, it is not understood when the CA domain functions as an ATPase or as a kinase. The C-terminal part of Spo0B is topologically similar to the CA domain of EnvZ but it lacks the conserved residues important for ATP-binding (Varughese *et al.*, 1998). This is explained with its distinct function: Spo0B is not a kinase; it just shuttles the phosphoryl group from the upstream to the downstream response regulator.

The structure of the **HPt domain** of ArcB was determined by Kato *et al.* (1997) (Fig. 1.4.). It is a monomeric protein consisting of six α-helices containing a four-helix bundle folding. The significance of a Zn^{2+} ion-binding site is not clear. The overall structure looks quite similar to the dimeric DHp domain, but the monomeric helix-bundle of the HPt domain is build up by a single polypeptide chain, and it is kidney shaped while the DHp domain is straight. A common feature of the HPt and DHp domains is that the position of the conserved His717 is located in the middle of an α-helix, and that its side chain is solvent exposed. However, the HPt monomer contains only one phosphorylatable His, whereas the dimeric DHp domain has two. The structure of the HPt domain of CheA also includes an antiparallel four-helix bundle with the same topology as that of EnvZ (Zhou *et al.*, 1995).

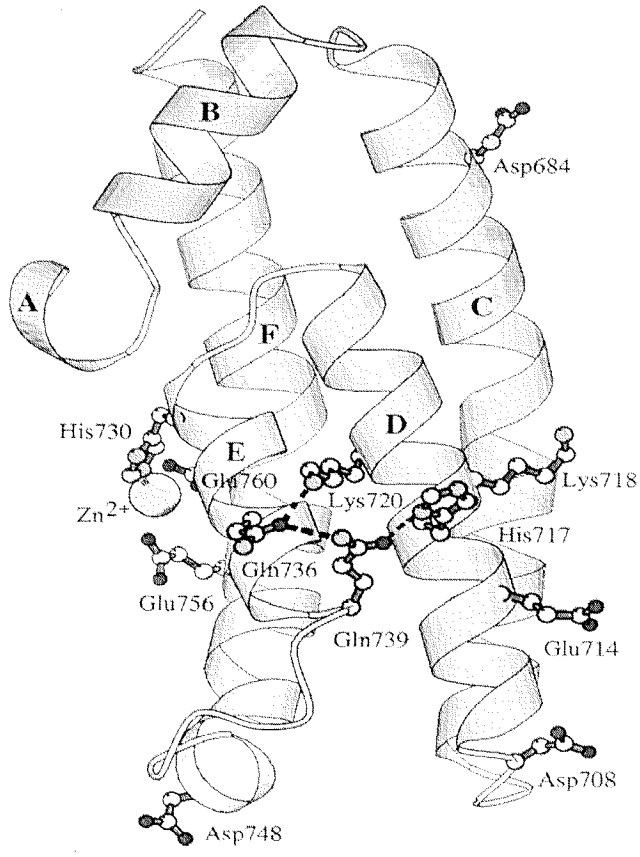


Fig. 1.4. Structure of the HPt domain of *E. coli* ArcB. The six α-helices forming the HPt domain are denominated A to F. The active residue His717 is located on the surface of helix D, which lies in the internal curvature of the kidney-shaped molecule. The side chain of the active His717 is shown as a ball-and-stick model together with those of Gln736, Gln739, Glu714, Lys718 and Lys720 at the active site. Conserved residues Asp684 and Asp708 are also shown. The Zn^{2+} ion is shown with its coordinated residues (Kato *et al.*, 1997).

The structure of the mutant histidine kinase CheAΔ289 (residues 290-671) from *Thermotoga maritima* was solved by Bilwes *et al.* (1999). The determined structure comprises the D-, CA- and R-domains. The CheAΔ289 protein is a dimer with each subunit comprising the three distinct domains linked by hinges (Fig. 1.5.). The dimerization domain is located in the center, and it is similar to the DHp domain of EnvZ, except for the missing phosphorylated histidines. The two kinase and regulatory domains are located around the D domain providing no further contacts between the two subunits. The CA domain is a two-layered α-β sandwich made of six α-helices and a five-stranded β-sheet and is highly homologous to the CA domain of EnvZ. The regulatory domain displays two twisted five-stranded β-barrels. It regulates the kinase activity by interacting with the transmembrane chemosensors through the adaptor protein CheW (Bourret *et al.*, 1993).

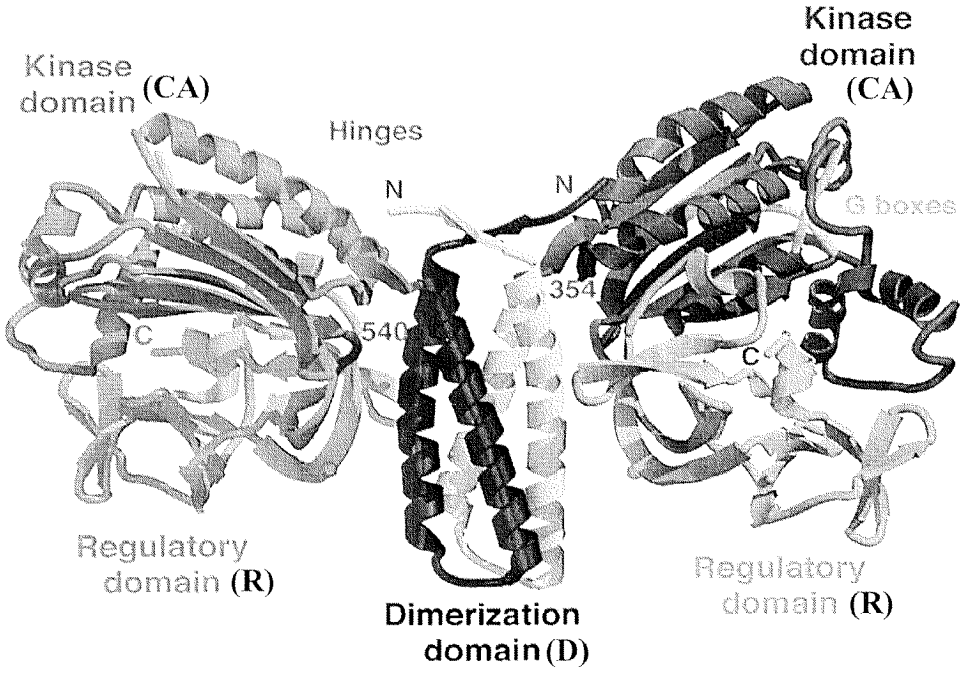


Fig. 1.5. Overall structure of the CheAΔ289 (residues 290-671) dimer. The two subunits of the CheA dimer each contain separate dimerization, kinase, and regulatory domains. The dimer associates by a central four-helix bundle and places the 2 ATP-binding sites (indicated by the G boxes) 90 Å away from each other (Bilwes *et al.*, 1999).

The structural analysis of the histidine kinases discussed above revealed striking similarities with respect to the structure of the domains involved in kinase activity, dimerization and regulation. By contrast, much less is known how the signal is detected or what the exact nature of the signal is. The signal is sensed either directly (e.g. in FixL; discussed below) or via a distinct sensor protein, like the Tar receptor of *E. coli*. Tar is a membrane protein involved in chemotaxis and senses the presence of Asp, Glu and maltose. When Tar is activated by binding of Asp, the signal is transmitted to CheA which is bound to Tar via the adaptor protein CheW. CheA then autophosphorylates and transduces the signal further downstream in the regulatory cascade (Aizawa *et al.*, 2000; Levit *et al.*, 1998).

FixL ist the best studied histidine kinase with respect to signal detection. FixL is an oxygen sensor which regulates nitrogen fixation gene expression in rhizobia (Agron & Helsinki, 1995). FixL comprises three domains: a membrane-anchored domain, a PAS domain comprising a heme cofactor and a kinase domain with the phosphorylated histidine. FixL autophosphorylates only under low-oxygen conditions (Lois *et al.*, 1993). The inhibition of its kinase activity by oxygen was found to be dependent on the conversion of the heme iron from the high- to the low-spin state

(Gilles-Gonzales *et al.*, 1995). This spin state change is accompanied by movement of the heme iron in and out of the porphyrin ring, which was documented by the crystal structure of the *B. japonicum* FixL PAS domain in the presence of cyanide, oxygen, imidazole and nitric oxide (Gong *et al.*, 1998, 2000). The binding of dioxygen to the heme alters the nonplanarity of the heme resulting in a shift of the positions of the two heme propionate side chains (Fig. 1.6.).

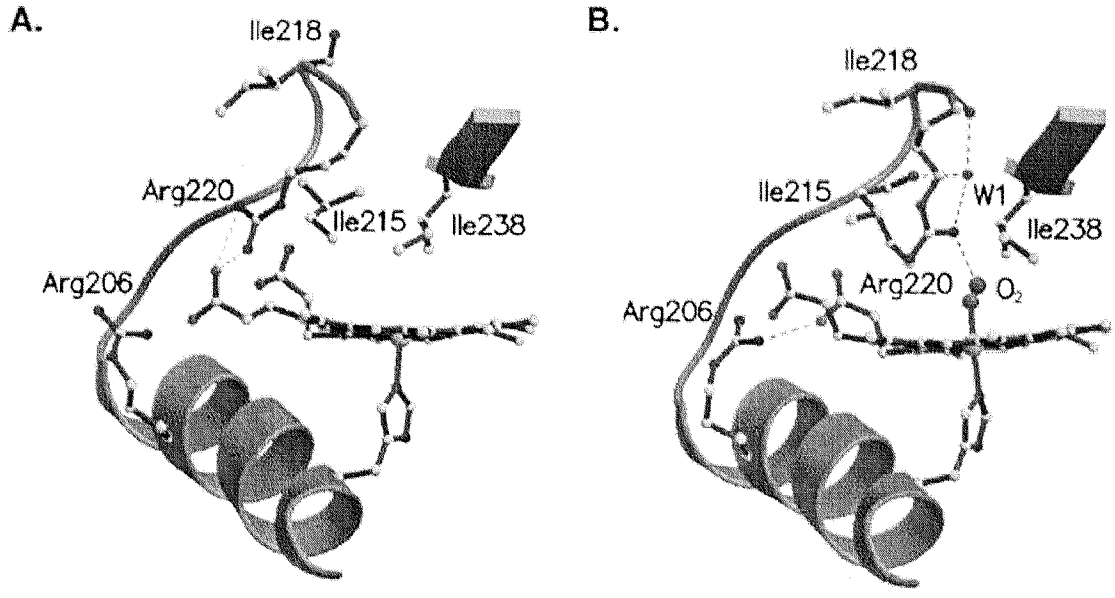


Fig. 1.6. Ribbon and ball-and-stick model diagrams of the *B. japonicum* FixL heme binding pocket. (A) met(Fe^{3+})-BjFixL and (B) oxy-BjFixL. W1 is a water molecule (Gong *et al.* 2000). For further details see text.

Upon dioxygen binding, Arg220 loses its salt bridge to heme propionate 7, rotates ~170° around its C_α-C_β bond and forms a hydrogen bond with the bound dioxygen ligand. Arg206 moves to form a salt bridge to heme propionate 6. These two movements result in a conformational change of a loop that provokes a rotation of Ile218 which then forms a hydrogen-bond with a water molecule which, in turn, forms a hydrogen bond with Arg220. These conformational changes are thought to alter the interaction of the PAS domain with the kinase domain and therefore abolish the autokinase activity of FixL.

Further examples of histidine kinases where binding of signaling molecules was demonstrated biochemically are CitA from *Klebsiella pneumonia* and PhoQ from *Salmonella typhimurium* (Kaspar *et al.*, 1999; Vescovi *et al.*, 1997). In both cases, the periplasmic domains were used to show *in vitro* binding of citrate to CitA and

Mg^{2+}/Ca^{2+} to PhoQ. However, it is not known yet how binding of the signal molecule to the periplasmic domain affects the function of the rest of these proteins.

1.1.2. Response regulators: structures and mechanisms of target gene activation

Upon autophosphorylation of the sensor kinase the phosphoryl label is transferred to the response regulator. The phosphorylated response regulator transmits the signal further downstream by regulating gene expression (most response regulators), binding to additional proteins (CheY) or acting as a methylesterase (CheB). Response regulators can be divided into four families based on similarities in their domains (Volz, 1995). The CheY family consists of one domain whereas the other three families are multidomain proteins. Members of the NtrC family typically consist of about 460 amino acid residues and have two additional domains following the receiver domain. Regulators belonging to the OmpR family which includes OmpR, PhoB and VirG, are about 230 amino acids in length and consist of two domains. Proteins of the FixJ family contain sequences with one extra FixJ-type domain on their C-terminus and are around 220 residues long. Domain structures of members of each class have been solved and will be discussed in the following.

Tab. 1.1. The four families of resonse regulators and selected examples. At: *Agrobacterium tumefaciens*; Bs: *Bacillus subtilis*; Bj: *Bradyrhizobium japonicum*; Bp: *Bordetella pertussis*; Ec: *E. coli*; Ef: *Enterococcus faecium*; Rc: *Rhodobacter capsulatus*; Sm: *Sinorhizobium meliloti*; Vf: *Vibrio fischeri*; v: various species.

Family	Selected examples
CheY	CheY (v), SpoOF (Bs)
OmpR	ArcA (Ec), KdpD (Ec), OmpR (Ec), PhoB (v), PhoP (v), TorR (Ec), VanR (Ef), VirG (At)
FixJ	ActR (Sm), BvgA (Bp), DegU (Bs), EvgA (Ec), FixJ (Bj), LuxR (Vf), NarL (Ec), NarP (Ec), NodW (Bj), RegA (Rc), RegR (Bj)
NtrC	DctD (Sm), NtrC (v)

The first structure was obtained from crystallized CheY of *Salmonella typhimurium* (Stock *et al.*, 1989; Volz & Matsumura, 1991). Fig. 1.7. shows CheY from *E. coli*, which is a single-domain protein with a doubly wound five-stranded α/β motif. Five β-strands form a central parallel β-sheet which is flanked on both sides by a total of five α-helices. Hydrophobic amino acids are located at the internal faces of

the helices that pack against the β-sheet. The phosphorylated residue Asp57 is located in an acidic pocket formed by loops at the C-terminal ends of two β-strands. This structure is observed in many other receiver domains of response regulators, for example NarL, FixJ, Spo0A and PhoB (Baikalov *et al.*, 1996; Birck *et al.*, 1999; Lewis *et al.*, 1999; Solà *et al.*, 1999).

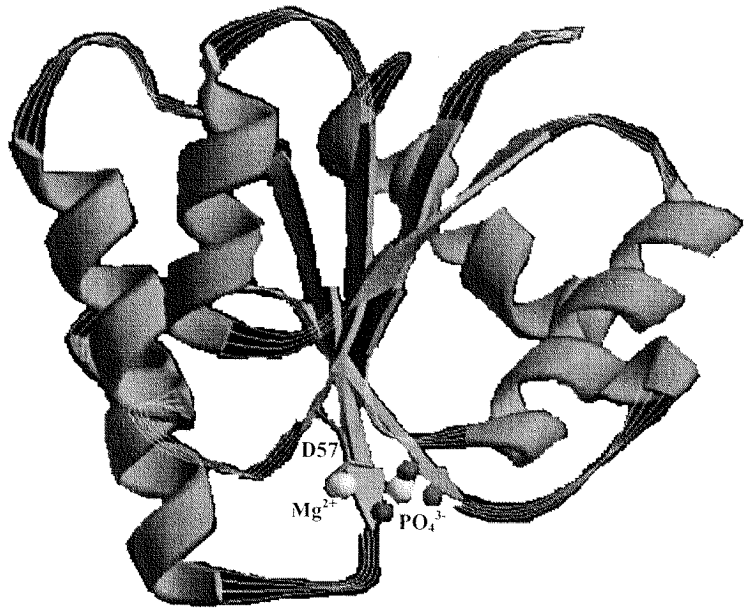


Fig. 1.7. Structure of CheY of *E. coli*. The site of phosphorylation (Asp57) and the putative positions of Mg^{2+} and PO_4^{3-} ions are represented as a ball-and-stick model. The figure was obtained from http://www.expasy.ch/cgi-bin/get-sw3d-entry?P06143.

NarL belongs to the FixJ family. Its crystal structure was solved by Baikalov *et al.* (1996). NarL is composed of two domains: a N-terminal receiver domain which shows the same structure as CheY and a C-terminal DNA-binding domain (Fig. 1.8.). The DNA-binding domain is a compact bundle of four α-helices, of which the middle two form a helix-turn-helix motif. The two domains are connected by an α-helix and a flexible linker. In this unphosphorylated form, the C-terminal domain is turned against the receiver domain which precludes DNA-binding.

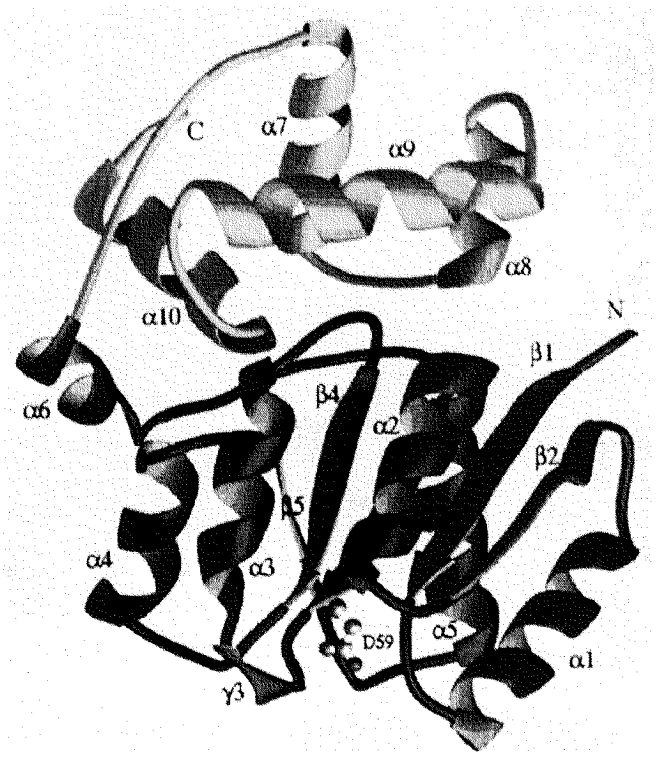


Fig. 1.8. Structure of NarL of *Escherichia coli*. The N-terminal domain is shown in black and the C-terminal domain in grey. A ball-and-stick representation is given for the site of phosphorylation, Asp59 (Baikalov *et al.*, 1996). Helices α8 and α9 form the helix-turn-helix motif of the C-terminal DNA-binding domain.

How does phosphorylation activate response regulators? Two different mechanisms were proposed (reviewed by Egger *et al.*, 1997). Phosphorylation of the N-terminal domain either induces dimerization of the regulator, which is a prerequisite for transcriptional activation (e.g. NtrC; Fiedler & Weiss, 1995), or it relieves the intrinsically inhibitory effect of the N-terminal domain on the C-terminal activation domain (e.g. FixJ; Da Re *et al.*, 1994). As mentioned above, NarL uses the second type of mechanism because the unphosphorylated N-terminal domain is orientated against the C-terminal domain. To describe more precisely the mechanism of activation, the structures of phosphorylated Asp, activated response regulators and conformational changes induced by phosphorylation will be discussed in the following part.

The structural analysis of most activated response regulators has been hindered because aspartyl-phosphate in response regulators has a short half-life. The use of beryllofluoride (BeF_3^-) which forms a persistent complex with response regulators to yield an acyl phosphate analog solved this problem and led to the determination of the

NMR structure of activated NtrC and CheY (Yan *et al.*, 2000; Cho *et al.*, 2000). An interesting new aspect concerning the control of response regulators was recently described by Ames *et al.* (1999). They showed that DNA-binding of OmpR stimulates its phosphorylation. The authors suggest that response regulators may be phosphorylated while bound to their target DNA. Whether this observation is valid also for other response regulators needs to be investigated.

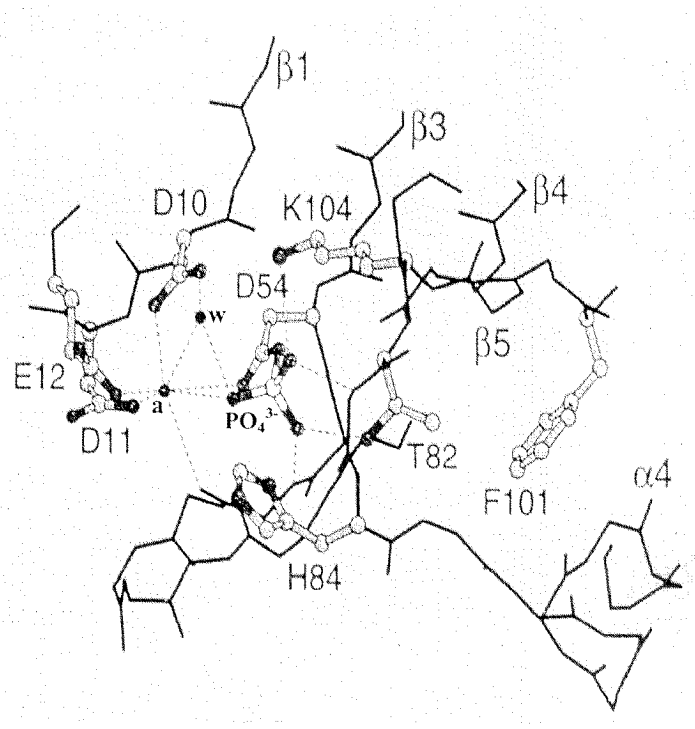


Fig. 1.9. Active site of phosphorylated FixJN. The hydrogen-bonding interactions made by the acyl phosphate group (PO_4^{3-}), a water molecule (w) and an ammonium ion (a) are represented by dotted lines (Birck *et al.*, 1999).

Phosphorylation of FixJ of *S. meliloti* and of its separated N-terminal domain (FixJN) induces dimerization (Da Re *et al.*, 1999). The crystal structures of phosphorylated and unphosphorylated FixJN were solved by Birck *et al.* (1999). The receiver domain shows the same overall structure as CheY. The phosphoryl group is covalently bound to the invariant Asp54 interacting via its oxygen atoms with a water molecule, an ammonium ion, and main-chain and side-chain atoms of two residues highly conserved in response regulators (Thr82 and Lys104; Fig. 1.9.). The formation of a strong hydrogen bond between the acyl phosphate group and the Thr82 side chain induces a significant modification of the main-chain dihedral angle of this residue, which affects the loop that connects β4 and α4. In turn, the N-terminal part of helix α4 is pushed away from the active site. This conformational change leads to the

molecular definition of the dimer interface provided by helix α4 and strand β5 from each receiver domain. This documents nicely how phosphorylation of FixJN leads to dimerization. These results also show that the two models for the activation of response regulators, relief of inhibition versus oligomerization, are not mutually exclusive. The implications for gene activation will be discussed below.

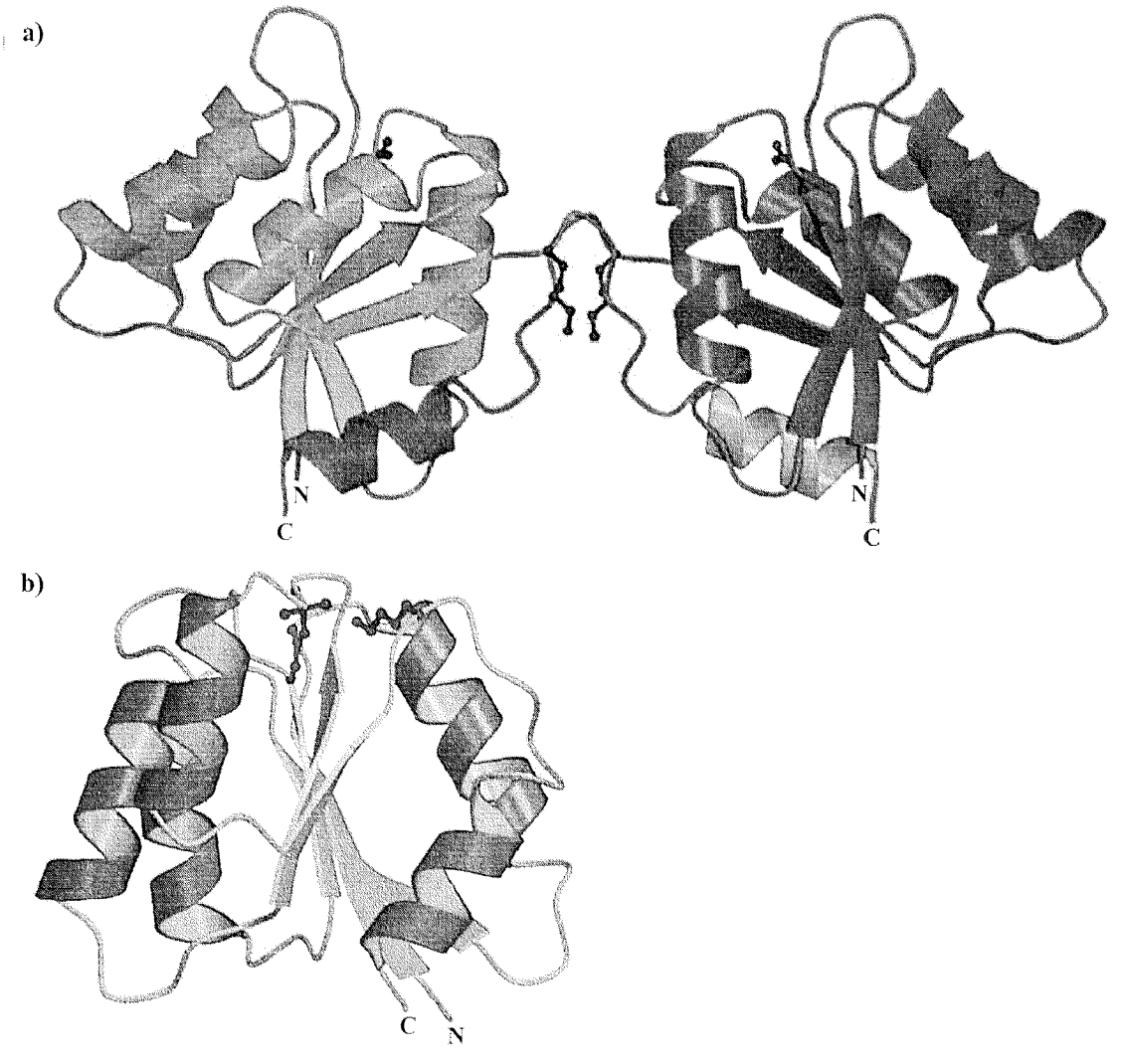


Fig. 1.10. Ribbon presentation of N-Spo0A (a) and N-Spo0A~P (b). Unphosphorylated N-Spo0A is a dimer formed by the interaction of the protruding α-helix with the β-sheet of the other subunit. In the monomeric N-Spo0A~P all α-helices are packed around the β–sheet. The side chains of Lys106 and Asp55 (phosphoryl-Asp55 in N-Spo0A~P) are indicated as ball-and-stick models. (Lewis *et al.*, 2000).

The crystal structures of the unphosphorylated and the phosphorylated receiver domain of Spo0A of *Bacillus stearothermophilus* (N-Spo0A) revealed a different mechanism for activation (Lewis *et al.*, 1999, 2000). Again, the phosphoryl group is covalently bound to the invariant Asp55, and the phosphoryl oxygen atoms make

hydrogen bonds with the side-chains of Thr84 and Lys106, the main-chain amide groups of Ile56, Ile57 and Ala85, a water molecule, and a calcium ion. The relative position of the Ca^{2+} ion is the same as that of the ammonium ion in FixJN and the Mg^{2+} ion in CheY (Birck *et al.*, 1999; Stock *et al.*, 1993). However, the unphosphorylated N-Spo0A is a dimer, whereas N-Spo0A~P is a monomer (Fig. 1.10.). Dephosphorylation of N-Spo0A leads to a *cis-trans* isomerization of the Lys106-Pro107 peptide bond. This results in a "3-D domain-swapping" mechanism. In the phosphorylated monomer, helix α5 packs intramolecularly onto the β-sheet. After dephosphorylation and "domain-swapping", helix α5 projects away from the molecule to mediate the formation of a dimer by packing onto the β-sheet of the partner subunit. However, it should be noted that the dimer formation in the crystals was observed at pH 4, and thus, the relevance of this process at more physiological conditions remains to be tested.

The question remains how response regulators control gene expression. The first step of activation involves specific DNA-binding. DNA-binding occurs via a helix-turn-helix motif which was found in most response regulators on the basis of sequence analysis and which is present in all DNA-binding domain structures of response regulators solved so far (NarL, OmpR and NtrC; Baikalov *et al.*, 1996; Martinez-Hackert & Stock, 1997; Pelton *et al.*, 1999). Detailed DNA-binding and transcription studies were made with NtrC of *E. coli* and *S. typhimurium* and with FixJ of *S. meliloti*. The respective results are summarized below.

The enhancer-binding protein NtrC (also called NRI) is the response regulator of the NtrBC two-component regulatory system. It activates transcription by the σ^{54}-holoenzyme of RNA polymerase (reviewed by Porter *et al.*, 1995). Binding of NtrC occurs at two DNA sites about 100 to 140 nucleotides upstream of the transcription start site (Fig. 1.11.). DNA-binding does not depend on phosphorylation but phosphorylation increases cooperative binding to both binding sites (Weiss *et al.*, 1992). It was shown that NtrC forms large oligomers which activate transcription (Wyman *et al.*, 1997). Phosphorylated NtrC oligomers contact RNA polymerase by means of a DNA loop formation. Upon hydrolysis of ATP by phosphorylated NtrC, it catalyzes the isomerization of closed, double-stranded complexes to transcriptionally productive open complexes which are characterized by locally denatured, single-stranded DNA stretches around the transcriptional start site (Popham *et al.*, 1989;

Weiss *et al.*, 1991). A region of the central domain of NtrC (residues 206-220) was shown to be essential for interaction with the RNA polymerase and/or for coupling the energy derived from ATP hydrolysis to a change in DNA conformation (Yan & Kustu, 1999).

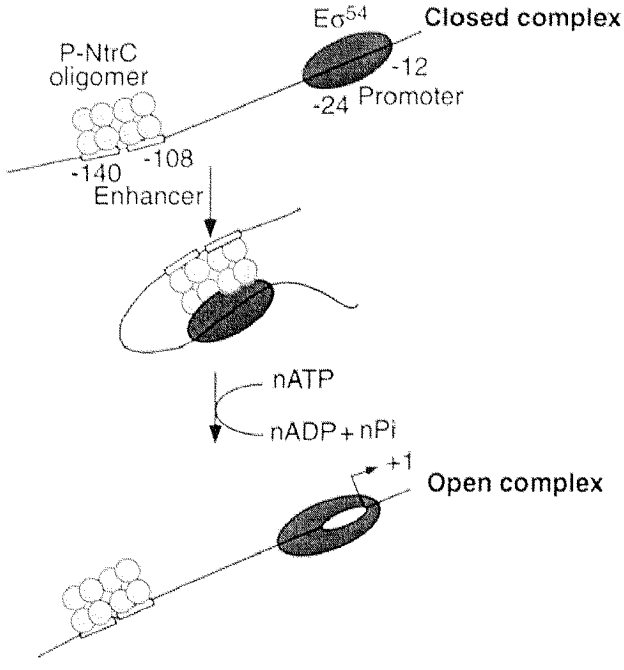


Fig. 1.11. Transcriptional activation by NtrC at the *glnA* promoter of *S. typhimurium*. Conserved promoter sequences recognized by α^{54}-holoenzyme ($E\alpha^{54}$) lie at sites -12 and -24 with respect to the start site of transcription at +1. Boxes represent the two 17-bp NtrC-binding sites that constitute the *glnA* enhancer; they are centered at -108 and -140. (Top) $E\alpha^{54}$ by itself can bind to the *glnA* promoter in a closed recognition complex, in which the DNA remains double-stranded. NtrC binds to the enhancer, but only the phosphorylated form (P-NtrC) can activate transcription. Active oligomers of P-NtrC must contain not only the two dimers bound to the enhancer but an additional dimer or dimers bound to these by protein-protein interactions. (Middle) P-NtrC contacts $E\alpha^{54}$ by means of a DNA loop. (Bottom) In a reaction that requires hydrolysis of ATP, P-NtrC catalyzes the isomerization of closed complexes between polymerase and the promoter to open complexes, in which the DNA around the transcriptional start site is locally denatured and the correct strand can be used as template (Wyman *et al.*, 1997).

Phosphorylation of FixJ enhances its DNA-binding and *in vitro* transcription activity (Agron *et al.*, 1993; Galinier *et al.*, 1994). As indicated above, phosphorylation of FixJ induces its dimerization. However, dimerization is not a prerequisite for activation as the *nifA* promoter can be activated by the isolated FixJC domain and, to a lesser extent, also by full-length FixJ, which are both monomers (Da Re *et al.*, 1994, 1999). The major role of the dimerization is probably to enhance the affinity and specificity of binding to target sites. This is supported by the fact that FixJ

mutant proteins which cannot dimerize but can be phosphorylated showed 10-fold lower affinity for the *fixK* promoter than FixJ~P (Da Re *et al.*, 1999).

Some response regulators are special in the sense that they can activate or repress transcription depending on the respective target gene(s) or that both the phosphorylated and the unphosphorylated form can activate transcription. For example, NarL~P of *E. coli* is an activator of the genes encoding nitrate and nitrite reductase, and it represses the genes encoding fumarate reductase and alcohol dehydrogenase (Stewart & Rabin, 1995). The DegU protein of *B. subtilis* activates late competence genes in its unphosphorylated form and genes encoding degradative enzymes in the phosphorylated form (Msadek *et al.*, 1995). Some response regulators are able to interact with different sigma factors. For example, Spo0A~P of *B. subtilis* interacts with σ^A, the vegetative sigma factor, and with σ^H, a sigma factor required for expression of genes in stationary phase and during sporulation (Hoch, 1995). Finally, the efficiency of some response regulators (e.g. NtrC, RegA) is enhanced by additional proteins (e.g. IHF; Santero *et al.*, 1992; Kirndörfer *et al.*, 1998).

1.2. The two-component regulatory systems FixLJ and RegSR of *B. japonicum*

The two-component regulatory systems FixLJ and RegSR of *B. japonicum* are essential for nitrogen fixation. The regulatory network comprising the FixLJ-$FixK_2$ cascade and the RegSR-NifA cascade is shown in Fig. 1.12.

The FixLJ-$FixK_2$ cascade senses low-oxygen conditions via FixLJ (section 1.1.2.). Active FixJ activates transcription of $fixK_2$ whose product is a positive regulator of genes needed for microaerobic life style, e.g. the *fixNOQP* genes encoding the high-affinity cbb_3-type cytochrome oxidase and $rpoN_1$, one of the two genes encoding the alternative sigma factor σ^{54} which is required for NifA-mediated activation in the RegSR-NifA cascade (Anthamatten & Hennecke, 1991; Anthamatten *et al.*, 1992; Nellen-Anthamatten *et al.*, 1998). The oxygen-responsive element of this cascade is the NifA protein which senses oxygen or redox conditions presumably via a metal cofactor bound to essential cysteine residues (Fischer & Hennecke, 1987; Fischer *et al.*, 1989). NifA is encoded in the *fixR-nifA* operon, which is preceded by two disparately regulated promoters ($fixRp_1$, $fixRp_2$; see Fig. 2.1.). Under aerobic conditions, low-level expression from $fixRp_2$ depends on the response regulator RegR which acts in concert with a still unknown σ factor (Bauer *et al.*, 1998). The *regR* gene was originally cloned on the basis of N-terminal amino acid sequence information derived from purified RegR protein (Kaspar, 1997; Bauer *et al.*, 1998). It is associated with the *regS* gene, and the deduced amino acid sequences suggested that RegR and RegS represent a two-component regulatory system (Bauer *et al.*, 1998). Most recently, it was shown that expression of *cbbP'-'lacZ* and *cbbL'-'lacZ* fusions is decreased in a *regR* mutant (Bauer, 2000). Currently it is not known if RegR regulates directly or indirectly the *cbbFPTALSXE* operon, encoding the structural genes required for CO_2 fixation. The implications are discussed in chapters 6 and 8. So far, it is not known, whether RegR-dependent expression is independent of the oxygen concentration, because a $rpoN_{1/2}$ mutant showed an increased *fixR'-'lacZ* expression under anaerobic conditions compared to that under aerobic condition (Barrios *et al.*, 1995). However, different media were used under these two oxygen conditions, which could result in a different expression pattern. A *nifA* mutant did not show a different *fixR'-'lacZ* expression under aerobic or anaerobic growth conditions. Presumably, the RNA-polymerase containing σ^{54} still binds to $fixRp_1$ and blocks

RegR-dependent transcription from *fixRp*$_2$. Under low-oxygen conditions, NifA enhances expression of the *fixR-nifA* operon via activation of *fixRp*$_1$ (Thöny *et al.*, 1989). The NifA protein in concert with the σ^{54} RNA polymerase activates the genes of the NifA regulon, e.g. the nitrogenase structural genes, *nifDK* and *nifH* (Fischer *et al.*, 1986; see Fig. 8.1.). In conclusion it is worth noting that two different signals are transduced by this regulatory network: oxygen at the levels of FixLJ and NifA and a yet unknown signal acting via RegSR.

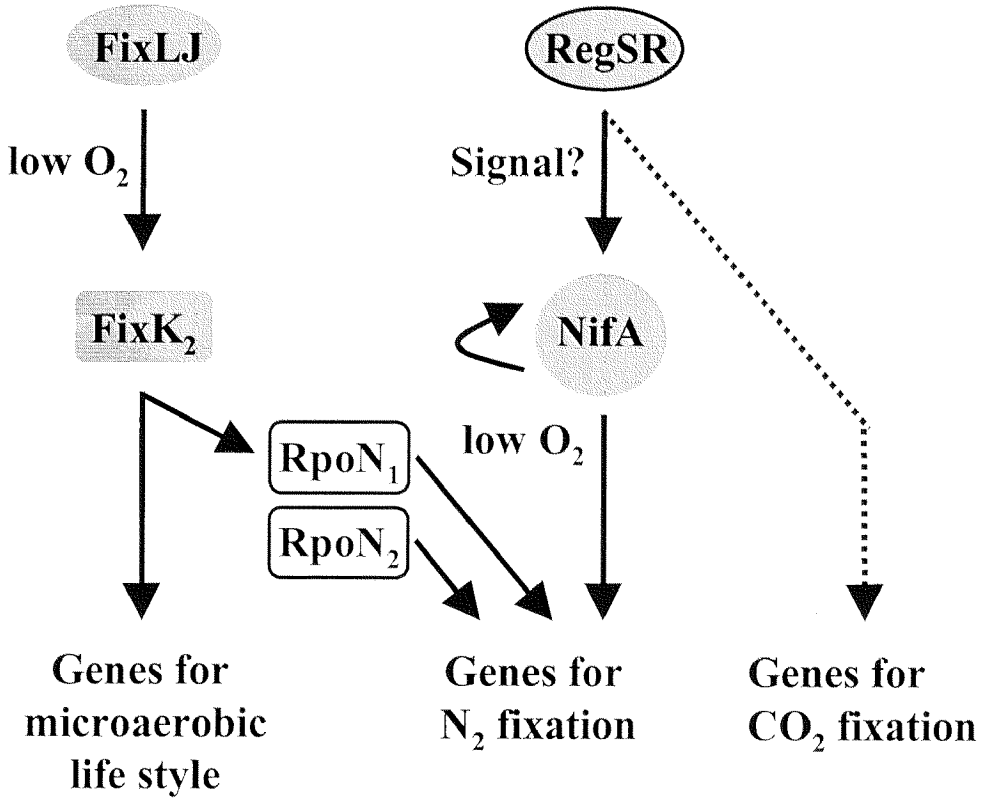


Fig. 1.12. Model of the regulatory network controlling microaerobic life style, nitrogen fixartion and carbon dioxide fixation in *B. japonicum*. For details see text. Direct gene activation (positive control) is indicated by solid arrows. The dotted arrow indicate that it is currently not known how RegR regulates the genes for CO_2 fixation.

The aim of the present work was to investigate whether RegS and RegR indeed represent a functional pair of a sensor kinase and a response regulator constituting a two-component regulatory system. The biochemical analysis and their function *in vitro* and *in vivo* are described in chapters 2, 3 and 4. So far, little was known about the DNA sequence determinants required for the interaction of RegR with the upstream activating sequence of the *fixR-nifA* operon. A detailed analysis of this

DNA-region (chapter 5) led to the definition of a RegR box which allowed the identification of new RegR-binding sites (chapter 6). The pronounced structural similarily of the RegSR proteins to RegBA of the purple photosynthetic bacteria *Rhodobacter capsulatus*, *Rhodovulum sulfidophilum*, *Roseobacter denitrificans* and *Rhodobacter sphaeroides* and ActSR of *Sinorhizobium meliloti* raised the question of whether the respective response regulators are functionally equivalent. Evidence that RegR, RegA and ActR are indeed functionally similar is presented in chapter 7. In chapter 8, a survey of the current knowledge about the *B. japonicum* RegR regulon is presented, and speculations about the identity of possible signals transduced by RegSR are made.

Chapter 2

Phosphorylation, dephosphorylation and DNA-binding of the *Bradyrhizobium japonicum* RegSR two-component regulatory proteins

Ralf Emmerich, Kelnor Panglungtshang, Philipp Strehler, Hauke Hennecke and Hans-Martin Fischer

European Journal of Biochemistry (1999) **263**, 455-463

2.1. Abstract

Micro-oxic induction of many genes required for nitrogen fixation in *Bradyrhizobium japonicum* depends on the redox-responsive transcriptional activator NifA which is encoded in the *fixR-nifA* operon. Basal expression of this operon depends on the response regulator RegR and a DNA element located around position –68 in the *fixR-nifA* promoter region. To investigate the functional properties of RegR and the interaction with its putative, cognate kinase RegS, we overproduced and affinity-purified RegR and a truncated, soluble variant of RegS ($RegS_C$), both as N-terminally His_6-tagged proteins. $RegS_C$ autophosphorylated when incubated with [γ-^{32}P]ATP, and it catalyzed the transfer of the phosphoryl label to RegR. The phosphorylated form of $RegS_C$ exhibited phosphatase activity on RegR-phosphate. Chemical stability tests and site-specific mutagenesis identified amino acids H219 and D63 of RegS and RegR, respectively, as the phosphorylated residues. Competition experiments with isolated domains demonstrated that the N-terminal but not the C-terminal domain of RegR interacts with $RegS_C$. Band-shift experiments revealed that phosphorylated RegR had at least eight-fold enhanced DNA-binding activity as compared with dephosphorylated RegR or the mutant protein RegR-D63N which cannot be phosphorylated. In conclusion, the RegSR proteins of *B. japonicum* exhibit functional properties *in vitro* that are typical for two-component regulatory systems.

Abbreviations: PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; NTA, nitrilotriacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

2.2. Introduction

Biological nitrogen fixation in diazotrophic bacteria is strictly regulated by environmental conditions. Critical signals controlling synthesis and activity of the nitrogen-fixing apparatus include both low-nitrogen and low-oxygen conditions in free-living diazotrophs whereas "low oxygen" is the predominant signal in symbiotic diazotrophs. In the latter group of organisms, species-specific regulatory circuits consisting of homologous regulators control the expression of numerous genes whose products are either directly involved in nitrogen fixation or in functions associated with the microaerobic life-style in symbiosis (reviewed by Fischer 1994, 1996; Kaminski *et al.* 1998). In *Bradyrhizobium japonicum*, the nitrogen-fixing root-nodule symbiont of soybean, transcriptional control of these genes is mediated by two oxygen-responsive cascades, one composed of the FixLJ and $FixK_2$ proteins and the other of the RegSR and NifA proteins.

The FixLJ-$FixK_2$ cascade responds to low oxygen by the FixLJ two-component regulatory system. The primary target of FixLJ-mediated control in *B. japonicum* is *fixK*$_2$ which encodes another transcriptional regulator required for control of diverse, microaerobically induced functions (Nellen-Anthamatten *et al.*, 1998). The oxygen-responsive component of the RegSR-NifA cascade in *B. japonicum* is the NifA protein which, under microaerobic or anaerobic conditions, activates transcription of many *nif* and *fix* genes in concert with RNA polymerase containing the sigma factor σ^{54}. Oxygen or redox conditions are sensed directly by NifA presumably via a metal cofactor bound to essential cysteine residues (Fischer & Hennecke, 1987; Fischer *et al.*, 1989).

The NifA protein of *B. japonicum* is encoded in the promoter-distal gene of the bicistronic *fixR-nifA* operon (Fig. 2.1.) which is preceded by two disparately regulated promoters (*fixRp*$_1$, *fixRp*$_2$). Low-level expression under aerobic conditions originates from the *fixRp*$_2$ promoter and depends on an upstream DNA region (UAS) located around position −68 (Thöny *et al.*, 1989; Barrios *et al.*, 1995). Under low-oxygen conditions, *fixR-nifA* expression is enhanced approx. fivefold by activation of the σ^{54}- and NifA-dependent *fixRp*$_1$ promoter which overlaps with *fixRp*$_2$. The *fixRp*$_1$-dependent transcript starts only two nucleotides upstream of the major transcript originating from *fixRp*$_2$. The UAS element represents a binding site for a protein

required for activation of $fixRp_2$. We have recently purified this UAS-binding protein and cloned the corresponding gene which we named *regR* (Bauer *et al.*, 1998). An additional gene, *regS*, was identified immediately upstream of *regR* (Fig. 2.1.). Null mutations in the *regR* gene led to a complete loss of aerobic *fixR-nifA* expression, and the mutants also displayed drastically decreased anaerobic *fixR-nifA* expression and nitrogen fixation activity. Furthermore, free-living growth of *regR* mutants under anaerobic conditions with nitrate as the terminal electron acceptor was severely impaired.

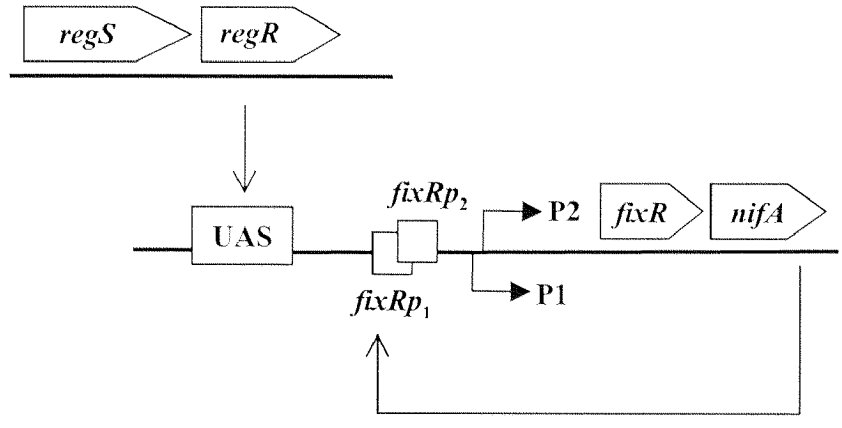


Fig. 2.1. The role of *regSR* in the regulation of the *B. japonicum fixR-nifA* operon and domain structure of the RegSR proteins. Regulatory elements controlling expression of the *fixR-nifA* operon. Horizontal arrows at P1 and P2 indicate the start sites of transcripts that originate from the overlapping promoters $fixRp_1$ and $fixRp_2$, respectively. For further details see text.

The predicted amino acid sequences of RegS and RegR showed that they belong to the family of two-component regulatory proteins. This family consists of sensor proteins that are autophosphorylated at a conserved histidine residue in response to a signal perceived by the N-terminal domain, and response regulators having a phosphorylation site at a conserved aspartate in the N-terminal domain and a proposed helix-turn-helix DNA-binding motif in the C-terminal domain (Stock *et al.*, 1995; Swanson *et al.*, 1994; Egger *et al.*, 1997). These structural features, including the phosphorylation sites H219 and D63, are conserved in the putative sensor RegS and response regulator RegR, respectively. Yet, the signal to which the *B. japonicum* RegSR system responds is presently not known.

Surprisingly, the phenotypic properties of *regS* mutants were largely indistinguishable from the wild type with regard to anaerobic growth and nitrogen

fixation activity. This raised the question whether RegS is a functional histidine kinase capable of autophosphorylation and, if the answer is yes, whether it is able to transfer a phosphoryl group to RegR. Here we report the construction of suitable *regS* and *regR* expression plasmids and the purification of the respective gene products which were then used for *in vitro* phosphorylation studies. In addition, we have analyzed the functional properties of separately expressed RegR domains, and have addressed the effect of RegR protein phosphorylation on DNA-binding activity.

2.3. Materials and Methods

2.3.1. Bacterial strains and growth conditions

Escherichia coli strains DH5α (Gibco-BRL) and BL21(DE3)/pLysS (Studier *et al.*, 1990) were used for routine cloning experiments and for expression of His_6-tagged proteins, respectively. *E. coli* cells were grown in Luria-Bertani medium (Miller, 1972). Where appropriate, selective antibiotics were added at the following concentrations (μg/ml): ampicillin (200), chloramphenicol (20) and kanamycin (30).

2.3.2. Recombinant DNA work and construction of expression plasmids

Routine genetic manipulations were performed as described elsewhere (Sambrook *et al.*, 1989). Nucleotide and amino acid positions correspond to those published by Bauer *et al.* (1998). Plasmid DNA was isolated from *E. coli* strains using Qiagen columns and procedures (Qiagen AG). Plasmid pRJ2803 directs the synthesis of a cytosolic, His_6-tagged C-terminal fragment of RegS (amino acids T183 to G440; $RegS_C$); for its construction, a 846-bp *Bst*EII-*Bam*HI-fragment from pRJ2413 (E. Bauer, unpublished; with the *Bst*EII-site made blunt) was cloned into a *Sca*I-digested derivative of pET28b(+) (Novagen) into which a *Sca*I-linker had been inserted at the blunt-ended *Not*I site. For reasons of clarity, the prefix "H_6-", refering to His_6-tagged derivatives, will hereafter be omitted from all protein designations. Plasmid pRJ2454 overproduces the His_6-tagged RegR protein (amino acids N2 to R184); it was constructed by insertion of a 540-bp *Nde*I-*Bam*HI-Fragment, generated by polymerase chain reaction (PCR) and a 1.1-kb *Bam*HI-*Eco*RI-Fragment from pRJ2403 into pET28a(+) (Novagen). Plasmid pRJ2805 overproduces a His_6-tagged N-terminal fragment of RegR (amino acids N2 to A129; $RegR_N$); it was constructed by insertion of a 399-bp *Nde*I-*Hin*dIII-PCR-fragment generated by into pET28a(+). Plasmid pRJ2807 overproduces a His_6-tagged C-terminal fragment of RegR (amino acids S126 to R184; $RegR_C$); for its construction, a 390-bp *Not*I-*Sal*I-fragment from pRJ2403 was subcloned into the *Sca*I-digested pET28b(+)-derivative described above. Plasmid pRJ2853 overproduces the mutant protein $RegS_C$-H219D; it carries the 905-bp *Xho*I-*Bam*HI-fragment from pRJ2803 that was subcloned into pBluescript II KS+. It was then mutated using the QuikChange site-directed mutagenesis kit (Stratagene GmbH) according to the procedure suggested by the manufacturer. The mutagenic primers regSMut3 (5'-C_{1178}GGCGTGCCGAGTTCATCCGCGGCCGCGGC-3') and

regSMut4 (5'-G_{1207}CCGCGGCCG-CGGATGAACTCGGCACGCCG-3') led to a nucleotide sequence change from C_{1190}ACGAG to GATGAA. The resulting 905-bp *Xho*I-*Bam*HI-fragment carrying the desired mutation was used to replace the corresponding fragment in pRJ2803 yielding plasmid pRJ2853. Plasmid pRJ2825 overproduces the mutant protein RegR-D63N; it was constructed by insertion of two PCR fragments into the *Nde*I-*Bam*HI-digested plasmid pRJ2454 which led to a change from G_{2148}AT to AAC. The first of the two fragments was obtained by *Nde*I-*Hpa*I digestion of a PCR-amplified 216-bp fragment from plasmid pRJ2451 with primers M13 and regRmut2 (5'-G_{2162}CCGAGGCGCAAGTTAACCACGGCG-3'). The second one was generated by *Hpa*I-*Bam*HI digestion of a 408-bp PCR product obtained from the same plasmid template with primers M13rev and regRMut3 (5'-C_{2138}GCCGTGGTTAACTTGCGCCTCGGC-3').

The nucleotide sequence of all DNA regions subjected to *in vitro* mutagenesis was confirmed by using the chain termination method of Sanger (Sanger *et al.*, 1977).

2.3.3. Purification of the His_6-tagged proteins

Freshly transformed cells of *E. coli* BL21(DE3)/pLysS carrying either plasmids pRJ2803, pRJ2853, pRJ2454, pRJ2825, pRJ2805 or pRJ2807 were grown at 37 °C in 1 l LB medium containing kanamycin and chloramphenicol. When the cultures had reached an optical density at 600 nm of 0.8, production of recombinant proteins was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and a decrease of the temperature to 30 °C. Cells were harvested after 2 h, resuspended in 20 ml TEPDM buffer (Bauer *et al.*, 1998) and disrupted in a French pressure cell (Bauer *et al.*, 1998). The lysate was centrifuged at 13,500 rpm in a Sorvall SS-34 rotor for 30 min at 4 °C, and the supernatant was further centrifuged at 25,000 rpm in a Beckman TST 28.38 rotor for 1 h at 4 °C. The supernatant was loaded onto a 3.5 ml Ni^{2+}-nitrilotriacetic acid (NTA) agarose column (Qiagen), and affinity chromatography was performed as specified in the manual of the pET expression system (Novagen) with the following modifications: the concentration of imidazole in the elution fractions was 400 mM for $RegS_C$, $RegS_C$-H219D and $RegR_C$, 300 mM for RegR and RegR-D63N, and 150 mM for $RegR_N$. Protein-containing fractions were desalted and buffer-exchanged by passing them through a prepacked Sephadex G-25 M column (PD-10; Pharmacia) equilibrated with TEDM-buffer (TEPDM buffer without phenylmethyl sulfonyl fluoride). Glycerol (10% [v/v]) was added to the

eluates before storage at –20 °C. Protein concentrations were determined by the Bradford method (Bradford, 1976).

2.3.4. *In vitro* phosphorylation assays

Autophosphorylation of $RegS_C$ was assayed at room temperature in TEDM buffer. $RegS_C$ was diluted to a final concentration of 5 µM, and the reaction was started by addition of [γ-^{32}P]ATP (6 Ci/mmol; Hartmann Analytic GmbH) to a final concentration of 36 µM. Samples were removed at different intervals, the reaction stopped by adding 5 x sodium dodecyl sulfate (SDS) sample buffer (10% [w/v] SDS, 50% glycerol, 300 mM Tris hydrochloride, pH 6.8, 200 mM dithiothreitol, 0.05% bromphenol blue), and the samples were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE), using 14% separating acrylamide gels that were then dried and exposed on a phosphoimager screen (Molecular Dynamics). To test the stability of the phosphorylated sensor, $RegS_C$ (4 µM) was first incubated with [γ-^{32}P]ATP (36 µM) for 10 min. Unlabeled ATP (3.3 mM) was then added, and samples were taken at different times. After stopping reactions with 5 x SDS sample buffer, the samples were subjected to SDS-PAGE and the gels exposed to a phosphoimager screen. To study phosphoryl transfer, $RegS_C$ (5 µM) was first phosphorylated for 10 min in a 60 µl volume in the conditions described above. Subsequently, 25 µl of this reaction were added to RegR (2.5 µM) or $RegR_N$ (2.5 µM) in a final volume of 50 µl. Samples of 4 µl were removed at different times and analyzed by SDS-PAGE and autoradiography. Phosphorylation assays with the $RegS_C$-H219D mutant protein (8.8 µM) and with $RegS_C$ (8.8 µM) were performed in a 40 µl volume applying the phosphorylation conditions described above. Two-µl samples were taken after 5 and 10 min, denatured with 5 x SDS sample buffer and stored on ice. Ten min later, further 25 µl of the reactions were added to RegR (0.5 µM) in a final volume of 50 µl. Samples of 4 µl were removed 1 and 5 min later, reactions stopped with 5 x SDS sample buffer, and all samples were analyzed by SDS-PAGE. The gel was stained with Coomassie Blue and exposed to a phosphoimager screen. To analyze the transfer of phosphoryl label from sensor to regulator, $RegS_C$ (12.9 µM) was phosphorylated for 10 min as described above in a volume of 30 µl. Ten µl of this reaction were then added to RegR (4.3 µM) or RegR-D63N (4.3 µM) in a final volume of 30 µl. Samples were taken 1 and 5 min

thereafter, denatured in 5 x SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

2.3.5. Dephosphorylation assay

$RegS_C$ (3 μM) was incubated with [γ-^{32}P]ATP (150 μM) and RegR (15 μM) for 2.5 h at room temperature in a volume of 30 μl. Subsequently, 5.33 μl were withdrawn and added to preincubated (10 min) solution containing either ATP (1 mM), ATP (1 mM) plus $RegS_C$ (6 μM), or ATP (1 mM) plus RegR (6 μM) in a final volume of 80 μl. Ten-μl samples taken at suitable intervals were denatured, analyzed by SDS-PAGE, and the radioactivity of the RegR~P band was quantified by exposing the dried gel to a phosphoimager.

2.3.6. Chemical stability assays

Radioactively phosphorylated proteins were separated by SDS-PAGE and transferred to Hybond C membranes (Amersham LIFE SCIENCE) by electroblotting. Four identical membranes with phosphorylated $RegS_C$ and RegR bound to them were treated individually for 30 min at room temperature with 1 M HCl, 50 mM Tris-HCl (pH 8.0), 1 M NaOH, or for 15 min at 30°C with 0.8 M NH_2OH (Hokin *et al.*, 1965). The membranes were washed with destilled water, dried and exposed to a phosphoimager screen.

2.3.7. Phosphorylation competition experiments

Solutions containing $RegS_C$ (2 μM) phosphorylated with [γ-^{32}P]ATP (36 μM) for 10 min were prepared, and RegR (2 μM) was then added together with RegR-D63N or $RegR_N$ or $RegR_C$ at different concentrations in a total volume of 15 μl. The reactions were incubated at room temperature. Samples of 5 μl were taken after 1 and 5 min, the reactions were stopped with 5 x SDS sample buffer, and the products were analyzed by SDS-PAGE and autoradiography. Signal intensities were quantified with a phosphoimager and the program "IMAGEQUANT" (Molecular Dynamics).

2.3.8. RegR DNA-binding activity

DNA-binding activity was tested in a gel retardation assay as described previously (Bauer *et al.*, 1998) by using two complementary, HPLC-purified oligonucleotides (5'-GATCC$_{-83}$ATTCCGCGTGCGCGACATTAGGACGCAAA-AC$_{-52}$AGCT-3')

which correspond to the region from –83 to –52 upstream of the transcription start site P2 of the *fixR-nifA* operon (Barrios *et al.*, 1995). The 32-bp double-strand DNA fragment that resulted from appropriate annealing of the 40-mer oligonucleotides has four overhanging nucleotides at both 5' ends, which are not present in the *B. japonicum* UAS. For comparison of the DNA-binding activities of purified RegR and RegR~P, RegR (6 μM) was incubated either alone or with $RegS_C$ (6 μM) and ATP (1 mM) in a volume of 40 μl for 10 min at room temperature. To analyse the DNA-binding activity of dephosphorylated RegR and RegR~P, we incubated RegR (1 μM) alone or together with $RegS_C$ (24 μM) in a volume of 45 μl for 150 min at room temperature. Then 15 μl were withdrawn and ATP (1 mM) or water was added, and the samples were incubated for further 10 min. To compare the DNA-binding activity of RegR~P and RegR-D63N, RegR (3.8 μM) and RegR-D63N (27 μM) were incubated in separate reactions with $RegS_C$ (1.26 μM with RegR and 9 μM with RegR-D63N) and ATP (1 mM) in a volume of 60 μl at room temperature for 30 min. Appropriate samples of the reactions were analyzed for DNA-binding activity in a gel retardation assay.

2.4. Results

2.4.1. Overexpression and purification of the His_6-tagged proteins

Figure 2.2. shows a schematic representation of the RegS and RegR proteins and domains used in this study. Expression plasmids pRJ246 and pRJ2454 were constructed for overproduction and efficient purification of N-terminally His_6-tagged derivatives of the RegS and RegR proteins, respectively. The 22.3-kDa RegR protein synthesized from pRJ2454 was found to be soluble. Overexpression of the full-length *regS* gene resulted in the synthesis of insoluble protein (data not shown). Therefore, on the basis of the previously described RegS domain structure (Bauer *et al.*, 1998), we cloned the 3'-part of *regS* in plasmid pRJ2803 which ought to direct the synthesis of a cytosolic, carboxy-terminal part of RegS carrying an N-terminal His_6-tag ($RegS_C$). The 33.1-kDa $RegS_C$ protein lacks 182 amino acids that constitute the hydrophobic N-terminal domain of wild-type RegS. Using site-directed mutagenesis, we constructed two additional expression plasmids, pRJ2853 and pRJ2825, which directed the synthesis of the mutant proteins $RegS_C$-H219D and RegR-D63N (for details see Materials and Methods). These proteins were produced at levels comparable to the corresponding wild-type proteins. Finally, plasmids pRJ2805 and pRJ2807 were constructed for separate expression of His_6-tagged derivatives of the N- and C-terminal domains of RegR. The 15.6 kDa protein $RegR_N$ synthesized from pRJ2805 corresponded to the first 129 amino acids of RegR with an N-terminal His_6-tag, whereas the 11.9 kDa $RegR_C$ protein produced from pRJ2807 corresponded to amino acids S126 to R184 of the RegR protein with a His_6-tag at the N terminus. All six proteins were overproduced in *E. coli* BL21(DE3)/pLysS using the IPTG-inducible T7 RNA polymerase expression system and purified by Ni^{2+}-NTA affinity chromatography. The protein preparations were >95% pure as judged by SDS-PAGE (data not shown).

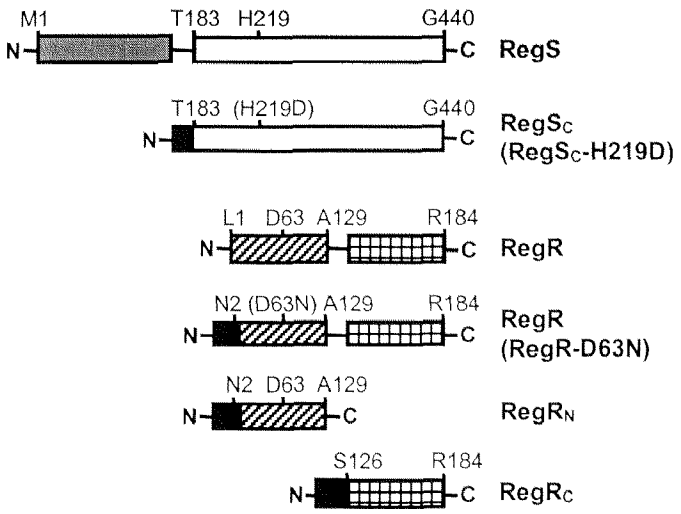


Fig. 2.2. Domain structure of the RegSR proteins. Schematic representation of RegS, RegR and derivatives used in this study. The predicted membane-spanning part and the putatively cytosolic part of RegS are marked by the grey and the white rectangle, respectively. The N-terminal domain of RegR is hatched, the C-terminal domain is cross-hatched. The black box represents the N-terminal His_6-tag.

2.4.2. Autophosphorylation of $RegS_C$ and stability of $RegS_C$~P

To test whether $RegS_C$ possesses autophosphorylation activity, purified $RegS_C$ protein was incubated with radioactive [γ-^{32}P]ATP. $RegS_C$ showed rapid progressive autophosphorylation over an initial period of 30 min followed by a further weak increase during the next 60 min (Fig. 2.3.A). The half-life of $RegS_C$~P, determined after chasing phosphorylated $RegS_C$ with an excess of cold ATP, was approx. 15 min (Fig. 2.3.B).

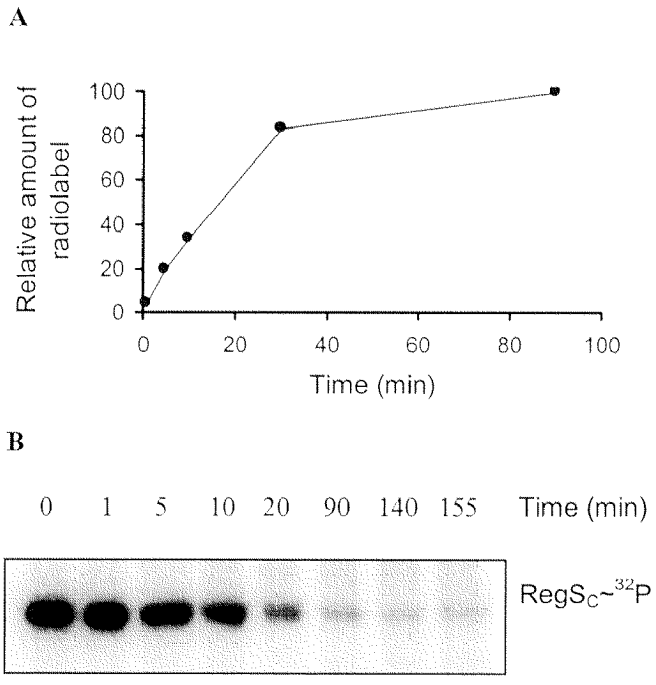


Fig. 2.3. Biochemical analysis of $RegS_C$. (A) Time-dependent autophosphorylation of $RegS_C$. To determine the relative levels of $RegS_C$ phosphorylation over time, $RegS_C$ (5 μM) was incubated with [γ-^{32}P]ATP (36 μM) at room temperature in TEDM buffer. Samples were removed at 1, 5, 10, 30, and 90 min and subjected to SDS-PAGE. Dried gels were analyzed with a phosphoimager. The radioactivity present in the $RegS_C$ band was quantified and presented as relative amount of labeled protein *versus* time. (B) Stability of phosphorylated $RegS_C$. $RegS_C$ (4 μM) was phosphorylated with [γ-^{32}P]ATP (36 μM) for 10 min at room temperature. A 1000-fold exess of unlabeled ATP was then added, and samples were taken at 0, 1, 5, 10, 20, 90, 140 and 155 min. Reactions were stopped with 5 x SDS sample buffer and the samples subjected to SDS-PAGE. Dried gels were analyzed with a phosphoimager.

2.4.3. Phosphotransfer from $RegS_C$~P to RegR and $RegR_N$

The ability of phosphorylated $RegS_C$ to donate a phosphate group to RegR was examined. $RegS_C$ was autophosphorylated with [γ-^{32}P]ATP for 10 min before RegR was added. The results showed that phosphorylation of RegR was detectable already after 15 sec and that it continued over the entire 16-min incubation period after which $RegS_C$~P was almost completely dephosphorylated (Fig. 2.4.A). A control experiment confirmed that RegR was not phosphorylated by [γ-^{32}P]ATP in the absence of $RegS_C$ (data not shown). Next we investigated whether the N-terminal domain of RegR would serve as a substrate for $RegS_C$~P-mediated phosphorylation in an experiment analogous to that performed with intact RegR (Fig. 2.4.B). In fact, $RegR_N$ was phosphorylated as rapidly as RegR, and complete dephosphorylation of $RegS_C$

occured even more rapidly (8 min) than in the reaction with full-length RegR. Hence, the amino acid residue within RegR which is phosphorylated by $RegS_C$ is located in the N-terminal domain.

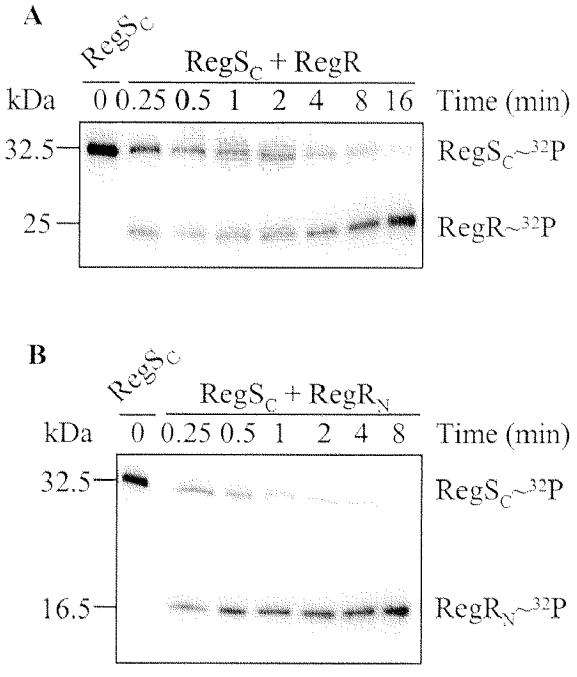


Fig. 2.4. Phosphotransfer from $RegS_C$~P to RegR and $RegR_N$. $RegS_C$ (2.5 μM) was phosphorylated as described in Materials and methods. After 10 min, RegR (2.5 μM) (A) or $RegR_N$ (2.5 μM) (B) was added. Samples were removed at the indicated times and analyzed by SDS-PAGE and autoradiography. Positions of protein size markers are shown on the left, the identity of proteins on the right.

2.4.4. Dephosphorylation of RegR~P

We examined whether $RegS_C$(~P) has dephosphorylation activity on RegR~P by monitoring the release of radioactivity from labeled RegR~P in the presence or absence of $RegS_C$(~P) (Fig. 2.5.). Since a mixture of $RegS_C$ and $RegS_C$~P was present in this experiment we could not distinguish whether the phosphatase activity originates from $RegS_C$ and/or $RegS_C$~P. Addition of $RegS_C$(~P) to prephosphorylated RegR and incubation for 162 min resulted in the loss of more than 90% of the radioactivity associated initially with RegR~P. Addition of ATP led to only a slight decrease of RegR~P presumably due to the phosphatase activity of $RegS_C$(~P) carried over from the preincuabtion step. When a six-fold excess of RegR was added to a control reaction, the radioactivity associated with the RegR band increased probably

as a consequence of the continued phosphotransfer from $RegS_C$~P to RegR. Thus, $RegS_C$(~P) exhibited phosphatase activity on RegR~P under the *in vitro* conditions applied in this experiment.

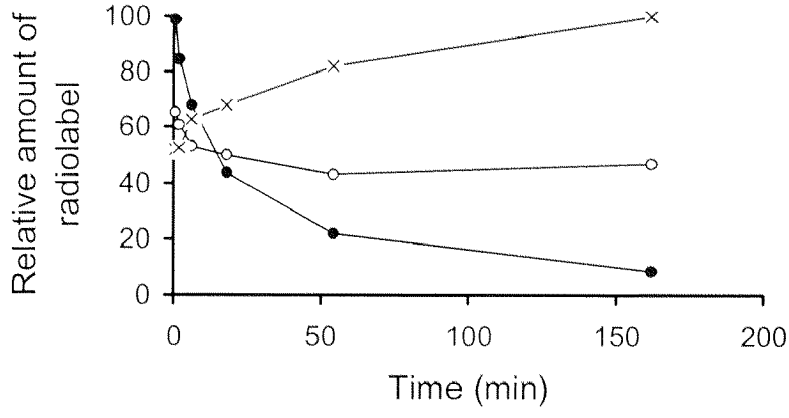


Fig. 2.5. Dephosphorylation of RegR~P by $RegS_C$~P. Phosphotransfer from 0.2 μM $RegS_C$ and 10 μM [γ-^{32}P]ATP to 1 μM RegR was allowed for 2.5 h. Then the reaction was chased with 1 mM ATP alone (○) or together with prephosphorylated 6 μM $RegS_C$ (six-fold molar protein excess; ●) or 6 μM RegR (×). After 0.5, 2, 6, 18, 54 and 162 min samples were denaturated, analysed by SDS-PAGE, the radioactivity present in the RegR~P protein band was quantified with a phosphoimager and presented as relative amount of labeled protein *versus* time.

2.4.5. Assessment of the chemical nature of the $RegS_C$ and RegR phospholigands

Chemical stability tests were performed to characterize the type of amino acid residues that might be phosphorylated in the $RegS_C$ and RegR proteins. In most prokaryotic two-component regulatory systems the sensor kinase is autophosphorylated at a conserved histidine residue, and it transfers the phosphoryl label to a conserved aspartate residue of the response regulator. Phosphoamidates are stable under alkaline conditions but sensitive to acidic conditions, whereas the opposite holds true for acyl phosphates (Hultquist, 1968; Fujitaki & Smith, 1984; Martensen, 1984; Weiss & Magasanik, 1988). Phosphate ester linkages to serine or threonine residues are stable under acidic but not under alkaline conditions, whereas phosphotyrosine linkages are acid- and base-stable (Martensen, 1984). Furthermore, unlike phosphate esters, acyl phosphates and phosphoamidates are susceptible to rapid

aminolysis at pH<5.5 in the presence of hydroxylamine (Hokin *et al.*, 1965; Bitte & Kabat, 1974). As shown in Fig. 2.6., the labeled phosphate group was lost from $RegS_C$~P when the latter was treated either with 1 M HCl or with 0.8 M hydroxylamine at pH 5.5, but it was retained in the presence of 1 M NaOH, which is the characteristic feature of a phosphoamidate. The binding of phosphate to RegR~P was more stable under acidic conditions than under alkaline conditions or in the presence of hydroxylamine, which is characteristic for an acyl phosphate.

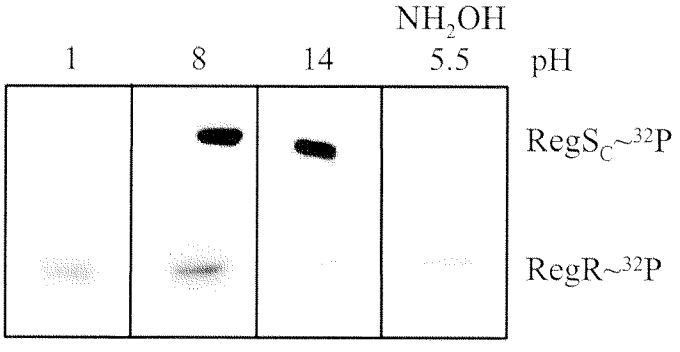


Fig. 2.6. Chemical stability of the phosphate bond in $RegS_C$~P and RegR~P. $RegS_C$ and RegR were phosphorylated as described in Materials and methods. Four identical samples containing a mixture of 150 pmol each of $RegS_C$ and RegR were separated by SDS-PAGE and transferred to Hybond C membranes. The membrane was cut into four strips which were incubated individually for 30 min at room temperature in either 1 M HCl, 50 mM Tris-HCl (pH 8.0), 1 M NaOH or for 15 min at 30°C with NH_2OH. Thereafter, the strips were washed with destilled water, dried and analyzed with a phosphoimager.

2.4.6. Lack of phosphorylation of mutant proteins $RegS_C$-H219D and RegR-D63N

Previous sequence alignments (Bauer *et al.*, 1998) and the stability tests described above strongly suggested that H219 of RegS and D63 of RegR are the residues to be phosphorylated. To test this inference, we have constructed by site-directed mutagenesis the mutant proteins $RegS_C$-H219D and RegR-D63N, respectively. Both proteins were overproduced, purified and analyzed with respect to phosphorylation (Fig. 2.7.). The presence of individual proteins in the samples was confirmed by Coomassie Blue staining of the gels before autoradiography (Fig. 2.7., the two bottom panels). $RegS_C$-H219D did not autophosphorylate and consequently did not transfer a phosphate label to RegR (Fig. 2.7.A, lanes 5-8). The analysis of the phosphotransfer reaction from $RegS_C$ to RegR-D63N and RegR is illustrated in Fig. 2.7.B. No

phosphate label could be transferred to RegR-D63N (lanes 4 and 5). Taken together, these results were in support of H219 of $RegS_C$ and D63 of RegR as the phosphorylated amino acid residues.

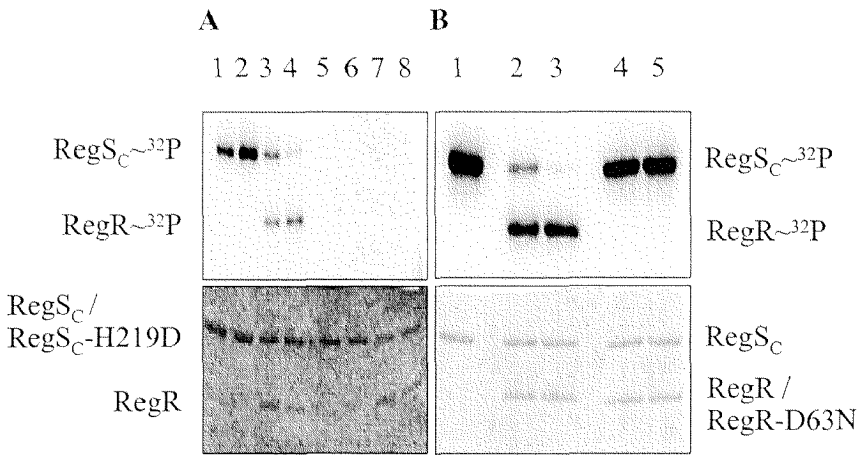


Fig. 2.7. Phosphorylation assays with mutant proteins $RegS_C$-H219D and RegR-D63N. (A) Analysis of $RegS_C$-H219D autophosphorylation. $RegS_C$-H219D (3.5 μM; lanes 5-8) and, as a control, $RegS_C$ (3.5 μM; 1-4) were incubated with [γ-^{32}P]ATP (36 μM), and samples were withdrawn after 1 (1, 5) and 10 min (2, 6). RegR (0.5 μM) was then added to the remainder of the reactions, and additional samples were taken 1 min (3, 7) and 5 min (4, 8) later. (B) Analysis of phosphotransfer from $RegS_C$~P to RegR-D63N. $RegS_C$ (4.3 μM) was phosphorylated for 10 min with [γ-^{32}P]ATP (36 μM) when a sample was taken (lane 1). Then, RegR (2, 3) or RegR-D63N (4, 5; both at 4.3 μM) were added, and samples were analyzed after 1 min (2, 4) and and 5 min (3, 5). Samples taken from the reactions at different times were stopped by addition of 5x SDS sample buffer and subjected to SDS-PAGE. Gels were stained with Coomassie Blue (bottom panels), dried and analyzed with a phosphoimager (top panels).

2.4.7. Does RegR-D63N interact with $RegS_C$?

Competition experiments were performed to probe the ability of mutant protein RegR-D63N to interact with $RegS_C$. We rationalized that the transfer of the phosphate group from $RegS_C$~P to RegR would be decreased if RegR-D63N was able to compete with RegR. To test this idea, $RegS_C$ was autophosphorylated with [γ-^{32}P]ATP before it was added to individual transfer reactions that contained constant amounts of RegR and variable amounts RegR-D63N.

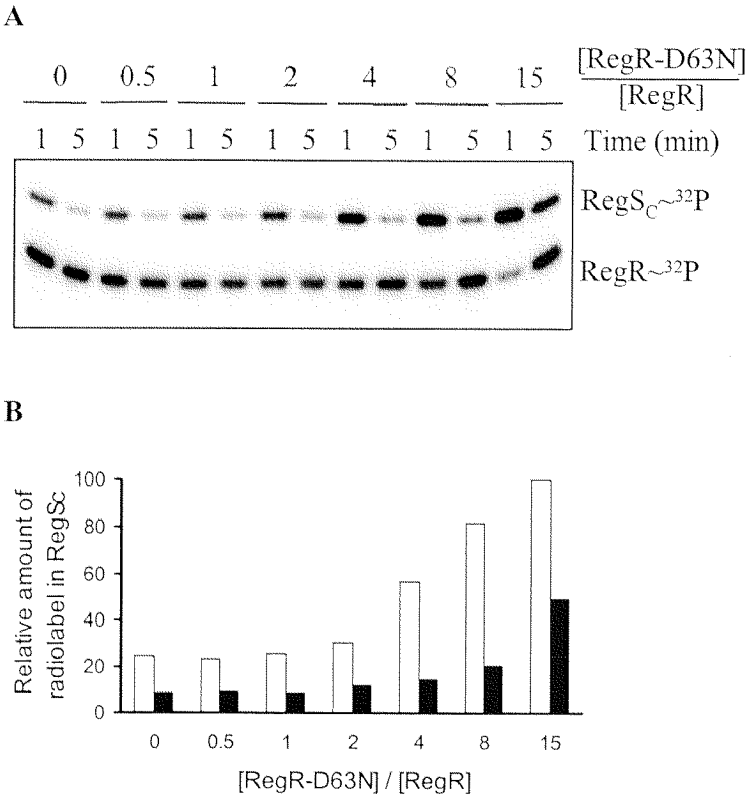


Fig. 2.8. Competitive phosphotransfer from $RegS_C$ to RegR and RegR-D63N. (A) Pre-phosphorylated $RegS_C$ (2 μM) was added to RegR (2 μM) and variable concentrations of RegR-D63N (0, 1, 2, 4, 8, 16, 30 μM) in individual reactions resulting in the ratios indicated. After 1 and 5 min samples were withdrawn, and denatured proteins were analyzed by SDS-PAGE. The radioactivity associated with $RegS_C$ and RegR was determined by autoradiography using a phosphoimager. (B) Quantification of the radioactivity in the $RegS_C$~P protein bands of panel A in samples taken 1 min (open bars) and 5 min (filled bars) after the start of the phosphotransfer reaction.

As shown in Fig. 2.8.A, the radioactivity associated with $RegS_C$ increased in those reactions in which the RegR-D63N-to-RegR ratio was >1. The effect was more obvious in the samples taken after 1 min, but it was also very pronounced in the 5-min samples taken from the reaction that contained a RegR-D63N-to-RegR ratio of 15. At this high RegR-D63N-to-RegR ratio, the phosphotransfer was drastically reduced such that only low amounts of phophorylated RegR protein were detected after 1 min. The result of this experiment, whose quantitative analysis is shown in Fig. 2.8.B, was interpreted to mean that the failure of the RegR-D63N mutant protein to become phosphorylated was not due to a dramatic structural change that might impair interaction with phosphorylated $RegS_C$, but was due rather to the absence of the acceptor amino acid for the phosphoryl group.

2.4.8. $RegR_N$, not $RegR_C$, interacts with $RegS_C$

The experiments described further above demonstrated that D63 in the amino-terminal domain of RegR is the phosphorylated amino acid residue. To analyze which domain of RegR is required for interaction with RegS, we performed phosphotransfer experiments from $RegS_C$ to RegR in the presence of increasing, competing amounts of either $RegR_N$ (Fig. 2.9.A) or $RegR_C$ (Fig. 2.9.B).

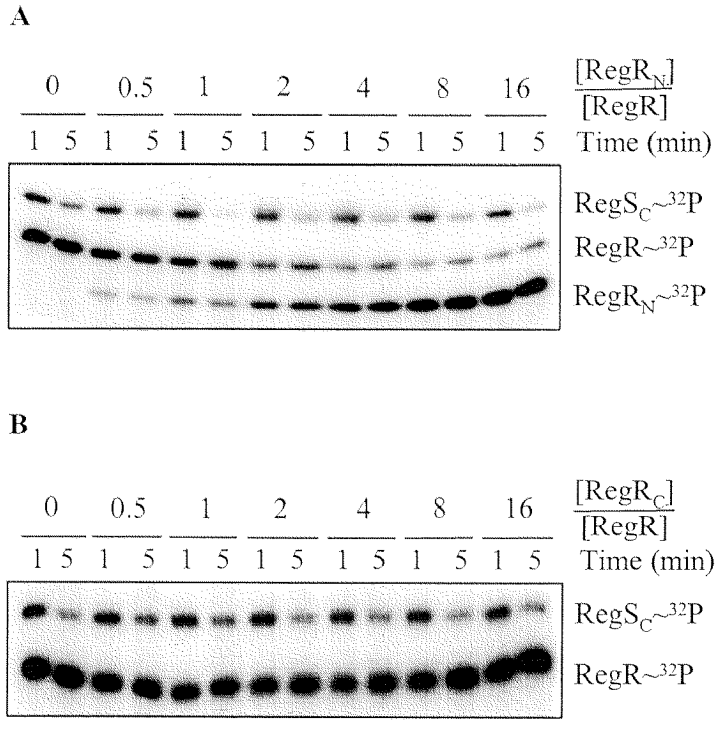


Fig. 2.9. Competition of the phosphotransfer from $RegS_C$ to RegR with $RegR_N$ and $RegR_C$. Pre-phosphorylated $RegS_C$ (2 μM) was added to RegR (2 μM) and variable concentrations of $RegR_N$ (A) or $RegR_C$ (B) (both at 0, 1, 2, 4, 8, 16 or 32 μM). After 1 and 5 min samples were withdrawn, and denatured proteins were analyzed by SDS-PAGE. The radioactivity associated with $RegS_C$, RegR and $RegR_N$ was detected by phosphoimager analysis of the dried gel.

$RegR_N$ was able to interact with $RegS_C$ as indicated by the decrease of radioactivity in the RegR~P band and the parallel increase in the $RegR_N$~P protein, using increasing ratios of $RegR_N$ to RegR (Fig. 2.9.A). By contrast, $RegR_C$ (or bovine serum albumin; data not shown) was not able to compete with the phosphotransfer from $RegS_C$ to RegR as deduced from the constant amount of radioactivity present in the $RegS_C$ protein over the whole range of $RegR_C$-to-RegR ratios tested (Fig. 2.9.B).

2.4.9. RegR~P has stronger DNA binding activity than RegR

The DNA-binding activities of purified RegR and RegR phosphorylated by $RegS_C$ and ATP were tested in gel retardation experiments (Fig. 2.10.A) using a 32-bp double-strand oligonucleotide that spans the *fixR-nifA* promoter-upstream region around position –68. The *fixR*-UAS oligonucleotide was shifted to a retarded electrophoretic mobility by binding of untreated RegR or RegR~P protein (lanes 2-5 and 6-9, respectively). The abundance of the retarded DNA-protein complex was dependent in both cases on the protein concentration used, and strong DNA binding was observed at concentrations ≥0.75 μM. Surprisingly, the DNA-binding activity of untreated RegR protein was only slightly enhanced by phosphorylation. The weak effect of *in vitro* RegR phosphorylation on DNA-binding activity could mean that the RegR protein had already been in a phosphorylated form after isolation from the overproducing *E. coli* strain. To test this hypothesis, we dephosphorylated purified RegR with $RegS_C$, and then rephosphorylated a sample of it in a separate reaction by the addition of ATP. All samples were finally subjected to a gel retardation assay (Fig. 2.10.B). There was no visible complex formation with dephosphorylated RegR (Fig. 2.10.B, lane 3) whereas rephosphorylated RegR showed strong complex formation (lane 4). Quantitative analysis of these results revealed DNA-binding of RegR to be enhanced at least eight-fold by phosphorylation. Thus, RegR phosphorylation strongly favors DNA-binding. Untreated RegR, as isolated from *E. coli* exhibited intermediate DNA-binding activity (lane 2). Finally, we also studied the DNA-binding properties of the mutant protein RegR-D63N (Fig. 2.10.C). In contrast to RegR~P, but similar to dephosphorylated RegR, hardly any complex formation was observed with RegR-D63N even at a concentration as high as 7.25 μM (Fig. 2.10.C, lanes 6-8; see also Discussion). Quantitative analysis revealed that the DNA-binding capacity of RegR~P was at least 15-fold higher than that of RegR-D63N.

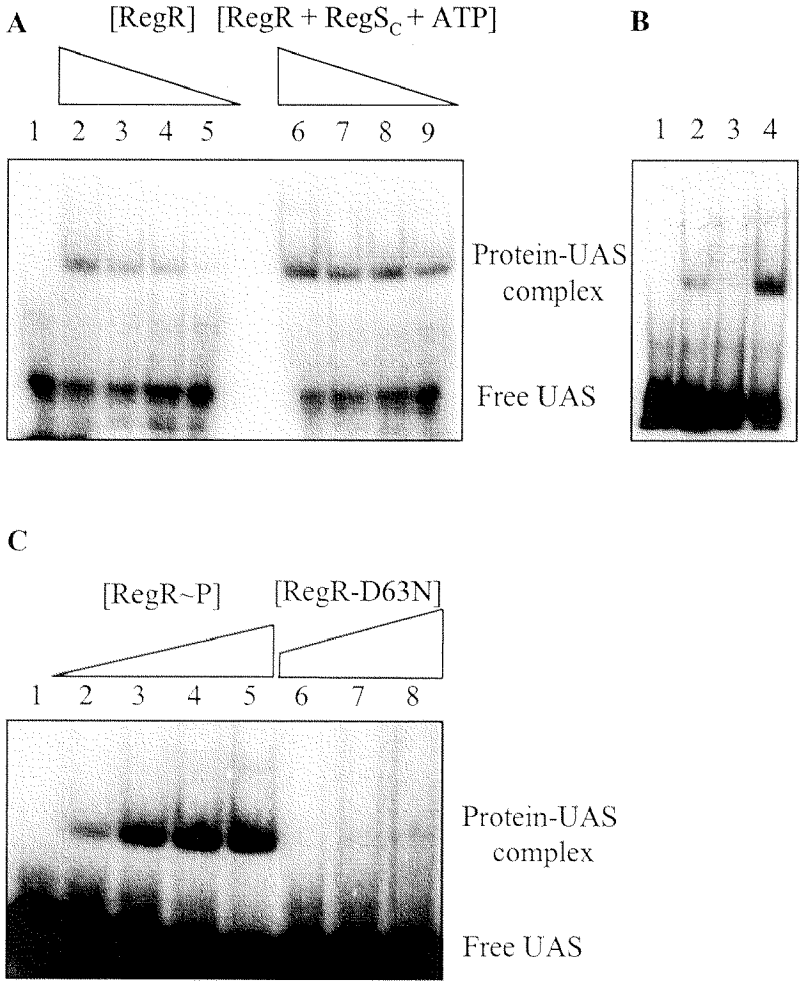


Fig. 2.10. Influence of RegR phosphorylation on DNA-binding. Gel retardation experiments were performed by incubating the protein samples with 0.1 ng of the ^{32}P-endlabeled, double-strand, 32-bp DNA-fragment corresponding to the *fixR-nifA* upstream region around position –68 (UAS) and poly(dI-dC) (1 μg) as nonspecific competitor. After 1 min incubation at room temperature loading dye was added and the samples were separated on a 6% nondenaturing polyacrylamide gel and visualized by phosphoimager analysis of the dried gel. No protein was added to the samples loaded in lane 1 of all panels. (A) Purified RegR (3.0, 1.5, 0.75 and 0.38 μM; lanes 2-5 and 7-10) was incubated alone (2-5) or with RegS$_C$ and ATP (7-10). (B) RegR (0.5 μM; lanes 3, 4) was dephosphorylated by RegS (12 μM; 3, 4). Then ATP (0.5 mM; 4) was added and the samples were analysed for DNA-binding activity. Untreated RegR (0.5 μM) was included as a control (2). (C) Purified RegR (0.38, 0.75, 1.75 and 3.0 μM; lanes 2-5) or RegR-D63N (1.81, 3.63 and 7.25 μM; 6-8) was preincubated with RegS$_C$ and ATP.

2.5. Discussion

This is a biochemical follow-up study on the *B. japonicum* RegS and RegR regulatory proteins which have been characterized previously only by genetic means (Bauer *et al.*, 1998). Upon overproduction and purification of His_6-tagged RegS and RegR derivatives, we have shown here that these proteins indeed exhibit the *in vitro* properties that are typical for two-component regulatory proteins, i.e., autophosphorylation, phosphoryl transfer and specific DNA binding.

RegS had to be purified as an N-terminally truncated derivative because the full-length protein is insoluble probably due to the hydrophobic N-terminal domain that presumably confers upon RegS the ability to associate with the membrane. The same strategy has been applied to overproduce soluble forms of other sensor kinases such as FixL, KdpD and RegB (Gilles-Gonzalez *et al.*, 1991; Nakashima *et al.*, 1993; Inoue *et al.*, 1995). The phosphorylation kinetics of $RegS_C$ and the half-life of $RegS_C$~P are comparable to the corresponding values determined for other sensor kinases. The autophosphorylation rate determined with $RegS_C$ might be an underestimation because membrane-bound KdpD, for example, was shown to be phosphorylated more rapidly than a soluble cytosolic version (Inoue *et al.*, 1995; Nakashima *et al.*, 1992). Presumably, the membane part of sensory kinases is not only involved in stimulus sensing but also contributes to the efficiency of autophosphorylation. Previous amino acid sequence comparisons between RegS and other sensor kinases like ActS of *Sinorhizobium meliloti* (Tiwari *et al.*, 1996), PrrB of *Rhodobacter sphaeroides* (Eraso & Kaplan 1994), and RegB of *Rhodobacter capsulatus* (Mosley *et al.*, 1994) implied that His219 is the phosphorylated amino acid (Bauer *et al.*, 1998). This was now strongly corroborated not only by chemical stability tests, which showed the phospholigand of $RegS_C$~P to be a phosphoamidate, but also by the failure of the $RegS_C$-H219D mutant protein to become phosphorylated.

The phosphoryl group is rapidly transferred from $RegS_C$~P to RegR at a rate similar to that observed in phosphotransfer reactions with other sensor-regulator pairs such as NtrBC (Keener & Kustu, 1988), NarXL (Walker & DeMoss, 1993), EnvZ/OmpR (Aiba *et al.*, 1989). Even the truncated $RegR_N$ protein is phosphorylated, proving that this domain carries the acceptor amino acid. Chemical stability tests with RegR~P suggested, and the amino acid exchange introduced into Reg-D63N finally confirmed, that D63 is the phosphorylated RegR residue as predicted from sequence

alignments (Bauer *et al.*, 1998). Moreover, the failure of Reg-D63N to become phosphorylated documents that, unlike in FixJ of *S. meliloti* (Reyrat *et al.*, 1994), there is no alternative phosphorylation site present in RegR-D63N. Interaction of RegR-D63N with $RegS_C$~P *per se* is not disturbed, as deduced from the ability of RegR-D63N to compete with RegR in the phosphotransfer reaction. These competition experiments as well as the phosphotransfer studies with the isolated N-terminal RegR domain indicate that this portion of RegR contains all of the determinants required for productive interaction with $RegS_C$~P. By contrast, we obtained no evidence for an interaction of $RegR_C$ with $RegS_C$~P. The likely function of the C-terminal RegR domain is DNA binding as inferred from the presence of a presumptive helix-turn-helix motif and its (weak) UAS-binding activity (data not shown).

Several sensor proteins including FixL (Lois *et al.*, 1993), EnvZ (Parkinson & Kofoid, 1992) and NarX (Schröder *et al.*, 1994) dephosphorylate their cognate response regulator. We used a pulse-chase assay to test the possible phosphatase activity of $RegS_C$(~P). In fact, our findings demonstrate that $RegS_C$(~P) catalyzes the release of the phosphoryl group from RegR~P. It should be noted, however, that it is unclear whether $RegS_C$ or $RegS_C$~P (or both) were responsible for this activity. Also, a six-fold molar protein excess of $RegS_C$ was needed, and it thus remains open whether the *in vitro* dephosphorylation activity of $RegS_C$(~P) is of physiological relevance. As mentioned above in connection with the autokinase activity of $RegS_C$, it is possible that the phosphatase activity of full-length RegS is higher than that of the truncated version used in the *in vitro* assay.

The −83 to −52 region of the *fixR-nifA* promoter was shown previously to be required for $fixRp_2$-dependent expression of the *fixR-nifA* operon (Thöny, *et al.*, 1989; Barrios *et al.*, 1995). We have now shown with gel retardation experiments that purified RegR protein specifically binds to that DNA region. The oligonucleotide includes the critical adenosine −68 and also guanosine −75 which Barrios *et al.* (1998) recently reported to be protected from *in vivo* methylation. What these authors have seen, might well be a protection mediated by RegR. Nevertheless, the critical nucleotides required for RegR binding remain to be elucidated. Attractive candidates include the partially overlapping inverted repeats (G_{-77}CGT-N_{13}-ACGC, T_{-74}GCG-N_{11}-CGCA and G_{-71}CGAC-N_5-GACGC) present in the *fixR-nifA* UAS. Interestingly,

the amino acid sequence of the predicted helix-turn-helix motif of RegR corresponds exactly to that of the corresponding motif found in ActR of *S. meliloti* and RegA of *R. capsulatus* proteins (Tiwari *et al.*, 1996; Phillips-Jones & Hunter, 1994). While no target sequence is known for ActR, evidence was recently reported for the interaction of a RegA variant with the promoters of the *puc* and *puf* operons of *R. capsulatus* on the basis of DNase I footprint analyses (Du *et al.*, 1998). Future studies should reveal whether the similarity between these proteins is also reflected by the nucleotide sequence of the DNA binding sites.

Although *in vitro* phosphorylation of RegR enhanced its DNA-binding activity, untreated RegR protein isolated from *E. coli* also showed significant binding activity. It thus appeared as if the RegR protein had been purified as an already partially phosphorylated form. The critical role of RegR phosphorylation was documented by the almost complete lack of DNA-binding activity of dephosphorylated RegR and of the RegR-D63N mutant protein. The competitive interaction of RegR-D63N with $RegS_C$ argues against a drastic structural distortion; yet we cannot formally rule out that the amino acid exchange has altered the overall structure of the mutant protein and, hence, its oligomerisation capacity or DNA binding activity.

Two distinct mechanisms have been proposed for activation of different classes of response regulators by phosphorylation (Egger *et al.*, 1997). In the first class, represented by NtrC and OmpR, phosphorylation of the N-terminal domain induces dimerization of the regulator, which is a prerequisite for transcriptional activation (Fiedler & Weiss, 1995). In the second class, exemplified by FixJ, phosphorylation of the N-terminal domain relieves its intrinsically inhibitory effect on the C-terminal activation domain, thereby promoting DNA binding and activation (Kahn & Ditta, 1991; Da Re *et al.*, 1994). Consequently, in regulators belonging to the latter class, deletion of the N-terminal domain results in constitutively active proteins. Since the *B. japonicum* $RegR_C$ protein exhibited only very weak DNA-binding activity (data not shown) we speculate that RegR of *B. japonicum* belongs to the first class of regulators; however, experimental evidence for RegR oligomerisation and the potential role of phosphorylation in this process is not yet available.

Although we have demonstrated that $RegS_C$ is both a functional autokinase and a kinase, the *in vivo* role of RegS in RegR-mediated transcriptional activation is still unclear. The facts that *regS* is dispensable *in vivo* and that only phosphorylated RegR binds to the UAS *in vitro* could mean that RegR is phosphorylated *in vivo* via cross

talk by another, functionally related kinase or by a low-molecular weight phosphodonor. Assuming that phosphorylation is required for *in vivo* RegR activity, the constitutive basal expression of the *fixR-nifA* operon suggests the permanent presence of at least some phosphorylated RegR protein in *B. japonicum*.

Acknowledgements: We thank A. Nocker for help in the competition experiments. This work was supported by the Swiss National Foundation for Scientific Research and the Federal Institute of Technology Zürich.

Seite Leer /
Blank leaf

Chapter 3

Further biochemical characterization of the RegSR proteins

This chapter summarizes unpublished results from the further biochemical characterization of the RegS and RegR proteins. A hydrophobicity plot of the deduced amino acid sequence of RegS (data not shown) indicated that RegS is a membrane protein. In order to corroborate whether full-length RegS is membrane associated, RegS was overproduced in *E. coli* and the membrane fraction was tested for specific phosphorylation activity (section 3.2.1.). *In vivo* data showed that RegR activates the *fixRp*$_2$ promoter (Bauer *et al.*, 1998). The capacity of RegR to activate *in vitro* transcription from *fixRp*$_2$ was analyzed as described in section 3.2.2. Section 3.2.3. presents additional tools for RegS and RegR characterization, e.g. the construction of a MalE-RegS$_C$ overproduction plasmid and the purification of RegR antibodies. Note that the RegS, RegS$_C$ and RegR proteins used here are His-tagged versions (except MalE-RegS$_C$).

3.1. Materials and Methods

3.1.1. Bacterial strains, plasmids, media and growth conditions

Bacterial strains and plasmids used in chapter 3 are listed in Table 3.1. *E. coli* strain BL21(DE3)/pLysS was used for expression of His$_6$-tagged proteins. *E. coli* cells were grown at 37°C in Luria-Bertani medium (Miller, 1972). Where appropriate, selective antibiotics were added at concentrations described in section 2.3.1.

Tab. 3.1. Bacterial strains and plasmids used in chapter 3.

Strains/plasmids	Relevant genotype or phenotype	Reference
E. coli		
BL21(DE3)/pLysS	F$^-$ Cam^r *ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3)	Studier *et al.*, 1990
Plasmids		
pBluescript SK$^+$ (pSK$^+$)	Ap^r (cloning vector)	Strategene, La Jolla, USA
pET28a(+)	Km^r (expression vector)	Novagen, Abingdon, UK
pMal-c	Ap^r (expression vector)	New England Biolabs, Beverly, USA
pRJ2413	Ap^r (pUR2) *B. japonicum regS* on a 3.1-kb *Eco*RI fragment	Bauer, 2000
pRJ2456	Km^r (pET28a(+)) overexpression of *regS*	K. Panglungtshang, unpublished

(continued)

(Tab. 3.1. continued)

Strains/plasmids	Relevant genotype or phenotype	Reference
pRJ2809	Ap^r (pSK^+) *fixR-nifA* promoter region (–606 to +64) on a 670-bp *Sac*II-*Bam*HI fragment upstream of *rrn*-terminator region	This chapter
pRJ2810	Ap^r (pSK^+) mutated *fixR-nifA* promoter region (–606 to +64; $TG_{-23}CT$) on a 670-bp *Sac*II-*Bam*HI fragment upstream of *rrn*-terminator region	This chapter
pRJ2811	Ap^r (pSK^+) mutated *fixR-nifA* promoter region (–606 to +64; $GC_{-10}AG$) on a 670-bp *Sac*II-*Bam*HI fragment upstream of *rrn*-terminator region	This chapter
pRJ2812	Ap^r (pSK^+) mutated *fixR-nifA* promoter region (–606 to +64; $A_{-66}C$) on a 670-bp *Sac*II-*Bam*HI fragment upstream of *rrn*-terminator region	This chapter
pRJ2813	Ap^r (pSK^+) *fixR-nifA* promoter region (–606 to +64) on a 670-bp *Sac*II-*Bam*HI fragment upstream of *rrn*-terminator region; generates extended transcript (see text)	This chapter
pRJ2830	Ap^r (pMal-c) truncated *regS* on a 2-kb *Bst*EII-*Eco*RI fragment (overproduction of MalE-$RegS_C$)	This chapter
pRJ8133	Ap^r (pUC19) *fixR-nifA* promoter region on a 837-bp *Eco*RI-*Bam*HI fragment	H. M. Fischer, unpublished
pRJ8134	Ap^r (pUC19) mutated *fixR-nifA* promoter region ($TG_{-23}CT$) on a 837-bp *Eco*RI-*Bam*HI fragment	H. M. Fischer, unpublished
pRJ8135	Ap^r (pUC19) mutated *fixR-nifA* promoter region ($GC_{-10}AG$) on a 837-bp *Eco*RI-*Bam*HI fragment	H. M. Fischer, unpublished
pRJ8136	Ap^r (pUC19) mutated *fixR-nifA* promoter region ($A_{-66}C$) on a 837-bp *Eco*RI-*Bam*HI fragment	H. M. Fischer, unpublished
pRJ9519	Ap^r (pSK^+) *rrn*-terminator region	Beck, 1998
pRJ9601	Ap^r (pSK^+) *rrn*-promoter and terminator region	Beck *et al.*, 1997

3.1.2. Western blot analysis

After SDS-PAGE, proteins were electroblotted onto a nitrocellulose membrane (Hybond-C, Amersham, Buckinghamshire, UK). The primary antibodies were used at the indicated dilutions in combination with an anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad Laboratories, Hercules, USA). Tetra-His antibodies were obtained from Qiagen. Bound secondary antibodies were visualized by chemiluminescence using a Western Blotting Kit from Boehringer (Mannheim, Germany).

3.2. Results and Discussion

3.2.1. RegS is membrane associated and active in *E. coli*

The expression plasmid pRJ2456 was constructed for overproducing an N-terminally His_6-tagged derivative of RegS. Membrane fractions from IPTG-induced *E. coli* BL21(DE3)/pLysS/pRJ2456 and *E. coli* BL21(DE3)/pLysS/pET28a(+) cells were prepared similar as published for $RegS_C$ (section 2.3.3.). Unlike for the preparation of soluble proteins, the pellets of the ultracentrifugation, containing the membrane-bound proteins, were resuspended in TEPDM buffer. Fractions of the resuspended membranes and the supernatant after ultracentrifugation were subjected to SDS-PAGE and Western blot analysis (Fig. 3.1.). RegS was detected only in the membrane fraction making it highly suggestive that RegS is a membrane protein.

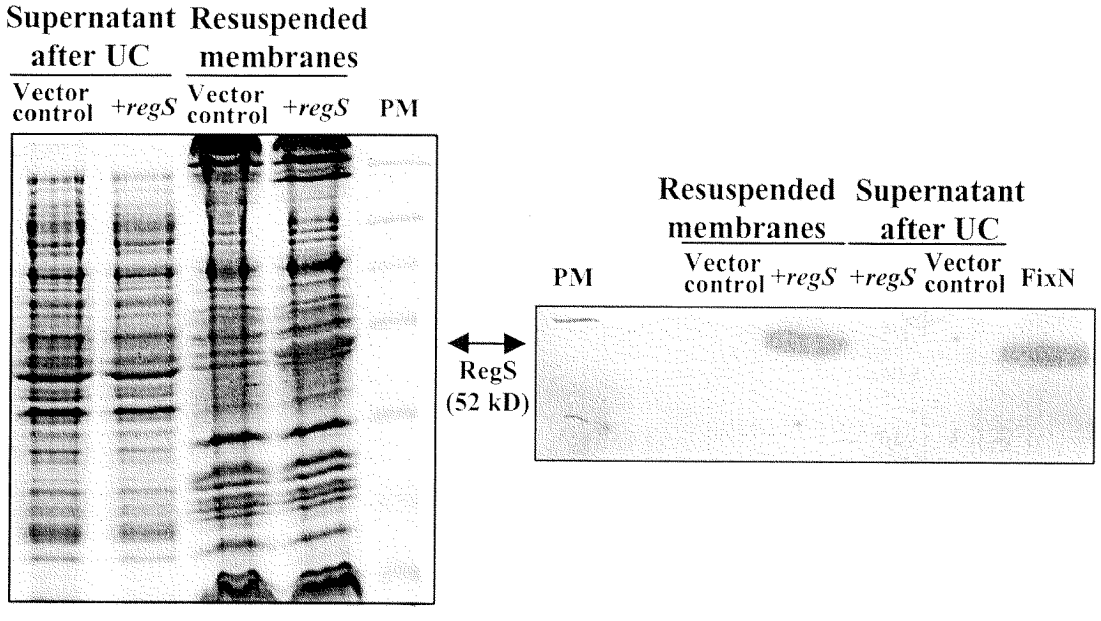


Fig. 3.1. Overproduced RegS is membrane associated in *E. coli*. Resuspended membranes (50 µg total protein) and supernatant after the ultracentrifugation step (UC; 50 µg total protein) of IPTG-induced cells of *E. coli* BL21(DE3)/pLysS with either pRJ2456 (*regS*) or pET28a(+) (vector control) were analyzed by SDS-PAGE (left panel) and Western blot analysis (right panel) using α-His_4 antibodies (0.1 µg/ml). Purified *B. japonicum* His-tagged FixN protein (obtained from E. Arslan; apparent molecular mass 47 kD) was used as a control. PM: protein size marker (175, 83, 62, 47.5, 32.5, 25 and 16.5 kD).

The ability of membrane-bound RegS to autophosphorylate was examined. RegS showed very rapid autophosphorylation with a maximum reached after about 60 s when incubated with [γ-^{32}P]ATP (Fig. 3.2.). The autophosphorylation rate of RegS is faster than that of $RegS_C$ (section 2.4.2.). Presumably, the membrane part contributes

to the efficiency of autophosphorylation (section 2.5.). When purified RegR was added, the phosphoryl label was transferred rapidly from RegS~P to RegR. Taken together, these experiments demonstrate that RegS is inserted in *E. coli* membranes in an enzymatically active state. An unknown phosphorylated *E. coli* protein with a higher electrophoretic mobility than RegS~P was detected in both membrane fractions. This protein seems to be membrane associated rather than tightly bound because it was detected in the membrane fraction as well as in the supernatant (data not shown).

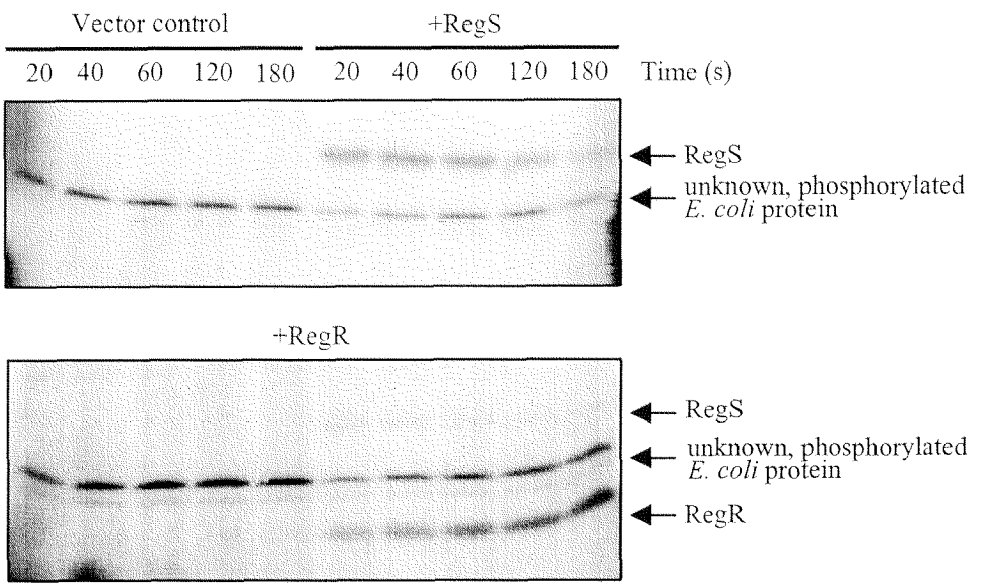


Fig. 3.2. Autophosphorylation of RegS and phosphotransfer from RegS~P to RegR. Resuspended membranes (50 µg total protein) containing (+RegS) or lacking (vector control) RegS were incubated with [γ-^{32}P]ATP (6 Ci mmol^{-1}). In the lower panel purified RegR (8 µM) was added prior to the addition of [γ-^{32}P]ATP. Samples were removed at the indicated times, subjected to SDS-PAGE and analyzed with a phosphorimager.

Kaplan and coworkers postulated that the *cbb*$_3$ oxidase generates a signal which inhibits the PrrBA system in *R. sphaeroides* (O'Gara *et al.*, 1998; Oh & Kaplan, 1999; Eraso & Kaplan, 2000; Oh & Kaplan, 2000). Although *E. coli* does not have a *cbb*$_3$-type oxidase, we analyzed if the general redox-state of the respiratory chain would affect RegS activity. Resuspended membranes (50 µg total protein) containing RegS were either oxidized by adding $KFe(CN)_6$ (10 µM) or reduced with NADH (1 mM). After 10 min, [γ-^{32}P]ATP (6 Ci mmol^{-1}) was added with or without purified RegR (8 µM), and samples were taken at appropriate intervals. Neither the autophosphorylation activity nor the phosphotransfer to RegR was influenced by the

redox state of the membranes (data not shown). *E. coli* possesses two *bd*-type oxidases and one bo_3-type oxidase (reviewed by Richardson, 2000). Presumably, only the cbb_3-type oxidase generates a specific inhibitory signal but not the three *E. coli* oxidases. Therefore, an analogous experiment should be performed with RegS overproduced in *B. japonicum* to analyze the potential role of the cbb_3 oxidase in controlling RegS activity.

3.2.2. Attempts to establish *in vitro* transcription assays with RegR

To analyze if RegR is able to activate transcription from the $fixRp_2$ promoter *in vitro* we performed transcription assays with purified components (RegR, RNA polymerase holoenzyme containing σ^{80} (Beck *et al.*, 1997)). The $fixRp_1$ promoter is activated by the σ^{54} RNA polymerase and NifA. Therefore, we did not expect transcripts originating from P1. Nethertheless we wanted to dissect the two promoters of the *fixR-nifA* operon, and therefore constructed the templates shown in Fig. 3.3. The plasmids pRJ2809, pRJ2810, pRJ2811, and pRJ2812 were constructed by insertion of the 670-bp *Sac*II-*Bam*HI fragments from pRJ8133, pRJ8134, pRJ8135, and pRJ8136 into *Sac*II-*Bam*HI-digested plasmid pRJ9519. The transcript lengths originating from $fixRp_1$- and $fixRp_2$-dependent transcription of these plasmids are expected to be 259 or 257 nucleotides, respectively. Plasmid pRJ2813 carries the wild-type promoters but generates extended transcripts of 263 or 261 nucleotides. It was constructed by filling in the overhanging ends of *Cla*I-digested pRJ2809. RegR-specific transcripts were expected from plasmids pRJ2809, pRJ2810 and pRJ2813 carrying the intact $fixRp_2$. We did not expect RegR-specific transcripts originating from plasmids pRJ2811 and pRJ2812 carrying a mutated $fixRp_2$ promoter or a mutated RegR-binding site, respectively.

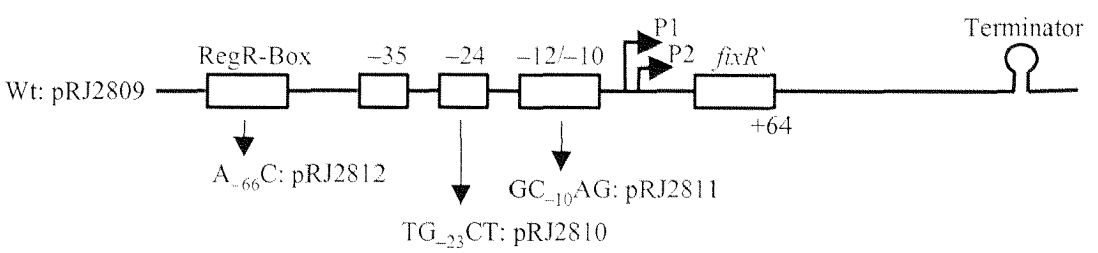


Fig. 3.3. Schematic representations of the plasmids used for *in vitro* transcription assays.

B. japonicum RNA polymerase was purified and the *in vitro* transcription experiments were performed as described by Beck *et al.* (1997). Buffers of purified RegR and $RegS_C$ were exchanged against the buffer utilized for *in vitro* transcription assays using gel filtration columns (Micro Bio-Spin Chromatography Columns P-6, Bio-Rad). In this buffer, RegR and $RegS_C$ remained fully active with respect to the (auto-)phosphorylation activity, yet RegR DNA-binding activity was decreased (data not shown). While the expected transcript resulting from the constitutive *B. japonicum rrn* promoter present on plasmid pRJ9601 and the uncharacterized reference transcript originating from an unknown vector sequence (Beck *et al.*, 1997) was reproducibly obtained, no $fixRp_2$-specific transcripts were observed. Numerous variations of the experimental conditions, i.e., different concentrations of proteins ($RegS_C$, RegR, RNA polymerase), preincubation of RegR with $RegS_C$ and ATP, different amounts of radioactivity or the use of *E. coli* RNA polymerase did not result in the formation of $fixRp_2$-specific transcripts. This could be due to the weak DNA-binding activity of RegR in the *in vitro*-transcription buffer or the absence in the preparation of the RNA polymerase of a specific sigma factor potentially required for recognition of $fixRp_2$. Using a constitutively active mutant variant of *R. capsulatus* RegA, RegA*, Bowman *et al.* (1999) showed by *in vitro* transcription assays that RegA* directly controls the activity of the housekeeping RNA polymerase of *R. capsulatus* at the *puc* and *puf* promoters. Both promoters were activated by RegB-phosphorylated RegA* which was previously shown to bind stronger to its DNA-targets than phosphorylated RegA (Bird *et al.*, 1999). RegA* also activated *in vitro* the *cycA* P2 promoter of the *R. sphaeroides* cytochrome c_2 gene in concert with the σ^{70} (housekeeping) RNA polymerase of *R. sphaeroides* or *E. coli* (Karls *et al.*, 1999). Therefore it seems plausible that the weak DNA-binding activity of RegR is responsible for our failure to detect *in vitro* transcription activity. However, the absence of the correct sigma factor in the *B. japonicum* RNA polymerase preparation used cannot be ruled out because it is possible that RegR and RegA activate their target promoters in concert with different RNA polymerase holoenzymes.

3.2.3. Additional tools for characterization of the RegSR proteins

3.2.3.1. Construction of the expression plasmid pRJ2830 and overproduction of MalE-$RegS_C$

Originally, the hybrid MalE-$RegS_C$ protein was constructed to facilitate the separation of RegR~P from MalE-$RegS_C$~P after the phosphotransfer reaction. Plasmid pRJ2830 directs the synthesis of a MalE-$RegS_C$ hybrid protein. For its construction, a 2-kb *Bst*EII-*Eco*RI fragment from pRJ2413 (with the *Bst*EII site made blunt) was cloned into *Stu*I-*Eco*RI-digested pMal-c. Overproduction of MalE-$RegS_C$ in *E. coli* BL21(DE3)/pLysS was performed as described for $RegS_C$ (section 2.3.3.). MalE-$RegS_C$ was efficiently overproduced as judged by SDS-PAGE (data not shown) and purified using an amylose resin (M. Meyer, personal communication). On the basis of the results from the RegS localization experiments described in section 3.2.1., the separation of RegR~P from RegS could now be achieved also via an alternative strategy, namely by using resuspended membranes containing full-length RegS~P and ultracentrifugation after phosphotransfer.

3.2.3.2. Preparation of α-RegR antibodies

Polyclonal RegR antibodies were generated to detect RegR in cell extracts. Purified RegR (0.5 mg) was subjected to SDS-PAGE and stained with 0.01% Coomassie brilliant blue R250. Gel pieces with the visualized RegR protein were excised and used to immunize a New Zealand white rabbit. The resulting antiserum was purified according to Smith & Fisher (1984). The purified antiserum was used at a dilution of 1:1000 to detect overproduced RegR in *E. coli* extracts (data not shown). However, it was not possible to detect RegR in *B. japonicum* crude extracts. The antiserum did not cross-react with RegA from *R. capsulatus*.

Chapter 4

In vivo properties of RegR-D63N

The failure of the mutant protein RegR-D63N to become phosphorylated *in vitro* was described in chapter 2. To analyze the *in vivo* properties of RegR-D63N, we studied *fixR-nifA* expression in (section 4.2.1.) and the symbiotic phenotype (section 4.2.2.) of a *B. japonicum* strain carrying the respective *regR* mutation (*regR**).

4.1. Materials and Methods

4.1.1. Bacterial strains, plasmids, growth conditions, DNA work and β-galactosidase assays

Bacterial strains and plasmids used in chapter 4 are listed in Table 4.1. *B. japonicum* cells were grown at 30°C in PSY medium (Regensburger & Hennecke, 1983) supplemented with 0.1% (wt/vol) arabinose. Appropriate concentrations of selective antibiotics were added as described (Narberhaus *et al.*, 1997). Routine genetic manipulations were performed as described elsewhere (Sambrook *et al.*, 1989). β-Galactosidase activities of aerobically grown *B. japonicum* cells harboring the *fixR'-'lacZ* fusion were measured as described previously (Thöny *et al.*, 1987).

Tab. 4.1. Bacterial strains and plasmids used in chapter 4.

Strains/plasmids	Relevant genotype or phenotype	Reference
B. japonicum		
7276B	Sp^r Km^r *fixR'-'lacZ* chromosomally integrated	Thöny *et al.*, 1989
2426R	Sp^r Km^r Sm^r *regR*::Ω *fixR'-'lacZ* chromosomally integrated	Bauer *et al.*, 1998
2832R	Sp^r Km^r Sm^r Tet^r *regR*::Ω *regR* fixR'-'lacZ* chromosomally integrated	This chapter
2833R	Sp^r Km^r Sm^r Tet^r *regR*::Ω *regR*$^+$ *fixR'-'lacZ* chromosomally integrated	This chapter
Plasmids		
pBluescript SK^+ (pSK^+)	Ap^r (cloning vector)	Stratagene, La Jolla, USA
pSUP202pol4	Tet^r (pSUP202) *oriT* from RP4	Fischer *et al.*, 1993
pRJ2403	Ap^r (pUC19) *B. japonicum regSR* on a 3.6-kb *Eco*RI fragment	Bauer *et al.*, 1998
pRJ2814	Ap^r (pSK^+) *B. japonicum regSR* on a 2.7-kb *Eco*RI-*Not*I fragment	This chapter
pRJ2825	Km^r (pET28a(+)) *regR** on a 1.7-kb *Nde*I-*Eco*RI fragment	section 2.3.2.
pRJ2827	Ap^r (pSK^+) *B. japonicum regS* and *regR** on a 2.7-kb *Eco*RI-*Not*I fragment	This chapter

(continued)

(Tab. 4.1. continued

Strains/plasmids	Relevant genotype or phenotype	Reference
pRJ2832	Tet^r (pSUP202pol4) *regS* and *regR** on a 1.6-kb *NotI* fragment	This chapter
pRJ2833	Tet^r (pSUP202pol4) *regS* and *regR* on a 1.6-kb *NotI* fragment	This chapter

4.1.2. Construction of a *B. japonicum regR** mutant strain

Plasmid pRJ2814 was constructed by insertion of the 1.1-kb *Eco*RI-*Not*I fragment and the 1.6-kb *Not*I fragment from pRJ2403 into pSK$^+$. The 550-bp *Nde*I-*Bam*HI fragment from pRJ2825 was ligated with the 5.2-kb *Nde*I fragment from pRJ2814, resulting in pRJ2827. The 1.1-kb *Eco*RI-*Not*I fragment from pRJ2827 and the 1.6-kb *Not*I fragment from pRJ2403 were cloned in pSUP202pol4, resulting in pRJ2832. Plasmid pRJ2832 carries *regS* and *regR** under the control of their genuine promoters. Similarly, plasmid pRJ2833 carries *regS* and *regR* under control of their original promoters; for its construction the 1.1-kb *Eco*RI-*Not*I and the 1.6-kb *Not*I fragments from pRJ2403 were inserted into pSUP202pol4. Plasmids pRJ2832 and pRJ2833 were introduced by conjugation (Hahn & Hennecke 1984) into *B. japonicum* strain 2426R (*fixR'-'lacZ regR*::Ω) and chromosomally integrated via homologous recombination yielding *B. japonicum* strains 2832R and 2833R, respectively. The genetic structure of the mutants was verified by appropriate Southern blot hybridizations of chromosomal DNA.

4.1.3. Determination of the leghemoglobin content in soybean root nodules

The preparation of leghemoglobin was performed as described by Appleby & Bergersen (1980). All steps were performed at 4°C. All nodules of a plant were homogenized in a potter with 2 ml of 0.1 M sodium/potassium phosphate (pH 7.4) containing 1 mM EDTA. The turbid red-brown solution was centrifuged for 15 min in an Eppendorf centrifuge at maximal speed. The clarified supernatant was mixed with 1 vol. alkaline pyridine reagent (4.2 M pyridine in 0.2 M NaOH). Leghemoglobin concentration (c_{LB}) was measured spectrophotometrically by recording the absorption difference between sodium-dithionite-reduced and potassium-hexacyanoferrate(III)-oxidized samples at 556 nm and 539 nm, using an extinction coefficient of 23.4 x 10^3 l mol^{-1} cm^{-1}. The leghemoglobin content (mg / g nodules) was calculated using:

leghemoglobin = c_{LB} (mM) x 16000 / (1000 x g nodules),

c_{LB} (mM) = ($\Delta OD_{556} - \Delta OD_{539}$) x 6 / 23.4

4.2. Results and Discussion

4.2.1. Expression of a *fixR'-'lacZ* fusion is strongly reduced in a *B. japonicum regR** mutant

In both mutant strains 2426R and 2832R, expression of the *fixR'-'lacZ* fusion was reduced at least 10-fold as compared with strains 2833R and 7276B (Tab. 4.2.). The high standard deviation of the β-galactosidase activity measured in strain 2832R makes it difficult to judge whether *fixR'-'lacZ* expression is as drastically reduced as in the *regR* mutant strain 2426R or whether it is slightly higher in the former strain. The *fixR'-'lacZ* expression in strain 2833R is 40% lower than in 7276B, possibly because of a reduced *regR* expression in strain 2833R due to the presence of the tetracycline resistance gene which is located downstream of *regR* and oriented oppositely to it. These results show that the RegR-D63N mutant protein, which cannot be phosphorylated, is unable to activate *fixR-nifA* expression to the same level as the wild-type RegR protein. This indicates that phosphorylation of RegR is required for efficient transcriptional activation on the $fixRp_2$ promoter.

Tab. 4.2. Aerobic expression of a chromosomally integrated *fixR'-'lacZ* fusion in a *B. japonicum regR*$^+$, regR$^-$* and *regR background.**

Strain	Relevant genotype	β-Galactosidase activity (Miller Units) [a]
7276B	*fixR'-'lacZ regR*$^+$	783 ± 183
2426R	*fixR'-'lacZ regR*::Ω	8.9 ± 2
2832R	*fixR'-'lacZ regR*::Ω *regR**	40.8 ± 34
2833R	*fixR'-'lacZ regR*::Ω *regR*$^+$	462 ± 47

[a] Numbers are mean values ± standard errors of at least two independent experiments. In each experiment at least two cultures of all strains were grown in parallel and assayed in duplicate. Bacteria were grown in aerobic PSY cultures to mid exponential phase.

4.2.2. Symbiotic phenotype of a *B. japonicum regR** mutant

The ability of the *regR** mutant to nodulate and to fix nitrogen in symbiosis with soybean was examined in a plant infection test (Tab. 4.3.). The mutants elicited about the same number of nodules as the wild type and showed no significant deviations from the nitrogen fixation activity of the wild type (deviations between 50% and 200% of wild-type fixation activities are not considered as significant). No significant alterations in the development of endosymbiotic bacteroids was observed after

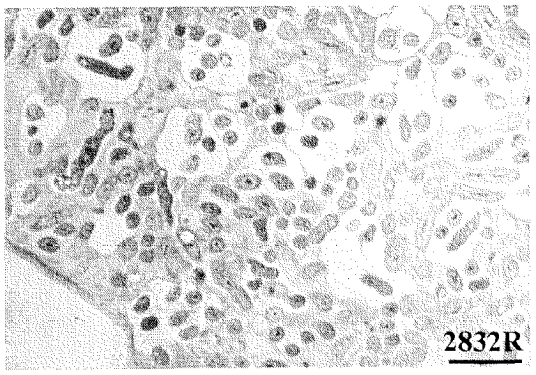
inspection of nodules by electron microscopy (Fig. 4.1.). Thus, we conclude that even though RegR-dependent expression of the *fixR-nifA* operon is reduced in the *regR** background sufficient amounts of NifA can be synthesized to enable microaerobic *nifA* autoactivation under symbiotic conditions, which is critical for an efficient symbiosis.

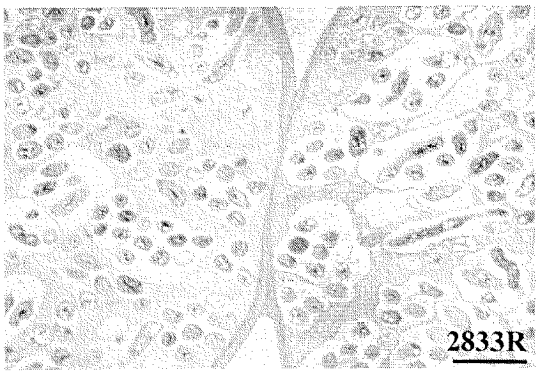
Tab. 4.3. Symbiotic phenotype of *regR mutants.**

		Characteristics [a]		
Strain	Relevant genotype	Number of nodules	Dry weight/nodule (mg)	Fix activity (% of wild type)
7276B	*regR*$^+$	22.6 ± 7	1.0 ± 0.3	100 ± 20
2426R	*regR*::Ω	39.9 ± 8.6	0.4 ± 0.1	0 ± 0
2832R	*regR*::Ω *regR**	31.3 ± 7	0.6 ± 0.1	67 ± 21
2833R	*regR*::Ω *regR*$^+$	23.3 ± 5.7	0.8 ± 0.3	160 ± 54

[a] Numbers are the mean values ± standard errors of at least ten individual plants. Fixation (Fix) activity was measured as the amount of C_2H_2 reduced per minute per milligram of nodule weight (dry weight).

The nodules elicited by the wild type and strain 2833R had a reddish interior. Interestingly, the nodules elicited by the *regR** mutant had the same greenish interior like those elicited by *regR* deletion mutants which are unable to fix nitrogen. To analyze whether the greenish interior of the *regR** mutants was due to reduced amounts of leghemoglobin, we determined the leghemoglobin content photospectrometrically. As shown in Table 4.4., leghemoglobin was detected in all nodule extracts. The amounts of leghemoglobin in nodules elicited by strains 2832R and 2833R are the same. This demonstrates that the greenish interior of the nodules elicited by the *regR** mutant is not due to the lack of leghemoglobin. There is no obvious explanation for this greenish color. One may speculate that the green color arises from higher amounts of biliverdin resulting from an increased degradation rate of leghemoglobin.





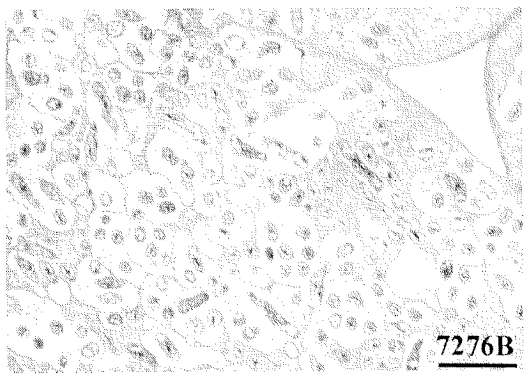


Fig. 4.1. Electron micrographs of infected soybean root-nodule cells. The nodules were elicited by *B. japonicum* strains 7276B (*regR*$^+$), 2832R (Δ*regR regR**) and 2833R (Δ*regR regR*$^+$). Bars = 1.6 μm.

Tab. 4.4. Amount of leghemoglobin in soybean root nodule extracts.

Strain	Relevant genotype	Leghemoglobin (mg / g nodules)[a]
7276B	wild type	4.34 ± 0.2
2832R	Δ*regR regR**	2.52 ± 0.0
2833R	Δ*regR regR*	2.58 ± 0.8

[a] Numbers are the mean values ± standard errors of two individual plants. Values are calculated from difference spectra (reduced minus oxidized) of clarified extracts of nodules (section 4.1.3.).

Acknowledgements: We are grateful to E. Wehrli for analyzing the nodule ultrastructure.

Seite Leer /
Blank leaf

Chapter 5

An imperfect inverted repeat is critical for DNA-binding of the response regulator RegR of *Bradyrhizobium japonicum*

Ralf Emmerich, Philipp Strehler, Hauke Hennecke and Hans-Martin Fischer

in press in *Nucleic Acids Research*

5.1. Abstract

RegR is the response regulator of the RegSR two-component regulatory system in *Bradyrhizobium japonicum*. The only target known so far is the *fixR-nifA* operon, encoding the redox-responsive transcription factor NifA which activates many genes required for symbiotic nitrogen fixation in soybean nodules. In previous *in vivo* studies, we identified a 32-bp upstream activating sequence (UAS) located around position –68, which is essential for RegR-dependent expression of the *fixR-nifA* operon. Here, we used an *in vitro* binding-site selection assay (SELEX) to more precisely define the DNA-binding specificity of RegR. The selected sequences comprised an imperfect inverted repeat (GCGGC-N_5-GTCGC) which is highly similar to an imperfect inverted repeat in the *fixR* UAS (GCGAC-N_5-GACGC). In a parallel approach, band-shift experiments were performed with oligonucleotides comprising defined point or deletion mutations in the *fixR* UAS. This led to the identification of 11 critical nucleotides within a 17-bp minimal RegR binding site centered at position –64 upstream of the *fixR-nifA* transcription start site. Notably, all 11 critical nucleotides were located either within the half sites of the inverted repeat (4 nucleotides in each half site) or in the 5-bp spacer which separates the half sites (3 nucleotides). Based on these results, we defined a DNA motif comprising those nucleotides which are critical for RegR binding (RegR box; 5'-GNG$^{A}_{G}$C$^{A}_{G}$TTNNGNCGC-3'). A comparison of the RegR box with functional bindings sites of the RegR-like regulator RegA of *Rhodobacter capsulatus* revealed considerable similarities. Thus, the RegR box may assist in the identification of new RegR target genes not only in *B. japonicum* but also in other α-proteobacteria possessing RegR-like response regulators.

5.2. Introduction

Regulation of nitrogen fixation in diazotrophic bacteria occurs at different levels including control of gene expression in response to various environmental signals. In most nitrogen-fixing proteobacteria, transcriptional control of genes required for nitrogen fixation is exerted by the NifA protein which activates transcription from –24/–12-type promoters that are associated with many *nif* and *fix* genes and recognized by the σ^{54} RNA polymerase (reviewed by Dixon, 1998; Fischer, 1994). In symbiotic rhizobia such as *Bradyrhizobium japonicum*, the root nodule endosymbiont of soybean, NifA is active only under microaerobic or anaerobic conditions. An increase of oxygen concentration is presumably sensed via a metal cofactor that is coordinated by essential cysteine residues in the NifA protein (Fischer *et al.*, 1989; Fischer & Hennecke, 1987).

NifA of *B. japonicum* is encoded in the bicistronic *fixR-nifA* operon which is under the control of two disparately regulated, overlapping promoters ($fixRp_1$, $fixRp_2$; Barrios *et al.*, 1995; Thöny *et al.*, 1989; Thöny *et al.*, 1987). The $fixRp_1$ promoter belongs to the –24/–12 class, and it is autoactivated only under low-oxygen conditions via NifA which binds to imperfect binding sites loacted approx. 100 nucleotides upstream of the transcription start site (Barrios *et al.*, 1998). The $fixRp_2$ promoter is active under both high and low oxygen conditions, and its activation is dependent on an upstream activating sequence (UAS) located around position –68. A protein which binds to the *fixR* UAS (RegR) was purified and the corresponding gene (*regR*) cloned (Bauer *et al.*, 1998). Null mutations in *regR* abolished aerobic *fixR-nifA* expression and also drastically reduced anaerobic expression. Moreover, those mutants showed only residual nitrogen fixation activity. An additional gene (*regS*) was found to be located upstream of *regR*, and we have recently shown that the products of *regS* and *regR* (RegS, RegR) are functional partners of a classical two-component regulatory system (Emmerich *et al.*, 1999). A purified His-tagged derivative of a soluble, cytoplasmic portion of the sensor kinase RegS ($RegS_C$) autophosphorylated *in vitro* and transferred the phosphoryl group to the His-tagged response regulator RegR. DNA-binding activity of RegR was demonstrated in gel retardation experiments using an oligonucleotide that spans the *fixR-nifA* UAS. Phosphorylation of RegR enhanced its DNA-binding activity substantially. Based on these data we proposed that RegR activates transcription from $fixRp_2$ under both aerobic and anaerobic conditions in

concert with a yet to be characterized RNA polymerase holoenzyme. Notably, the signal sensed and transduced by the RegSR system is also not known yet.

So far, the DNA target required for RegR binding was rather poorly defined by the endpoints of two deletion derivatives of the *fixR* UAS and a point mutation at position –68 (Thöny *et al.*, 1989). Accordingly, we have used in our previous gel retardation experiments a 32-bp oligonucleotide spanning the DNA region between positions –52 to –83 relative to the transcription start site of $fixRp_2$ (Bauer *et al.*, 1998; Emmerich *et al.*, 1999). In this study we have applied a variant of the SELEX strategy originally developed by Tuerk & Gold (1990) in order to learn more about the DNA determinants required for RegR binding. The results from this approach combined with those from binding assays and a series of specifically mutated oligonucleotides enabled us to define a RegR box including 11 critical nucleotides that are part of an imperfect inverted repeat and the intervening spacer.

5.3. Materials and Methods

5.3.1. Oligonucleotides and gel retardation assays

The double-stranded oligonucleotides FRPwt, FRP1 through FRP3, UBP-36M1, UBP-36M2 and P1 through P36, which correspond to the *fixR* UAS and deletion or point mutations thereof, were prepared as previously described (Emmerich *et al.*, 1999; Tab. 5.1.). Note that all oligonucleotides have single-strand overhangs consisting of four nucleotides at the 5' end of both strands. The sequence of the overhang in the top strand of all oligonucleotides is GATC and also in the bottom strand of oligonucleotides UBP-36M1 and UBP-36M2. All other oligonucleotides carry an AGTC overhang at the 5' end of the bottom strand. All oligonucleotides were purchased from Microsynth (Balgach, Switzerland).

The randomized oligonucleotide pool ran-UAS2 was generated by PCR amplification of the 100-bp template oligonucleotide 5'-GGG GGA TCC GTT ACG ATA CGT AAA CGT ACA TAT G$(N)_{32}$ CTG CAG AGT CGT TGG TCA ATC GCA CGA ATT CCC C-3' with primer 5 (forward primer; 5'-GGG GGA TCC GTT ACG ATA CGT AAA CGT A-3') and primer 6 (reverse primer; 5'-GGG GAA TTC GTG CGA TGA CCA ACG ACT-3'). Underlined nucleotides represent restriction enzyme recognition sites that are not present in the *B. japonicum* UAS. They were introduced for cloning purposes. The 100-bp oligonucleotide wt-UAS2 comprising 32 nucleotides between position –83 and –52 of the *fixR*-UAS flanked by 34 nucleotides on both sides was generated by PCR amplification of the template oligonucleotide 5'-CCC GCT AAG CTT TCG ATG TCA AAA CTA TCA TAT GC_{-83}A TTC CGC GTG CGC GAC ATT AGG ACG CAA AAC_{-52} CTG CAG GCA TGC ATA TCC GAA TGC GTC TAG AGG G-3' with primer 3 (5'-CCC GCT AAG CTT TCG ATG TCA AAA CTA T-3') and primer 4 (5'-CCC TCT AGA CGC ATT CGG ATA TGC ATG C-3').

His-tagged RegR protein was purified as reported by Emmerich *et al.* (1999). Labeling of the oligonucleotides and gel retardation assays were performed as described previously (Bauer *et al.*, 1998). Because we demonstrated previously that purified RegR protein is at least partially phosphorylated (Emmerich *et al.*, 1999) no extra *in vitro* phosphorylation step was applied. Intensities of radioactive signals in band shift gels were quantified with a phosphorimager and the program

IMAGEQUANT (Molecular Dynamics). RegR-binding activities were calculated as the ratio between the intensity of radioactive signals of the retarded DNA and the combined intensities of radioactive signals of retarded plus free DNA. Unspecific background noise was substracted from all signal intensities. Relative RegR-binding activities were calculated as the percentage of binding activity of specific oligonucleotides relative to that of the wild-type *fixR* UAS or the DIVS sequence (for dominant *in vitro* selected sequence; see Results). Wild-type *fixR* UAS or the DIVS sequence were present on each band shift gel to enable the calculation of RegR-binding activities independently of the specific experiment.

5.3.1. In vitro binding-site selection assay

Approx. 100 pg of the 5' end-labeled double stranded oligonucleotides ran-UAS2 and wt-UAS2 (approx. 50'000 cpm) were incubated in separate reactions with RegR (1.5-3 μM in the initial four selection cycles and 1.5 μM in later cycles) and subjected to a gel retardation assay. The gel was dried under vacuum on a Whatman 3MM filter and exposed to a phosphorimager screen. The gel region harboring putative ran-UAS2-RegR complexes was localized by comparison with the electrophoretic mobility of the wt-UAS2-RegR complex which was loaded on the adjacent control lane. An appropriate gel slice was excised, rehydrated by adding 250–500 μl of 2 mM ethylenediaminetetraacetic acid and 50-100 μl 3 M Na-acetate, and the DNA was extracted by the freeze-squeeze method (Beutel & Gold, 1992) without the filtration step described in the original protocol. The eluted DNA was amplified by PCR with primers 5 and 6, and the PCR products were purified by preparative 3% agarose gel electrophoresis. DNA fragments of an apparent length of 90 to 140 bp were isolated and subjected to a new cycle of labeling and gel retardation. Surprisingly, up to cycle 6, the major products obtained by amplification of ran-UAS2 had an apparent length of approx. 140 bp whereas those obtained with the control wt-UAS2 had the expected size of 100 bp (section 5.4.). After the 6^{th} cycle, two distinct DNA populations of approx. 100 and 140 bp size were observed with ran-UAS2, and both were processed separately in subsequent cycles. The 100-bp DNA population became dominant in the amplification products of cycles 10 and 11. Individual species of the 100-bp amplification products were cloned by digesting appropriate samples with *Eco*RI and *Bam*HI, ligation with *Eco*RI-*Bam*HI-digested pUC19 vector DNA and subsequent transformation into *Escherichia coli* DH5α using standard cloning procedures

(Sambrook *et al.*, 1989). Plasmid DNA was isolated from individual white colonies grown on Luria-Bertani medium plates (Miller, 1972) supplemented with isopropyl thio-β-D-galactoside (IPTG; 200 $\mu g \cdot mL^{-1}$), 5-bromo-4-chloro-3-indoxyl-β-D-galactoside (X-gal; 20 $\mu g \cdot mL^{-1}$) and ampicillin (200 $\mu g \cdot mL^{-1}$). Inserts were sequenced with the chain-termination method (Sanger *et al.*, 1977) using an ABI PRISM 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA) and M13 and M13reverse standard primers.

5.4. Results

5.4.1. *In vitro* selection of RegR binding sites

From our previous work it was known that purified RegR binds to a 32-bp oligonucleotide corresponding to positions –52 to –83 of the *fixR-nifA* promoter region (Bauer *et al.*, 1998; Emmerich *et al.*, 1999). In order to more precisely define the RegR binding site, we synthesized a population of oligonucleotides comprising 32 randomized nucleotide positions flanked by defined sequences of 34 bp on both sides (ran-UAS2), and used purified RegR protein to select high-affinity DNA targets as described in Materials and Methods. As a control, we used the oligonucleotide wt-UAS2 corresponding to the wild-type UAS flanked by similar border sequences (see Materials and Methods). After 11 cycles of selection, a distinct protein-DNA complex was detected which had the same electrophoretic mobility as the RegR-wt-UAS2 complex (Fig. 5.1.).

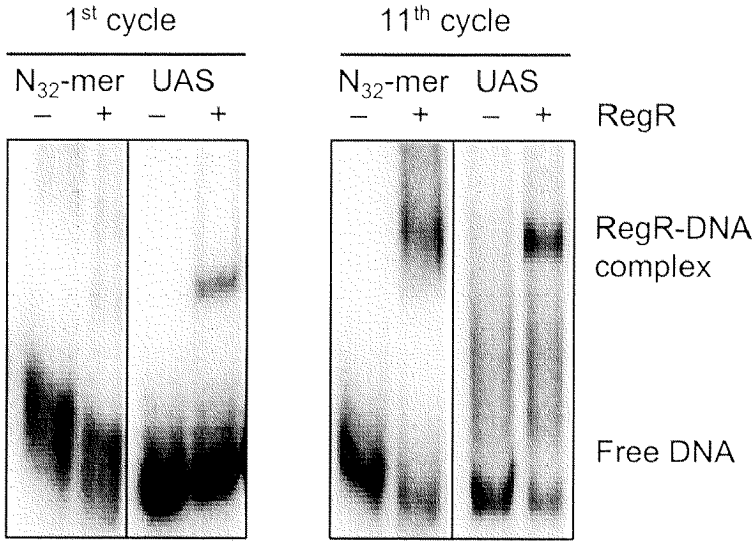


Fig. 5.1. Representative band shift experiments of the 1st and the 11th cycle of the SELEX procedure used for the enrichment of RegR binding sites. Approximately 100 pg (50'000 cpm) of end-labeled oligonucleotides ran-UAS2 (N_{32}-mer) and wt-UAS2 (UAS) were incubated with buffer (–) or with 3 μM (1st cycle) or 1.5 μM RegR protein (11th cycle). After separation on a 6% nondenaturating polyacrylamide gel, the gel was dried and bands were visualized by phosphorimager analysis.

Portions of the retarded DNA from cycles 9, 10 and 11 were amplified, cloned and characterized by DNA sequencing (Fig. 5.2.). Out of a total of 40 clones that were sequenced after the 10th and the 11th cycle, 30 clones had an identical nucleotide

sequence at those 32 positions which were randomized in the original ran-UAS2 pool (hereafter referred to as DIVS sequence [for dominant *in vitro* selected sequence]). Eight additional clones contained a similar sequence with deviations at one or two positions. Two clones contained a totally different sequence which did not bind RegR in subsequent experiments (not shown in Fig. 5.2.). Aligning the DIVS sequence with the *fixR* UAS revealed a total of 16 identical nucleotides most of which are located in the 5' portion of the DIVS sequence. Interestingly, related imperfect inverted repeats consisting of five nucleotides per half site and five intervening nucleotides are present in the region of maximal similarity between the *fixR* UAS and the DIVS sequence (GCGAC-N_5-GACGC [*fixR* UAS]; GCGGC-N_5-GTCGC [DIVS]; Fig. 5.2.). The inverted repeat of the DIVS sequence includes at its 5' end a guanosine residue which was not part of the randomized sequence portion but corresponds to the last position of the defined 5' sequence of ran-UAS2.

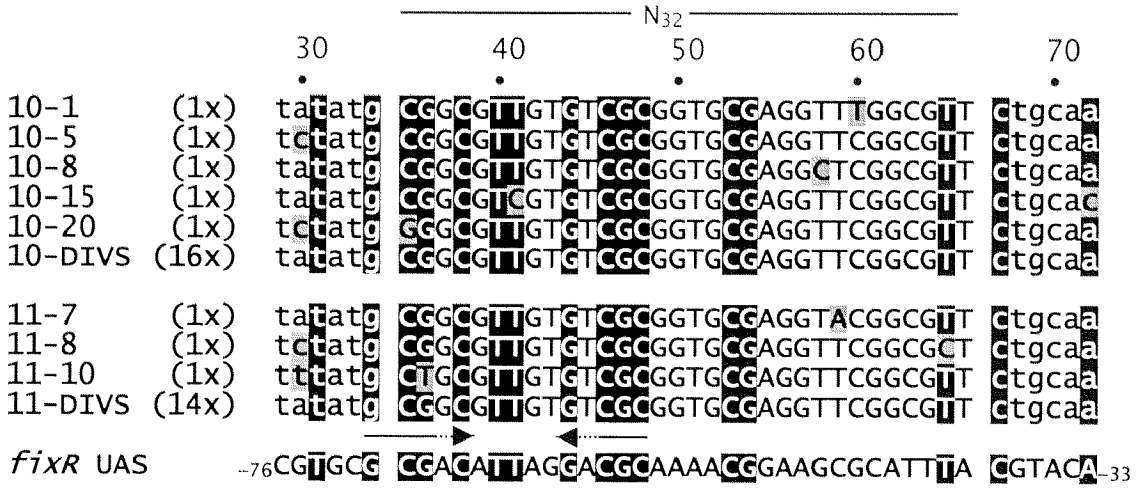


Fig. 5.2. Comparison of the *fixR* UAS with individual oligonucleotides selected after 10 (upper part) and 11 (lower part) cycles of the SELEX procedure. The alignment was created with the GCG "PILEUP" program of the software package (version 10.0) of the UWGCG (Genetics Computer Group of the University of Wisconsin, Madison, WI). Individual oligonucleotides are specified at the left margin with a code and, in parenthesis, with the number of their appearance in a total of 40 clones analyzed after 10 or 11 rounds of selection. Two clones showed a completely different sequence, and thus were not included in the figure. 10-DIVS and 11-DIVS refer to identical sequences which were found most frequently after round 10 and 11. Capital letters denote nucleotides located within the stretch of the 32 originally randomized nucleotides, and lowercase letters mark unique flanking sequences which were not covered by the primers used for amplification. Nucleotides shaded in black are common to all sequences shown while the nucleotides in the selected sequences which are shaded in grey specify positions which deviate from the DIVS sequence. Horizontal arrows mark an imperfect inverted repeat present in the *fixR* UAS and in the DIVS sequence. The numbering of the *fixR* UAS refers to the transcription start site P2 of the *fixR-nifA* operon (Barrios *et al.*, 1998). The numbers indicated at the top refer to position numbers of the 100-bp ran-UAS2 oligonucleotides.

Next we determined the binding activity of RegR for the *in vitro* selected oligonucleotides listed in Fig. 5.2. RegR bound equally well to the dominant DIVS sequence and the wt-UAS2 oligonucleotide comprising the wild-type *fixR* UAS (data not shown). Oligonucleotides 10-15 and 10-20 both carrying two alterations at positions 41 plus 72 and 30 plus 35, respectively, showed a slight reduction (15-20%) in binding activity relative to the DIVS sequence (Fig. 5.3., left panel). Binding of oligonucleotide 11-10 which differs only at position 36 from the DIVS sequence was greatly reduced (approx. 5% residual binding activity; Fig. 5.3., right panel, see also Discussion). RegR binding to the five remaining oligonucleotides 10-1, 10-5, 10-8, 11-7, 11-8 altered at positions 60, 30, 58, 59 and 30 plus 65, respectively, did not differ from binding to the DIVS sequence (data not shown).

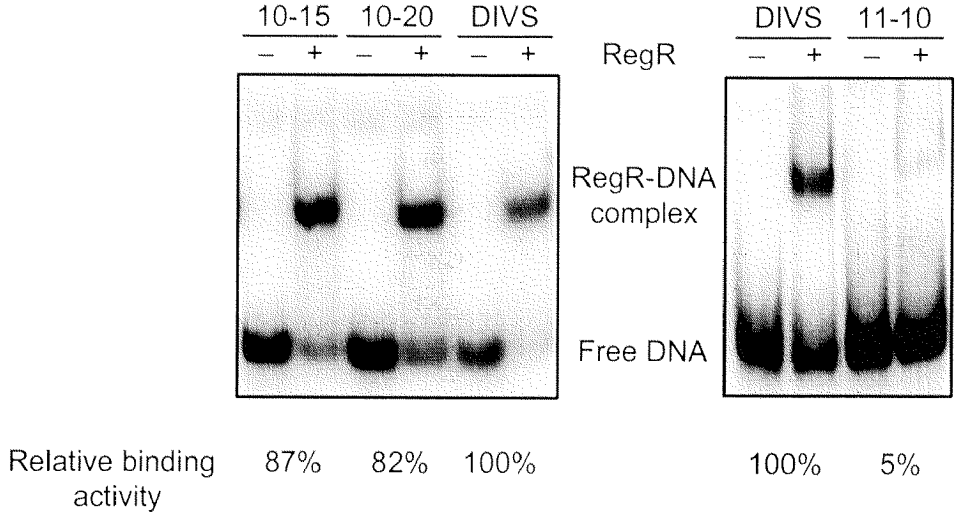


Fig. 5.3. RegR-binding activity of three *in vitro* selected sequences which deviate from the DIVS sequence at one or two positions (see Fig. 5.2.). Approximately 100 pg (50'000 cpm) of end-labeled oligonucleotides 10-15, 10-20, 11-10, and DIVS were incubated with buffer (–) or with 3 μM RegR protein (+). After separation on 6% nondenaturating polyacrylamide gels, the gels were dried and bands were visualized by phosphorimager analysis. Upon quantification of the signal intensities, relative binding activities were calculated as described in Materials and Methods.

To evaluate whether the DIVS sequence was already present after round 9, a portion of the retarded band of cycle 9 was cloned and 18 clones were analyzed by DNA sequencing. Five clones contained the DIVS sequence whereas the remaining 13 clones had other sequences. A compilation of all 18 sequences revealed that 31 of 44 nucleotide positions were biased in favour of the DIVS sequence (data not shown).

5.4.2. Determination of the minimal binding site of RegR

To identify the minimal binding site for RegR, we performed gel retardation experiments using a series of oligonucleotides carrying progressive deletions at one or both ends and determined their relative RegR-binding activity (Tab. 5.1.B). A 17-bp oligonucleotide (P20) still exhibited 70% relative binding activity as compared to the 32-bp reference oligonucleotide FRPwt which corresponds to the wild-type *fixR* UAS. Deletion of one additional nucleotide at each end (P24) abolished RegR binding almost completely. On the basis of these results we concluded that the minimal RegR-binding in the *fixR* UAS comprises 17 nucleotides from A_{-56} to C_{-72}.

5.4.3. Identification of nucleotides critical for RegR binding

To determine those nucleotides that are critical for binding of RegR to the minimal 17-bp element, we first performed gel retardation experiments with oligonucleotides comprising multiple exchanges at several (3 to 12) positions (FRP1, FRP2, FRP3, P5, P11 and P12; Tab. 5.1.C). All of these oligonucleotides were severely impaired in RegR binding. Next, we used oligonucleotides carrying single nucleotide exchanges. Oligonucleotides P7, P9, P13, P14, P15, P17 representing transversions G_{-71} to T, G_{-69} to T, A_{-66} to C, T_{-65} to G, T_{-64} to G, G_{-61} to T, respectively, showed strongly reduced relative binding activities between 0 and 29%. Relative binding activities between 32% and 51% were observed with oligonucleotides UBP-36M1, P28, P31, P10, P32 (A_{-68} to C, C_{-67} to A, C_{-59} to A, G_{-58} to A, C_{-57} to A). Exchanges at positions –73 (P6), –72 (P26), –70 (P27), –63 (P29), –60 (P30) and –56 (P33) had little effects on RegR binding. The transversions A_{-68} to C and A_{-66} to C resulted in a significant decrease of RegR-binding activity. In the DIVS sequence, guanosines are present at the positions corresponding to A_{-68} and A_{-66} in the *fixR* UAS. We therefore determined the binding activity of RegR to oligonucleotides P34, P35 and P36 carrying the transitions A_{-68} to G, A_{-66} to G and A_{-68} to G plus A_{-66} to G, respectively. RegR exhibited strong binding activity to all three oligonucleotides, which is in perfect accordance with the binding of RegR to the DIVS sequence. Based on these results, we defined a motif comprising the nucleotides critical for RegR binding as "RegR box" (5'-GNG$^{A}_{G}$C$^{A}_{G}$TTNNGNCGC-3'; Tab. 5.1.E). The RegR box comprises 15 nucleotides of which 11 are critical for RegR binding and eight form an imperfect inverted repeat.

Tab 5.1. Effect of mutations in the *fixR* UAS on RegR-binding activity. [a]

Oligonucleotides used in gel retardation experiments with RegR [b, c]		Relative RegR-binding activity [%]
Code	Structure [d]	
(A)	-80 -70 -60	
FRPwt	CATTCCGCGTGCGCGACATTAGGACGCAAAAC-52	100
(B)		
P1	----------ΔΔΔΔ	80
P2	----------ΔΔΔΔΔΔ	8
P3	-ΔΔΔΔΔ----------	104
P4	-ΔΔΔΔΔΔΔΔ----------	61
P18	-ΔΔΔΔΔΔΔΔ----------ΔΔΔΔ	99
P20	-ΔΔΔΔΔΔΔΔΔΔ----------ΔΔΔΔ	70
P24	-ΔΔΔΔΔΔΔΔΔΔΔΔ----------ΔΔΔΔΔ	4
P25	-ΔΔΔΔΔΔΔΔΔΔΔΔΔ----------ΔΔΔΔΔΔ	2
(C)		
FRP1	----------CTGA----------AATC----	10
FRP2	-Δ----------AATCACGGCTTC--------	0
FRP3	-Δ------CTGA----------	10
P5	----------AATC----	6
P11	----------CGG----------	10
P12	----------CTTC--------	1
UBP-36M2	--------T----------	167
P8	----------T-T----------	0
P6	----------T----------	78
P26	----------A----------	101
P7	----------T----------	5
P27	----------A----------	96
P9	----------T----------	3
UBP-36M1	----------C----------	40
P34	----------G----------	137
P28	----------A----------	32
P13	----------C----------	0
P35	----------G----------	139
P36	----------G-G----------	113
P14	----------G----------	29
P15	----------G----------	2
P29	----------C----------	110
P16	----------T----------	354
P17	----------T----------	11
P30	----------C--------	72
P31	----------A--------	46
P10	----------A----------	37
P32	----------A----------	51
P33	----------C----	96
(D)		
P21	-ΔΔΔΔΔΔΔΔΔΔ----------T----ΔΔΔΔ	100
P22	-ΔΔΔΔΔΔΔΔΔΔT----------T----ΔΔΔΔ	111
P23	-ΔΔΔΔΔΔΔΔΔΔT---------T-T----ΔΔΔΔ	155
P19	----------T----------T--------	302
(E)		
RegR box	GNG$^{A}_{G}$C$^{A}_{G}$TTNNGNCGC	

[a] Oligonucleotides were incubated with 3 μM RegR and separated on a 6% nondenaturating polyacrylamide gel. The RegR-binding activity of individual oligonucleotides was determined as described in Materials and Methods, and it is indicated as percentage of the binding activity of the FRPwt oligonucleotide (A) which corresponds to the wild-type *fixR* UAS.
[b] Oligonucleotides are listed in the following groups: (A), wild-type *fixR* UAS serving as a reference in all band shift assays; (B), deletion derivatives used for determination of the minimal RegR-binding site; (C), oligonucleotides used for determination of positions critical for RegR binding; (D), oligonucleotides carrying an improved inverted repeat. Deletions (Δ) and exchanges are indicated in the mutant oligonuceotides while unaltered positions are symbolized by a dash; (E), RegR box.
[c] Numbering of positions in (A) refers to the transcription start site P2 of the *fixR-nifA* operon (Barrios *et al.*, 1998).
[d] Single nucleotide exchanges, which resulted in more than 48% reduction of RegR-binding activity are shaded in black.

5.4.4. Improvement and/or extension of the imperfect inverted repeat enhances the RegR-binding activity

Two mutations (G_{-75} to T in UBP-36M2 and G_{-62} to T in P16) strongly increased RegR binding. The G_{-62} to T transversion in P16 results in the addition of a sixth pair of complementary nucleotides at the proximal borders of the imperfect inverted repeat. Similarly, the G_{-75} to T mutation in UBP-36M2 extends the complementarity of the half sites at their distal ends (positions –75/–74 and –54/–53). The additional complementary positions, however, are separated by two non-complementary positions (positions –73/–72 and –56/–55) from the core elements of the imperfect inverted repeat. These observations prompted us to study in more detail the effects of improved complementarity between the half sites of the imperfect inverted repeat on RegR binding with the help of the oligonucleotides listed in section D of Tab. 5.1. RegR-binding activity was progressively increased by step-wise improving and extending the complementarity of the imperfect inverted repeat in the minimal RegR-binding site (cf. oligonuclotides P20, P21, P22 and P23 in Tab. 5.1.). Maximal enhancement of RegR-binding (approx. 3-fold) was observed when the optimized inverted repeat consisting of 6 nucleotides per half site was flanked by the genuine sequences present in the *fixR* UAS (oligonucleotide P19).

5.5. Discussion

In the present study, we have defined the binding site for the response regulator RegR from *B. japonicum* by applying two different *in vitro* approaches: (i) a SELEX strategy by which a RegR-binding site was selected from a pool of randomized oligonucleotides, and (ii) directed mutagenesis of the *fixR* UAS, the only RegR-binding site known so far in *B. japonicum*.

The SELEX strategy yielded a dominant oligonucleotide species (DIVS sequence) with RegR-binding abilities comparable to the *fixR* UAS and eight additional species of different RegR-binding activities, which deviated from the DIVS sequence at one or two positions. Surprisingly, oligonucleotide 11-10 showed only very weak RegR-binding activity although it apparently passed through 11 cycles of selection. It is possible, however, that the point mutation which distinguishes this oligonucleotide from the DIVS sequence was introduced during the PCR amplification after the final round of selection. Similarly, PCR errors are the likely cause of the nucleotide sequence variability detected at positions 30 and 72 which both belong to the region with a defined sequence in the original pool of oligonucleotides (ran-UAS2). After all, PCR errors may have stimulated the SELEX procedure by increasing the complexity of the oligonucleotide pool.

The functional role of the highly similar imperfect inverted repeats present in the *fixR* UAS and the DIVS sequence ($GCG^{A}_{G}C\text{-}N_5\text{-}G^{A}_{T}CGC$) was studied by analyzing the RegR-binding activity of a collection of oligonucleotides carrying progressive deletions at their borders or nucleotide exchanges. The minimal-length oligonucleotide still exhibiting RegR-binding activity (P20) perfectly coincided with the location of the inverted repeat. On the basis of our mutational analysis, we conclude that the half sites of the imperfect inverted repeat are necessary but not sufficient for RegR-binding. Critical nucleotides also include the spacer region between the two half sites as documented by the effects of exchanges at positions A_{-66}, T_{-65}, and T_{-64}. The combined results from the SELEX approach and the directed mutagenesis led us to define the RegR box (5'-$GNG^{A}_{G}C^{A}_{G}$TTNNGNCGC-3'). Formally, this definition of the RegR box should not be regarded as invariant because certain individual positions were not exchanged by all three alternative nucleotides. The type of exchange may indeed be important as documented by our finding that A to G transitions at positions

–68 and –66 did not affect RegR binding whereas A to C transversions at these positions strongly reduced it.

In previous studies, the role of the *fixR* UAS had been studied *in vivo* by genomic footprinting (Barrios *et al.*, 1998) and mutagenesis (Thöny *et al.*, 1989). Remarkably, G_{-75} which was protected from *in vivo* methylation is not part of the minimal RegR-binding site and its mutation does not interfere with RegR binding *in vitro*. This could mean that RegR protects its target DNA to a larger extent than is required for binding. Conversely, no protection of guanosins at positions –71, –69, –62, –61 and –58 was observed even though exchanges at these positions affected RegR binding to a variable extent. It seems obvious, however, that contacts of RegR with critical nucleotides does not automatically prevent their modification with the small molecule dimethyl sulphate used for *in vivo* methylation. Alternatively, RegR could become re-positioned on the DNA *in vivo* by other transcription factors. An A_{-68} to C mutation reduced *fixR-nifA* expression by more than 90% in aerobically grown cells. By contrast, oligonucleotide UBP-36M1 which carries the very same exchange still exhibited considerable RegR-binding activity (40%). This may indicate that RegR-mediated transcriptional activation *in vivo* requires strong DNA-binding. Alternatively, it is possible that the A_{-68} to C mutation, though it still enables DNA binding by RegR, affects productive interaction of RegR with the RNA polymerase, e.g., by altering the topology of the protein-DNA complex.

B. japonicum RegR is highly similar to RegA, the response regulator of the *Rhodobacter capsulatus* RegBA two-component system (80% similarity; reviewed by Sganga & Bauer, 1992; Bauer *et al.*, 1999). Most notably, a C-terminal region of 23 amino acids comprising the putative helix-turn-helix motif is completely conserved in the two proteins suggesting that they bind to comparable DNA target sequences. In fact, we recently demonstrated that RegR binds to oligonucleotides comprising binding sites for the constitutively active RegA variant, RegA* (chapter 7). The RegA-binding sites in the promoter regions of the *R. capsulatus puc-*, *puf-* and *senC-regA-hvrA*-operons and of the *Rhodobacter sphaeroides cycA* gene had been characterized by DNase I footprinting (Mosley *et al.*, 1994; Du *et al.*, 1998, 1999; Karls *et al.*, 1999). Inspection of the regions protected by RegA indeed revealed similarities to the RegR box (Fig. 5.4.). All RegA-binding sites include an inverted repeat which differ from each other by the length of the half sites (3 to 8 bp) and the extend of the intervening spacer DNA (1 to 7 bp). The variations in the spacing

between half sites may indicate that RegR and RegA can bind to one half site only or that they bind as monomers. Moreover, it should be noted that unlike all other examples listed in Fig. 5.4. the promoter of *R. capsulatus senC* is negatively controlled by RegA, which may be reflected by a distinct architecture of the corresponding binding site. The half sites of the inverted repeats exhibit variable but substantial sequence similarity to the RegR box which is most conserved in the *R. capsulatus puc* and *puf* promoter regions. It seems well possible that optimal DNA-binding sites for RegR and RegA show subtle differences even though both proteins share identical helix-turn-helix motifs.

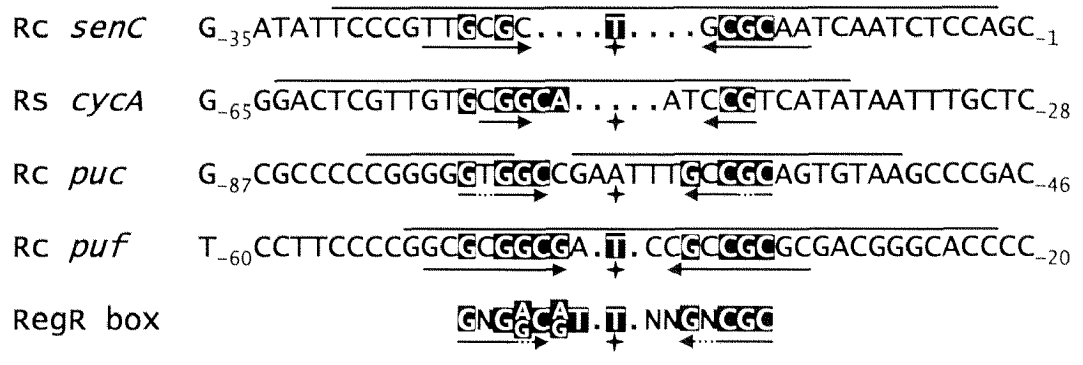


Fig. 5.4. Comparison of the *B. japonicum* RegR box and known DNA-binding sites of *R. capsulatus* RegA*, a constitutively active variant of RegA (Du *et al.*, 1998). Black horizontal lines mark the DNA regions upstream of the *R. capsulatus puf-* (Rc *puf*), *puc-* (Rc *puc*), and *senC-regA-hvrA*-operons (Rc *senC*) and of the *R. sphaeroides cycA* gene (Rs *cycA*) which are protected from DNase I attack by RegA* (Du *et al.*, 1998; Du *et al.*, 1999; Karls *et al.*, 1999). Position numbers are indicated relative to the transcription start site of the respective gene or operon. Nucleotides shaded in black are identical in the RegR box and in the RegA*-binding sites. Horizontal arrows denote the half sites of (imperfect) inverted repeats centered at the positions marked by a plus sign. Gaps were introduced manually to align the center of the inverted repeats.

In conclusion, we believe that the RegR box defined in this work will assist in the identification of new binding sites for RegR in the genome of *B. japonicum* or even for RegR homologues in other α-proteobacteria such as ActR of *Sinorhizobium meliloti* (Tiwari *et al.*, 1996), PrrA of *Rhodobacter sphaeroides* (Eraso *et al.*, 1994) and RegA of *R. capsulatus*, *Rhodovulum sulfidophilum* and *Roseobacter denitrificans* (Inoue *et al.*, 1995; Masuda *et al.*, 1999) all of which possess identical helix-turn-helix DNA-binding domains.

*Acknowledgements:*We thank Enrique Morett and Humberto Barrios for providing plasmids containing mutated RegR-binding sites, which were used in initial experiments.We also appreciate the valuable comments of an anonymous reviewer. This work was supported by the Federal Institute of Technology Zürich.

Seite Leer /
Blank leaf

Chapter 6

Identification of new RegR-binding sites

6.1. RegR binding to a RegR box in front of the *regR* gene

In order to find new potential RegR target genes, we searched the sequenced symbiotic region of *B. japonicum* (Göttfert *et al.*, manuscript submitted) for RegR box-like elements. A potential RegR-binding site showing high similarity to the RegR box defined in section 5.4.3. was found in the *regR* promoter region located between *regS* and *regR* (Fig. 6.1.A). Gel retardation experiments revealed that RegR binds specifically to an oligonucleotide comprising nucleotides –90 to –59 upstream of the *regR* transcription start site (Fig. 6.1.B). RegR showed higher binding activity to the *regR*-oligonucleotide than to the *fixR*-oligonucleotide. This observation can be explained by the fact that the RegR box-like element in front of the *regR* promoter carries a perfect inverted repeat whereas the RegR box of the *fixR-nifA* operon comprises an imperfect inverted repeat. Previously, it was shown that improvement of the imperfect inverted repeat enhances RegR binding activity (section 5.4.4.).

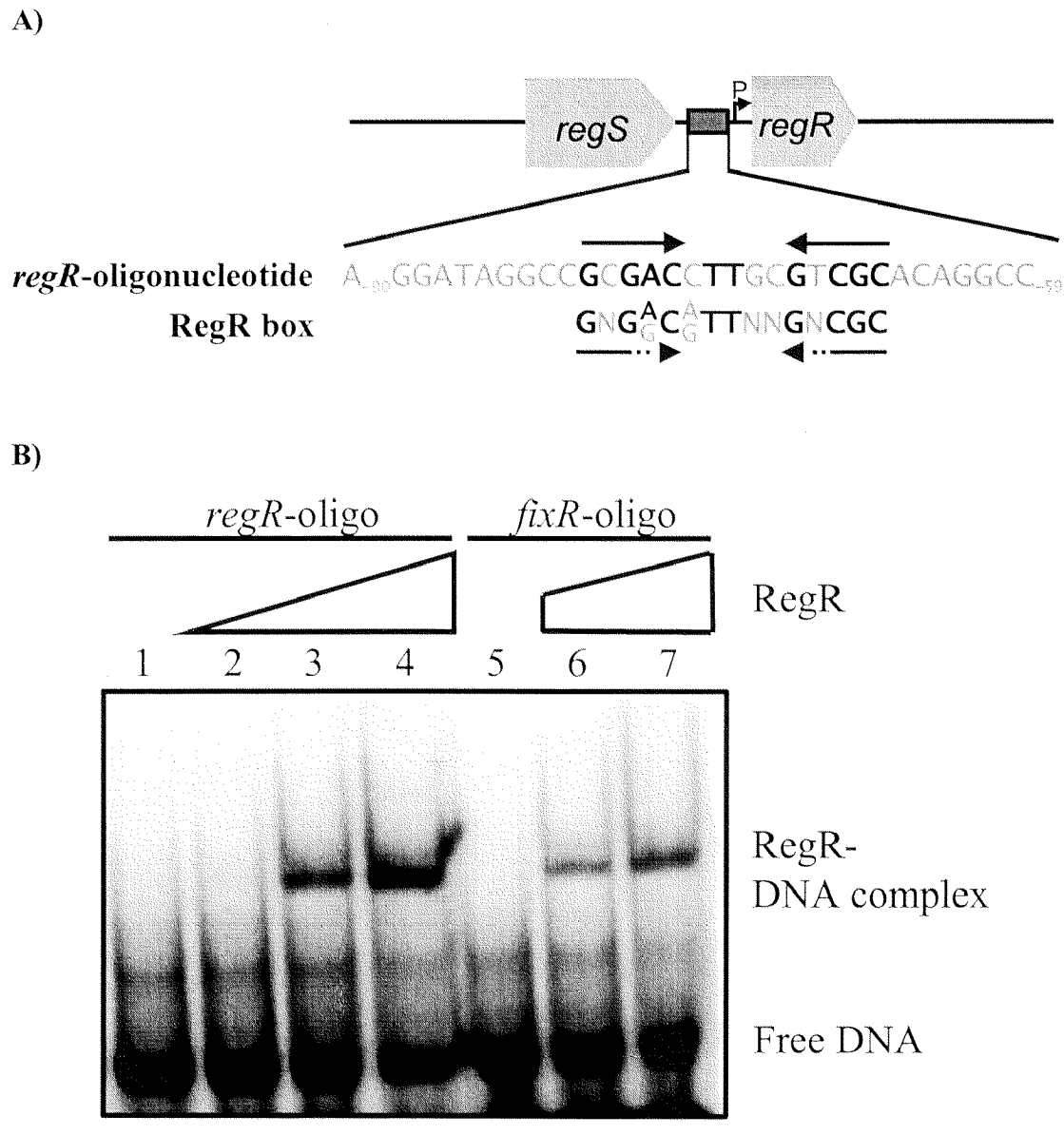


Fig. 6.1. Binding of RegR to the promoter region of the *regR* gene. A) Location of the newly identified RegR box-like element in front of the *regR* transcription start site (P) and alignment with the RegR box as defined in section 5.4.3. The identical nucleotides (G_{-80} to C_{-76} and T_{-74} to C_{-66}) are shown in black bold letters. The numbering refers to Bauer *et al.* (1998). The indicated genes and the RegR binding site are not drawn to scale. B) The gel retardation experiment was performed by incubating purified RegR (0.3, 0.9, 2.7, 0.9 and 2.7 μM; lanes 2, 3, 4, 6, 7) with the [^{32}P]end-labeled, double stranded 32-bp DNA fragment (0.1 ng) corresponding to the *regR* upstream region around position –74 (*regR*-oligo; see panel A) or the *fixR-nifA* upstream region around position –68 (*fixR*-oligo; chapter 2), and poly(dI-dC) (1 μg) as non-specific competitor. After 1-min incubation at room temperature, loading dye was added and the samples were separated on a 6% nondenaturating polyacrylamide gel. Bands were visualized by phosphorimager analysis of the dried gel. No protein was added to the samples loaded in lanes 1 and 5.

6.2. Expression of *regR'-'lacZ* is reduced in a *B. japonicum regR* mutant

The finding of a RegR-binding site upstream of the *regR* gene suggested that *regR* is autoregulated. To analyze if the *in vivo* expression of *regR* indeed depends on RegR, a *regR'-'lacZ* fusion was chromosomally integrated in the *B. japonicum regR* mutant 2426. Plasmid pRJ2430 (Bauer, 2000) was introduced by conjugation into *B. japonicum* 2426, yielding strain 2426-30. The genetic structure of the mutant was verified by appropriate Southern blot hybridization of chromosomal DNA.

Expression of *regR* in the *regR* mutant was reduced under aerobic and microaerobic conditions by about 40% as compared with the wild type (Tab. 6.1.), which implies that RegR indeed activates its own expression, albeit weakly. This finding is different from the situation in *R. capsulatus* where RegA represses its own transcription (Du *et al.*, 1999). The difference between the two systems is reflected by the location of the respective binding sites for RegR and RegA. The RegR box-like element of the *B. japonicum regR* gene is located around position –74 upstream of the *regR* transcription start site whereas in *R. capsulatus* RegA binds directly between the –10 and the –35 region of the *senC-regR-hvrA* promoter. The physiological relevance of the different autoregulation of *regR* in *B. japonicum* and *regA* in *R. capsulatus* is unclear. Also, it should be noted that the significance of the reduction of *regR'-'lacZ* expression in the *B. japonicum regR* mutant must be verified using an appropriate control, e.g. with a *lacZ* fusion whose expression is not affected by a *regR* mutation.

Tab. 6.1. Expression of a chromosomally integrated *regR'-'lacZ* fusion in a *B. japonicum regR* mutant.

Strain	Relevant genotype	β-Galactosidase activity (Miller Units)[a]	
		Aerobic	Microaerobic
2430	*regR'-'lacZ*	355 ± 156	584 ± 121
2426-30	*regR'-'lacZ regR*::Ω	224 ± 35	338 ± 36

[a] Numbers are mean values ± standard errors of at least two independent experiments. In each experiment at least two cultures of the strains were grown in parallel and assayed in duplicate. Bacteria were grown in PSY medium in aerobic or microaerobic (0.5% oxygen) cultures to mid exponential phase.

6.3. Further potential RegR boxes

Two more potential RegR targets located upstream of the *rpoN*$_1$ and *cbbL* genes resulted from the search for new RegR box-like element in the *B. japonicum* genome (Fig. 6.2.). *rpoN*$_1$ encodes the alternative sigma factor σ^{54} (σ^N) (Kullik *et al.*, 1991). *cbbL* is part of the *cbbFPTALSXE* operon encoding the structural genes required for CO_2 fixation (Bauer, 2000; H. M. Fischer, personal communication). However, gel retardation experiments did not reveal RegR-binding activity to an oligonucleotide comprising the nucleotides of the putative RegR-binding site upstream of *rpoN*$_1$ (data not shown). The RegR box-like element upstream of *cbbL* is located in the coding region of *cbbA*. An oligonucleotide including the nucleotides of this region indeed showed RegR-binding activity (Bauer, 2000).

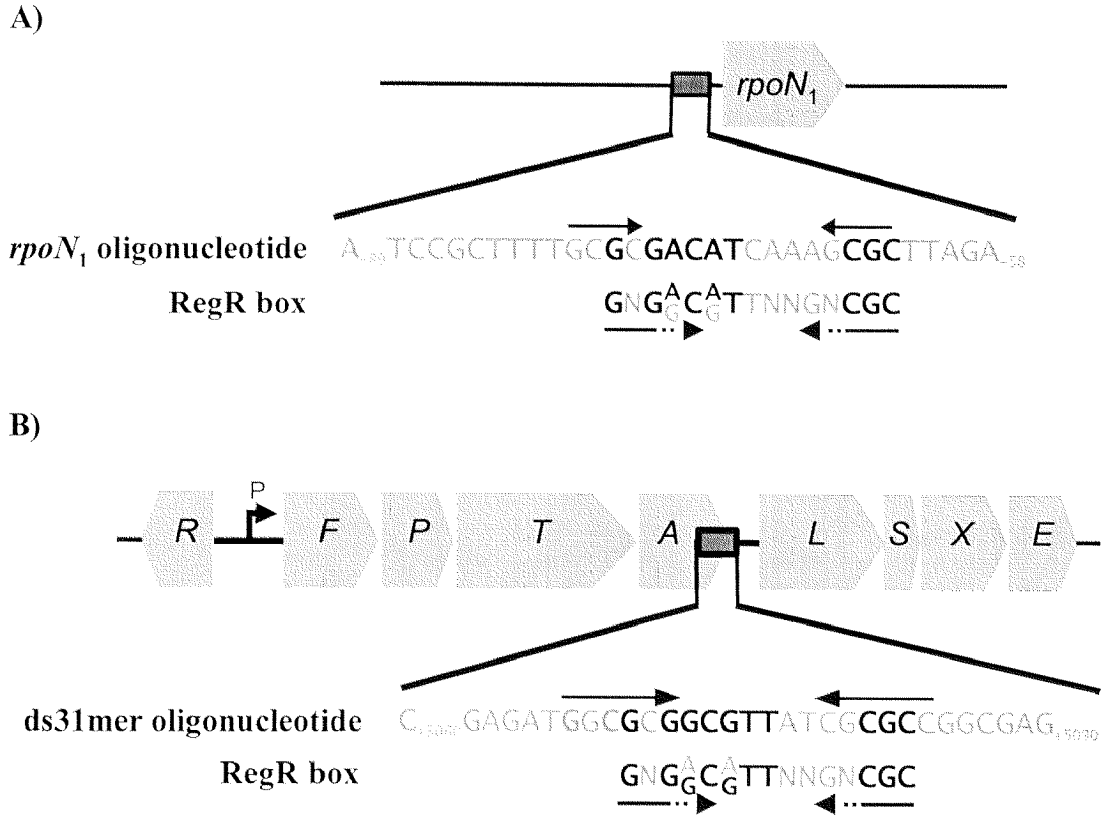


Fig. 6.2. Two RegR box-like elements upstream of the *rpoN*$_1$ (A) and *cbbL* (B) genes. The oligonucleotides used for gel retardation assays are shown and aligned with the RegR box. Identical nucleotides are shown in black bold letters. Position numbers are indicated relative to the translation start site of *rpoN*$_1$ (Kullik, 1992) and relative to the transcription start site upstream of *cbbF* (P; H. M. Fischer, unpublished data).

The failure of RegR to bind to the *rpoN*$_1$ oligonucleotide might be explained by the fact that two nucleotides of the RegR box are not present in the *rpoN*$_1$ oligonucleotide (C_{-70} and A_{-67} instead of T and G, respectively). Another possible explanation is the extended length of the spacing region between the half sites of the inverted repeat. The spacer comprises nine nucleotides, and thus it is by 4 nucleotides longer than the spacing region of the RegR box. The RegR-binding sequence upstream of *cbbL* contains a perfect inverted repeat of six nucleotides separated by seven nucleotides. The half sites of the inverted repeats in different RegR- and RegA-binding sites are separated by one to seven nucleotides (section 5.5.). Possibly, RegR cannot bind to inverted repeats with half sites separated by eight or more base pairs. Thus, future searches of RegR binding sites in the genome of *B. japonicum* and in other α-proteobacteria should consider both the sequence conservation and the spacing of half sites of potential RegR-binding sites.

Preliminary data obtained with *lacZ* fusions indicated that the expression of the *B. japonicum cbbFPTALSXE* operon is reduced in a *regR* mutant (Bauer, 2000). However, there is no evidence for a promoter upstream of *cbbL*, which could be activated by binding of RegR to the RegR box-like element shown above. On the other hand there is no obvious RegR-binding site upstream of the recently mapped transcription start site upstream of *cbbF* (H. M. Fischer, unpublished data). Thus, the functional role of the RegR binding site identified in the *cbbFPTALSXE* operon is questionable. Regulation of the *cbbFPTALSXE* operon is likely to include the adjacent gene, *cbbR*, encoding a putative LysR-type activator which was shown to activate expression of the *cbbFPTALSXE* operon, e.g. in *Ralstonia eutropha* under inducing conditions (Kusian *et al.*, 1995; Jeffke *et al.*, 1999). In fact, binding of RegA* and CbbR to the *cbb* promoter regions of *R. sphaeroides* and *R. capsulatus* was recently shown (Dubbs *et al.*, 2000; Vichivanives *et al.*, 2000). Hence, one may speculate that also in *B. japonicum* RegR acts in concert with CbbR to control *cbbFPTALSXE* expression.

Chapter 7

Evidence for a functional similarity between the two-component regulatory systems RegSR, ActSR, and RegBA (PrrBA) in α-Proteobacteria

Ralf Emmerich, Hauke Hennecke and Hans-Martin Fischer

in press in *Archives of Microbiology*

7.1. Abstract

The symbiotic bacteria *Bradyrhizobium japonicum* and *Sinorhizobium meliloti*, and the purple photosynthetic bacteria *Rhodobacter capsulatus*, *Rhodovulum sulfidophilum*, *Roseobacter denitrificans* and *Rhodobacter sphaeroides* possess homologous two-component regulatory systems, namely RegSR, ActSR, RegBA and PrrBA. The respective response regulators of these bacteria control expression of different regulons that are involved in N_2 fixation, CO_2 fixation, photosynthesis or acid tolerance. We therefore asked whether the regulators are functionally exchangeable or whether they have disparate functions in the different species, despite the amino acid sequence similarity. In this study, we showed that purified *B. japonicum* RegR bound *in vitro* to genuine DNA targets for *R. capsulatus* RegA, and that RegA was phosphorylated *in vitro* when $RegS_C$ (a soluble variant of the sensor kinase RegS) was added to an *Escherichia coli* extract containing overexpressed RegA. *In vivo*, RegA and *S. meliloti* ActR activated transcription of the *B. japonicum fixR-nifA* operon, normally a target for RegR. The genes for both regulators, *regA* and *actR*, were able to complement a *B. japonicum regR* mutant with respect to the formation of a nitrogen-fixing symbiosis with soybean. Vice versa, RegR activated in *R. capsulatus* the expression of the photosynthesis operon *puc*, normally a target for RegA. In conclusion, the results show that *B. japonicum* RegR, *R. capsulatus* RegA, and *S. meliloti* ActR are functionally similar.

Key words acid tolerance • *Bradyrhizobium japonicum* • carbon dioxide fixation • nitrogen fixation • photosynthesis • *Rhodobacter capsulatus* • *Sinorhizobium meliloti* • symbiosis

7.2. Introduction

Four two-component regulatory systems in six different α-proteobacterial species are well conserved, namely RegSR in *Bradyrhizobium japonicum*, RegBA in *Rhodobacter capsulatus*, *Rhodovulum sulfidophilum* and *Roseobacter denitrificans*, PrrBA in *Rhodobacter sphaeroides*, and ActSR in *Sinorhizobium meliloti* (Bauer *et al.*, 1998; Masuda *et al.*, 1999). The response regulators of these systems control the expression of genes involved in different processes. RegR in *B. japonicum* activates transcription of the *fixR-nifA* operon encoding NifA, a redox-responsive transcription regulator (Bauer *et al.*, 1998). NifA activates many genes required for symbiotic nitrogen fixation in soybean nodules (Fischer, 1994). The synthesis of the photosystem and of RegBA itself is controlled by RegA in *R. capsulatus*, *Rhodovulum sulfidophilum* and *Roseobacter denitrificans* (Sganga & Bauer 1992; Du *et al.*, 1999; Masuda *et al.*, 1999). Recently, it has been shown that the uptake hydrogenase and the molybdenum-containing nitrogenase in *R. capsulatus* are co-regulated by RegBA (Elsen *et al.*, 2000). In *R. sphaeroides*, PrrBA activates the expression of photosynthesis and Calvin cycle CO_2 fixation operons and the cytochrome c_2 gene (Eraso & Kaplan, 1994; Qian & Tabita, 1996; Karls *et al.*, 1999). ActR regulates the synthesis of proteins involved in CO_2 fixation, and the formate, formaldehyde and methanol dehydrogenases in *S. meliloti* (Fenner *et al.*, 2000). Why ActR also confers acid tolerance (Tiwari *et al.*, 1996) is not known.

Amino acid sequence alignments have shown that the response regulators RegR, RegA, PrrA and ActR share identical putative helix-turn-helix motifs (Bauer *et al.*, 1998; Masuda *et al.*, 1999). This suggested that they bind to DNA regions with similar nucleotide sequence motifs. DNA binding has been demonstrated *in vitro* with purified RegR to the upstream activating sequence (UAS) of the *fixR-nifA* operon (Emmerich *et al.*, 1999). Purified RegA*, a constitutively active mutant derivative of RegA, binds to the promoter regions of the *R. capsulatus puc*, *puf* and *hupSLC* operons, the *regB* gene, the *nifA2* gene, and the *R. sphaeroides cycA* gene (Du *et al.*, 1998, 1999; Karls *et al.*, 1999; Elsen *et al.* 2000). A consensus RegR binding motif (RegR box) was proposed based on an alignment of the RegR and RegA* binding sites (section 5.4.3.). This implies that the DNA binding sites for at least two of the four response regulators are conserved. Therefore, it appeared as if researchers were dealing with functionally equivalent proteins, despite the four different names used

for them. In this work, we tested whether the proteins can functionally replace each other. We focused our investigations on RegSR, ActSR and RegBA; the PrrBA system was excluded because it appears to play a role in *R. sphaeroides* similar to that of RegBA in *R. capsulatus* (Karls *et al.*, 1999; Dubbs *et al.*, 2000).

7.3. Materials and methods

7.3.1. Bacterial strains, plasmids, growth conditions, and DNA work

The *Escherichia coli*, *B. japonicum* and *R. capsulatus* strains and plasmids used in this study are listed in Table 7.1. *E. coli* was grown in Luria-Bertani medium (Miller, 1972) at 37°C. *B. japonicum* strains were grown at 30°C in PSY medium (Regensburger & Hennecke, 1983) supplemented with 0.1% (w/v) arabinose. *R. capsulatus* strains were grown at 34°C in PYS medium (Young *et al.*, 1989). Photosynthetic cells were grown anaerobically in the light at 3000 lux in filled screw-capped tubes. Appropriate concentrations of antibiotics were added as described previously (Narberhaus *et al.*, 1997; Sganga & Bauer, 1992). Routine genetic manipulations were performed as described elsewhere (Sambrook *et al.*, 1989).

Tab. 7.1. Bacterial strains and plasmids used in this study.

Strains/plasmids	Relevant genotype or phenotype	Reference
E. coli		
DH5α	*supE44 ΔlacU169*(Φ*80lacZ*ΔM15) *hsdR17 recA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories, Gaithersburg, Md., USA
S17-1	Sm^r Sp^r *hsdR* (RP4-2 *kan*::Tn7 *tet*::Mu chromosomally integrated)	Simon *et al.*, 1983
BL21(DE3)/pLysS	F^- Cm^r *ompT* $hsdS_B(r_B^-m_B^-)$ *gal dcm* (DE3)	Studier *et al.*, 1990
B. japonicum		
110*spc*4	Sp^r wild type	Regensburger & Hennecke, 1983
7276B	Sp^r Km^r *fixR'-'lacZ* chromosomally integrated	Thöny *et al.*, 1989
2426R	Sp^r Km^r Sm^r *regR*::Ω *fixR'-'lacZ* chromosomally integrated	Bauer *et al.*, 1998
2846R2	Sp^r Km^r Sm^r Tc^r *regR*::Ω *fixR'-'lacZ* P_{aphII}-*regA*$^+$ chromosomally integrated	This work
2866R2	Sp^r Km^r Sm^r Tc^r *regR*::Ω *fixR'-'lacZ* P_{aphII}-*actR*$^+$ chromosomally integrated	This work
R. capsulatus		
SB1003	Rif^r wild type	Yen & Marrs, 1976
MS01	Rif^r Km^r *regA*::*aphII*	Sganga & Bauer, 1992
Plasmids		
pUC19	Ap^r (cloning vector)	Norrander *et al.*, 1983
pBSL15	Ap^r Km^r (pBSL10, cloning vector)	Alexeyev, 1995
pET28(+)	Km^r (expression vector)	Novagen, Abingdon, UK
pSUP202pol4	Tc^r (pSUP202 *oriT* from RP4)	Fischer *et al.*, 1993

(continued)

(Tab. 7.1. continued)

Strains/plasmids	Relevant genotype or phenotype	Reference
pRK290	Tc^r	Ditta *et al.*, 1980
pRK290X	Tc^r (pRK290 with *Xho*I site)	Alvarez-Morales *et al.*, 1986
pLHIIZ	Sp^r *puc'-'lacZ*	Sganga & Bauer, 1992
pSP72::*regA*	Ap^r (pSP72) *R. capsulatus regA* on an 857-bp *Bam*HI-*Sal*I fragment	Inoue *et al.*, 1995
pRT546-11	Tc^r (pSW213) *S. meliloti actR* on a 3.7-kb *Eco*RI fragment	Tiwari *et al.*, 1996
pRJ2403	Ap^r (pUC19) *B. japonicum regSR* on a 3.6-kb *Eco*RI fragment	Bauer *et al.*, 1998
pRJ2454	Km^r (pET28a(+)) (overexpression of *regR*)	Emmerich *et al.*, 1999
pRJ2816	Ap^r (pBSL15) P_{aphII}-*regA*	This work
pRJ2818	Ap^r (pBSL15) P_{aphII}-*regR*	This work
pRJ2820	Tc^r (pRK290) *B. japonicum regSR* on a 3.6-kb *Eco*RI fragment	This work
pRJ2822	Tc^r (pRK290X) P_{aphII}-*regA*	This work
pRJ2823	Tc^r (pRK290) P_{aphII}-*regR*	This work
pRJ2846	Tc^r (pSUP202pol4) P_{aphII}-*regA*	This work
pRJ2849	Ap^r (pBSL15) P_{aphII}-*actR*	This work
pRJ2866	Tc^r (pSUP202pol4) P_{aphII}-*actR*	This work

7.3.2. Complementation of *B. japonicum regR* mutant strains

The 960-bp *Hin*dIII-*Nde*I fragment from pSP72::*regA*, and *Bgl*II-digested vector pBSL15, both made blunt by filling in overhanging ends, were ligated. The resulting plasmid, pRJ2816, carries *regA* under the control of the constitutive *aphII* promoter. The 2.2-kb *Xba*I fragment of pRJ2816 was cloned into pSUP202pol4, resulting in pRJ2846. Plasmid pRJ2849, carrying *actR* under the control of the constitutive *aphII* promoter, was constructed by insertion of a 0.7-kb *Bam*HI-*Nco*I fragment, generated by PCR from pRT546-11, into pBSL15. The 1.3-kb *Xba*I fragment from pRJ2849 was cloned into pSUP202pol4, resulting in pRJ2866. Plasmids pRJ2846 and pRJ2866 were introduced by conjugation (Hahn & Hennecke, 1984) into *B. japonicum* strain 2426R (*fixR'-'lacZ regR*::Ω) and chromosomally integrated via homologous recombination between the *aphII* cassette present on the plasmids and in the chromosome of strain 2426R. The genetic structure of the mutants was verified by appropriate Southern blot hybridizations of chromosomal DNA. Plasmid pRJ2818 carries a form of *regR* that is preceded by the codons for a hexahistidine tag and is expressed from the constitutive *aphII* promoter. It was constructed by insertion of a 1.0-kb *Bgl*II fragment from pRJ2454 into the *Bgl*II site located in the *aphII* gene of

pBSL15. The 2.1-kb *Eco*RI fragment from pRJ2818 was cloned into pRK290, resulting in pRJ2823. Plasmid pRJ2823 was introduced into *B. japonicum* strain 2426R by conjugation (Hahn & Hennecke, 1984) using its tetracycline resistance gene as selection marker.

7.3.3. Complementation of *R. capsulatus* MS01

Plasmids pRK290, pRJ2820, pRJ2822 and pRJ2823 were introduced into *R. capsulatus* MS01 by conjugation (Hahn & Hennecke, 1984) and selected by their resistance to tetracycline. pRJ2820 was constructed by inserting the 3.6-kb *Eco*RI fragment from pRJ2403 into pRK290. The insertion of the 2.2-kb *Sal*I fragment from pRJ2816 into the *Xho*I-digested pRK290X resulted in pRJ2822.

7.3.4. β-Galactosidase assays and plant infection tests

β-Galactosidase activities of aerobically grown *B. japonicum* cells harboring the *fixR'-'lacZ* fusion and of photosynthetically grown *R. capsulatus* cells harboring the *puc'-'lacZ* fusions were measured as described previously (Thöny *et al.*, 1987). The *R. capsulatus* cultures were grown in PYS medium containing spectinomycin as the sole antibiotic. The symbiotic phenotypes of the *B. japonicum* strains were determined in soybean infection tests (Hahn & Hennecke, 1984; Göttfert *et al.*, 1990).

7.3.5. Gel retardation assays

Purification of His-tagged RegR, labeling of the oligonucleotides, and gel retardation assays were performed as described elsewhere (Emmerich *et al.*, 1999). The 32-bp oligonucleotide fixR corresponds to the region from –83 to –52 upstream of the transcription start site P2 of the *fixR-nifA* operon (UAS; Emmerich *et al.*, 1999). The 38-bp double-stranded DNA fragments, called puc and puf, which correspond to the regions from –84 to –47 and –55 to –18 upstream of the transcription start sites of the *R. capsulatus puc* and *puf* operons, respectively (Du *et al.*, 1998), resulted from annealing of the oligonucleotides
(5'-C_{-84}CCCCGGGGGTGGCCGAATTTGCCGCAGTGTAAGCCCG$_{-47}$-3',
5'-C_{-55}CCCGGCGCGGCGATCCGCCGCGCGACGGGCACCCCCT$_{-18}$-3'), and the respective complementary oligonucleotides.

7.3.6. Phosphorylation assays

His-tagged $RegS_C$, a truncated version lacking the N-terminal hydrophobic domain, was purified as described (Emmerich *et al.*, 1999). Methods for overexpression and preparation of soluble extracts of *E. coli* BL21(DE3)/pLysS carrying plasmids pET28(+), pSP72::*regA* or pRJ2454 were reported previously (Emmerich *et al.*, 1999). The supernatant of the second centrifugation step was dialyzed overnight against TEDM buffer (Emmerich *et al.*, 1999). Phosphorylation of RegA and RegR was assayed at room temperature in TEDM buffer. Dialyzed *E. coli* extracts (15 μg) were incubated with 36 μM [γ-^{32}P]ATP (6 Ci·mmol^{-1}; Hartmann Analytic GmbH, Braunschweig, Germany) with or without purified $RegS_C$ (2.5 μM). Samples were removed after 10 min and the reactions stopped by adding 5 x SDS sample buffer (Emmerich *et al.*, 1999). The samples were analyzed by SDS-PAGE, using separating acrylamide (14%) gels, which were then dried and exposed to a phosphorimager screen (Molecular Dynamics, Sunnyvale, Calif.).

7.4. Results

7.4.1. *B. japonicum* RegR binds *in vitro* to DNA targets for *R. capsulatus* RegA

Two well-defined targets for RegA in *R. capsulatus* are the *puc* and *puf* operons, encoding the reaction center and light-harvesting complexes I and II (Du *et al.*, 1998). The ability of purified, His-tagged RegR to bind to these genuine RegA targets was tested in a gel retardation assay (Fig. 7.1.). The two oligonucleotides puc and puf span the promoter-upstream regions of the *R. capsulatus puc* and *puf* operons around positions –66 and –37, respectively. RegR specifically shifted both oligonucleotides to a retarded electrophoretic mobility. The oligonucleotide fixR, comprising the UAS of the *B. japonicum fixR-nifA* operon, was used as a positive control for RegR binding. We concluded that the puc and puf oligonucleotides comprise DNA motifs which both RegR and RegA can bind.

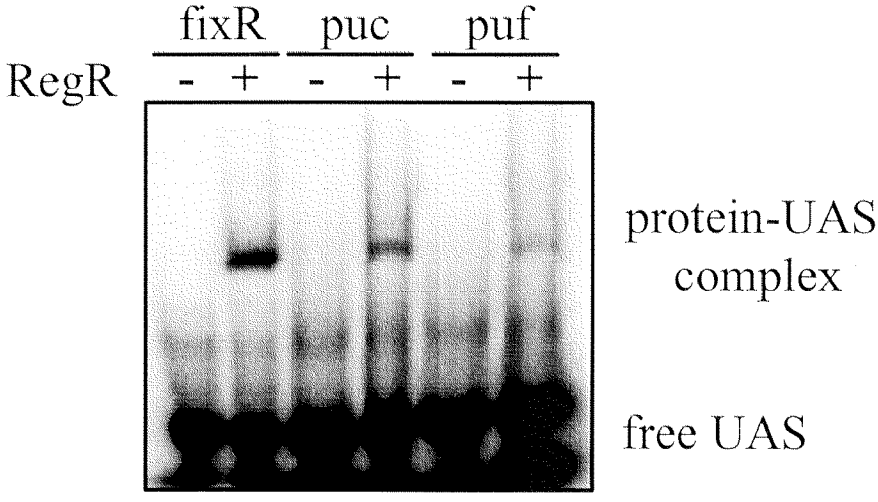


Fig. 7.1. RegR binding to the promoter regions of the *R. capsulatus puc* and *puf* operons. Gel retardation experiments were performed with DNA fragments corresponding to the *B. japonicum fixR-nifA* and *R. capsulatus puc* and *puf* promoter regions, around positions –68 (fixR), –66 (puc) and –37 (puf) upstream of the transcription start sites. ^{32}P-endlabeled DNA fragments (0.1 ng) and poly(dI-dC) (1 μg) as nonspecific competitor were incubated with (+) or without (–) 3 μM purified RegR. After 1-min incubation at room temperature, loading dye was added, and the samples were separated on a nondenaturing 6% polyacrylamide gel and visualized by phosphorimager analysis of the dried gel.

7.4.2. *B. japonicum* $RegS_C$ phosphorylates *R. capsulatus* RegA

A phosphorylation assay was performed to analyze whether RegA could be phosphorylated by the histidine kinase RegS from *B. japonicum*. Because purified RegA was not available to us, *E. coli* extracts containing overproduced RegA or RegR were used. Both proteins were phosphorylated when purified $RegS_C$ was added (Fig. 7.2.). Note that RegR has a lower electrophoretic mobility than RegA because of its additional His-tag. In the control strain, carrying only the expression vector, no phosphorylated protein was detected with an electrophoretic mobility comparable to that of RegA or RegR. An unspecifically phosphorylated protein appeared in each lane at a slightly higher electrophoretic mobility than $RegS_C$~P. The results shown in Fig. 7.2. suggest that both response regulators carry similar structural determinants needed for productive interaction with $RegS_C$.

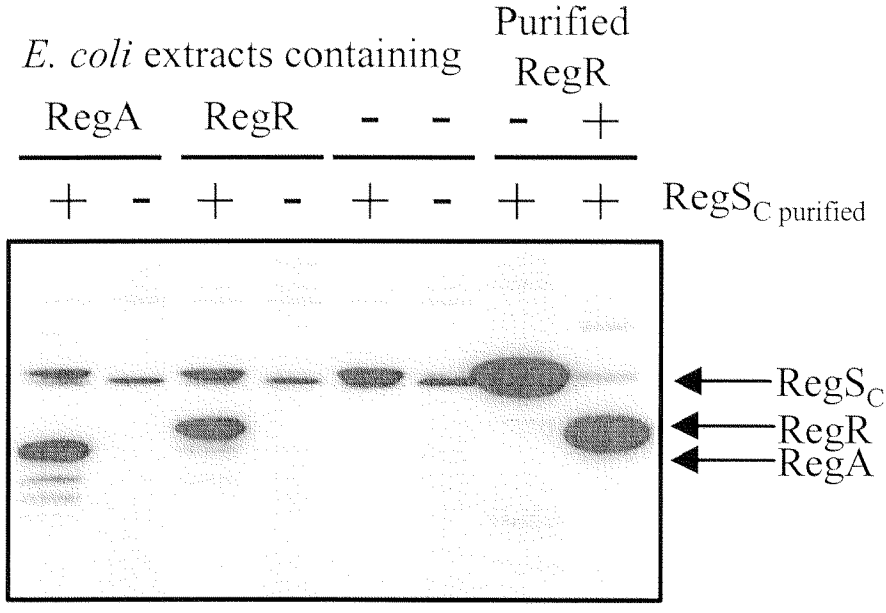


Fig. 7.2. Phosphotransfer from *B. japonicum* $RegS_C$ to *R. capsulatus* RegA. *E. coli* extracts containing overproduced *R. capsulatus* RegA (pSP72::*regA*) or *B. japonicum* RegR (pRJ2454) or, as a control, with the expression vector pET28a(+) were incubated together with [γ-^{32}P]ATP (36 μM) and with (+) or without (−) purified $RegS_C$ (2.5 μM). Samples were removed after 10 min and analyzed by SDS-PAGE. The radioactivity was detected by phosphorimager analysis of the dried gel. Phosphorylated forms of purified $RegS_C$ ($RegS_{C\ purified}$) and RegR proteins were used as markers in the two rightmost lanes.

7.4.3. *S. meliloti* ActR and *R. capsulatus* RegA activate *fixR-nifA* transcription in *B. japonicum*

Having demonstrated that RegR and RegA could replace each other *in vitro*, it was further analyzed if they could also functionally substitute each other *in vivo*. This comparison was extended by including the highly homologous response regulator ActR from *S. meliloti*. The genes *actR* and *regA*, under the control of the constitutive *aphII* promoter, were chromosomally integrated in strain 2426R. The expression of the *fixR'-'lacZ* fusion present in the resulting strains was determined (Table 7.2.). The *regA* and *actR* genes were both capable of complementing the *regR* mutation, suggesting that transcriptional activation of the *fixR-nifA* operon was enabled *in vivo* by RegA and ActR. The β-galactosidase activities of the complemented mutant strains were in the same range as in strain 2426R/pRJ2832, which harbors the *regR* gene expressed from the *aphII* promoter on a plasmid. The strains expressing the complementing gene from the *aphII* promoter showed approximately two-fold higher *fixR'-'lacZ* expression than the reference strain 7276B, which harbors the *regR* gene under control of its genuine promoter.

Tab. 7.2. Expression of a chromosomally integrated *fixR'-'lacZ* fusion in *B. japonicum* wild type and *regR* mutants carrying constitutively expressed *regR*, *regA* or *actR*. Note that unlike the P_{aphII}-$regA^+$ and P_{aphII}-$actR^+$ constructs, which both are chromosomally integrated, P_{aphII}-$regR^+$ in the control strain is plasmid-located.

Strain	Relevant genotype	β-Galactosidase activity (Miller Units)[a]
7276B	$regR^+$ (wild type)	265 ± 40
2426R	*regR*::Ω	6 ± 4
2426R/pRJ2823	*regR*::Ω P_{aphII}-$regR^+$	596 ± 37
2846R2	*regR*::Ω P_{aphII}-$regA^+$	745 ± 171
2866R2	*regR*::Ω P_{aphII}-$actR^+$	440 ± 90

[a] Numbers are mean values ± standard deviations of at least two independent experiments. In each experiment, at least two cultures of all strains were grown in parallel and assayed in duplicate. Bacteria were grown in aerobic PSY cultures to mid-exponential phase.

7.4.4. *S. meliloti actR* and *R. capsulatus regA* can replace *regR* in *B. japonicum* during symbiosis

B. japonicum mutant strain 2426R (*regR*::Ω *fixR'-'lacZ*) is not able to fix nitrogen symbiotically. Therefore, derivatives of strain 2426R that were complemented with *actR* or *regA* were tested for their ability to nodulate and fix nitrogen in a plant infection test (Tab. 7.3.). All strains elicited nodules on soybean. The interior of the nodules induced by the complemented mutants had the same reddish color as the wild type, in contrast to the greenish interior produced by *regR* mutants. Furthermore, the complemented mutants regained the ability to fix nitrogen (acetylene reduction) to comparable levels as the wild type.

Tab. 7.3. Symbiotic phenotype of *B. japonicum regR* mutants carrying constitutively expressed and chromosomally integrated *regA* or *actR*, compared with the wild type and a *regR* mutant.

Strain	Relevant genotype	Characteristics		
		Number of nodules	Dry weight/nodule (mg)	Fix activity [a] (% of wild type)
7276B	Wild type	20.5 ± 5.4	1.1 ± 0.3	100 ± 40
2426R	*regR*::Ω	22.1 ± 6.0	0.9 ± 0.2	0 ± 0
2846R2	*regR*::Ω P_{aphII}-*regA*$^+$	31.0 ± 11.8	0.6 ± 0.2	69 ± 31
2866R2	*regR*::Ω P_{aphII}-*actR*$^+$	22.4 ± 5.8	0.7 ± 0.1	84 ± 48

[a] Values are the mean ± standard deviations of at least ten individual plants. Fixation (Fix) activity was measured as the amount of C_2H_2 reduced per min per g of nodule dry weight.

7.4.5. *B. japonicum* RegR activates transcription of the *puc* operon in *R. capsulatus*

To analyze the properties of RegR in *R. capsulatus*, the *regA* mutant strain MS01 was complemented with pRK290 (control), pRJ2820, pRJ2822 and pRJ2823. The β-galactosidase activity expressed from a plasmid-borne *puc'-'lacZ* fusion was determined and compared with MS01 and wild type SB1003 (Tab. 7.4.). Constitutive expression of *regR* or *regA* enhanced the expression of the *puc'-'lacZ* fusion to wild-type levels. When pRJ2820, a plasmid that carries *regS* and *regR* under control of their cognate promoters, was present, expression of the *puc'-'lacZ* reporter fusion was also restored to wild-type levels.

Tab. 7.4. Expression of plasmid-borne *puc'-'lacZ* fusions in a *R. capsulatus regA* mutant complemented with *regR*.

Strain	Plasmids	Relevant genotype	β-Galactosidase activity (Miller Units) [a]
SB1003	pLHIIZ	wild type	4395 ± 751
MS01	pLHIIZ	*regA::aphII*	1597 ± 314
MS01	pLHIIZ pRK290	*regA::aphII*	1212 ± 143
MS01	pLHIIZ pRJ2822	*regA::aphII* P_{aphII}-*regA*$^+$	4641 ± 904
MS01	pLHIIZ pRJ2823	*regA::aphII* P_{aphII}-*regR*$^+$	4017 ± 1216
MS01	pLHIIZ pRJ2820	*regA::aphII regSR*$^+$	4103 ± 880

[a] Numbers are mean values ± standard deviations of at least two independent experiments. In each experiment, at least two cultures of all strains were grown in parallel and assayed in duplicate. Bacteria were grown photosynthetically for 20 h in PYS medium containing spectinomycin in screw-capped tubes at a light intensity of 3000 lux.

7.5. Discussion

The results of this study demonstrate that the respective response regulators RegR, RegA, and ActR from *B. japonicum*, *R. capsulatus*, and *S. meliloti* can functionally replace each other. It was shown that RegR binds *in vitro* to two genuine RegA DNA-targets. The observation that RegR and RegA bind to the same DNA target is reflected at the structural level by the high degree of similarity in the C-terminal domain of the respective proteins. The putative helix-turn-helix motifs are even identical. PrrA and ActR share this common helix-turn-helix motif as well, from which we predict that they also bind to the same targets. Whether or not similar regulatory proteins are present also in species other than those belonging to the α-Proteobacteria remains open. Our latest database search, using the amino acid sequence of the RegR protein as query sequence, revealed that the putative protein gcc_221 of *Caulobacter crescentus*, again an α-proteobacterium, carries the conserved helix-turn-helix motif of the RegR family.

Phosphorylation of overexpressed RegA present in *E. coli* crude extracts was dependent on *B. japonicum* $RegS_C$, the cytoplasmic domain of the RegR-phosphorylating histidine kinase. Albeit we cannot exclude the involvement of additional factors, it is highly likely that phosphorylation of RegA was catalyzed directly by $RegS_C$. This implies that RegA and RegR share the motifs needed for recognition by $RegS_C$. Previously, it was shown that the determinants required for productive interaction with $RegS_C$ are located in the N-terminal domain of RegR (Emmerich *et al.*, 1999) and the same holds probably true for RegA.

A natural target of RegR in *B. japonicum* is the *fixR-nifA* operon (Bauer *et al.*, 1998). Our *in vivo* experiments showed that *R. capsulatus* RegA and *S. meliloti* ActR complement a *B. japonicum regR* mutant with respect to *fixR-nifA* expression and establishment of a functional symbiosis with soybean. This demonstrates that RegA and ActR are both able to activate *in vivo* the *fixR-nifA* transcription in *B. japonicum*. Conversely, RegR activated transcription of the *puc* operon in the *R. capsulatus regA* mutant. We have not tested whether complementation by the heterologous activator proteins was dependent on the sensor proteins present in the complemented hosts. Notably, even the homologous activators seem to depend only partially on their cognate sensory proteins, as implied by their rather weak effects of mutations in the respective sensor genes (Bauer *et al.*, 1998; Mosley *et al.*, 1994), and *regR*-mediated

activation of the *puc'-'lacZ* fusion was comparable in the absence and presence of *regS*.

Four relevant regulons have been identified which are involved in N_2 fixation, CO_2 fixation and photosynthesis, or which include genes encoding three dehydrogenases. In none of the six α–proteobacterial species carrying either the RegSR, RegBA, PrrBA or ActSR systems have the same set of target genes been identified (see Introduction). This leads to two alternative scenarios: (1) The different species use orthologous two-component regulatory systems controlling the expression of different sets of genes. Then, the RegSR, RegBA, PrrBA, ActSR systems might stem from a common ancestral system, which was adapted in each case to meet special physiological requirements. In this scenario the respective sensors might detect different signals. (2) An ancestral form of the response regulator originally controlled the expression of the same set of genes. Some of the target genes might have been lost during subsequent evolution of the individual species, or alternatively, some target genes have yet to be discovered. This raises the question why these different regulons are connected via a common regulation. Joshi & Tabita (1996) proposed a model to explain why photosynthesis, CO_2 assimilation and N_2 fixation are linked. They postulated that the large amounts of reductant produced by photochemical reactions and organic carbon oxidation are removed via the nitrogenase system when the Calvin-Benson-Bassham pathway is blocked.

The nature of the physiological signal is unknown for all of the two-component regulatory systems addressed in this study. Attempts to identify the signal have been made recently in *R. sphaeroides* (O'Gara *et al.*, 1998; Eraso & Kaplan, 2000). An undefined inhibitory signal originating from the cbb_3-type oxidase affects expression of the target genes by acting via the PrrC protein through the PrrBA system.

An interesting parallel is that in nearly all cases a second activator regulates expression of the target genes in concert with the two-component regulatory system. The *fixR-nifA* operon of *B. japonicum*, besides being controlled by RegR, is autoactivated during microaerobic or anaerobic growth by NifA (Thöny *et al.*, 1989). In *R. capsulatus*, the *puc* and *puf* operons are further activated by HvrA under dim-light conditions (Buggy *et al.*, 1994). In *R. sphaeroides*, the *cbb* operon is additionally regulated by CbbR (Gibson & Tabita, 1993), and the *cycA* gene is further controlled by a member of the extracytoplasmic sigma factor family (Newman *et al.*, 1999). This

parallel indicates that RegSR, RegBA, PrrBA, and ActSR might act as more general global control systems side-by-side with other, more specific regulators.

Acknowledgements: We are grateful to Ravi Tiwari, Carl Bauer and Gabriele Klug for providing strains and plasmids. We thank Roger Santimaria for technical assistance. This work was supported by a grant from the Federal Institute of Technology Zürich.

7.5. Addendum: Correction of the growth defect of *R. sphaeroides* PRRA2 under photoheterotrophic conditions by RegR and RegA

To analyze if RegR and RegA can replace PrrA in *R. sphaeroides*, plasmids pRK290 (vector control), pRJ2820, pRJ2822 and pRJ2823 were introduced by conjugation into the *R. sphaeroides* PRRA2 mutant (Δ*prrA*; Eraso & Kaplan, 1995). *R. sphaeroides* strains were grown at 30°C in Sistrom's medium containing succinate as a carbon source (Cohen-Bazire *et al.*, 1957). For photoheterotrophic growth, cells were cultivated anaerobically in the same medium in the light (3000 lux) in filled screw-cap tubes. *R. sphaeroides* PRRA2 and PRRA2 containing pRK290 were not able to grow under photoheterotrophic conditions whereas the three complemented strains containing *regR* or *regA* were able to grow (data not shown). These data are consistent with those of Dubbs *et al.* (2000) who showed that *R. sphaeroides* CAC::*regA*Ω (*prrA* insertion mutant), which is not able to grow under phototrophic conditions, regained the ability to grow under both photoheterotrophic and photoautotrophic conditions when complemented with *regA*.

Seite Leer /
Blank leaf

Chapter 8

The RegR regulon,
a recapitulating discussion

8.1. The RegR regulon of *B. japonicum*

So far, the RegR regulon of *B. japonicum* comprises two direct targets: the *fixR-nifA* operon and the *regR* gene. In both cases, *in vitro* experiments demonstrated RegR binding to a RegR box-like element located about 70 nucleotides upstream of the transcription start site (chapters 5, 6). Furthermore, RegR was found to activate *in vivo* expression of *regR* and *fixR-nifA* (chapter 6; Bauer *et al.*, 1998). Probably, the *cbb* operon represents another RegR target, though it is not yet known whether RegR-mediated control is direct or indirect (Bauer *et al.*, 2000; section 6.3.). The components of the RegR regulon are depicted in Fig. 8.1. Three consequences of RegR-mediated control are discussed in the following.

First, the autoactivation of *regR* ensures a rapid accumulation of RegR. The activity of RegR is controlled by its phosphorylation state. When RegR is phosphorylated by RegS, it shows a higher DNA-binding activity to the *fixR* UAS (chapter 2) and consequently *fixR* transcription would increase. If this also holds true for the *regR* UAS, phosphorylation of RegR increases both its protein level and activity. A turn-off signal might result in the dephosphorylation of RegR~P by RegS. This inactivates RegR and, in consequence, decreases its own transcription rate and the expression of its target genes. In summary, the autoregulation of *regR* would ensure a rapid turn-on and turn-off of the RegR regulon in response to the signal transduced by RegSR. The identification of the signal is now of prime importance to obtain a hint as to why a fast response is advantageous for *B. japonicum*.

Second, RegR causes aerobic expression of the *fixR-nifA* operon (section 1.2.). Yet, NifA-dependent expression of *nif* and *fix* genes does not occur under these conditions because NifA is oxygen-sensitive. This raises the question why NifA is synthesized under aerobic conditions. One may argue that rapid activation of *nif* gene expression would be ensured if inactive NifA preformed under aerobic conditions could be reactivated upon a drop in oxygen concentration. However, it was shown in *E. coli* that NifA is irreversibly inactivated and degraded under aerobic conditions (Kullik *et al.*, 1989; Morett *et al.*, 1991). Yet, it cannot be excluded that in *B. japonicum* NifA reactivation may be possible with the help of a hypothetical (re)activating enzyme. If NifA is irreversibly inactivated in *B. japonicum*, *de novo* NifA synthesis is needed under microaerobic conditions to produce active NifA. It

remains open why basal *fixR-nifA* expression is dependent on RegR and not simply mediated by a constitutive promoter.

Third, expression of the *cbbFPTALSXE* operon depends on RegR. The assumption that RegR activates indirectly the *cbbFPTALSXE* operon is based on the fact that *cbbP'-'lacZ* expression is reduced in a *regR* mutant (Bauer, 2000), but no obvious RegR-box was found upstream of the *cbb* promoter (H. M. Fischer, unpublished data; section 6.3.). An indirect regulation of the *cbbFPTALSXE* operon could occur via CbbR which is an activator of the *cbb* operons in *R. eutropha*, *R. sphaeroides* and *R. capsulatus* (Kusian *et al.*, 1995; Jeffke *et al.*, 1999; Dubbs *et al.*, 2000; Vichivanives *et al.*, 2000). If RegR regulates the expression of *cbbR* in *B. japonicum* this would result in an indirect regulation of the *cbbFPTALSXE* operon. Why should RegR regulate *cbbR* expression? In *R. eutropha*, the activity of CbbR is inhibited by the effector phosphoenolpyruvate (Grzeszik *et al.*, 2000). If this also holds true for *B. japonicum* CbbR, it would mean that RegR ensures the synthesis of CbbR which, in turn, would detect the effector molecule. This model suggests a similar role of RegR in the regulation of *cbbR* and *nifA* in the sense that RegR controls the synthesis of another regulatory protein (CbbR, NifA) which then serves as sensor for an additional signal (phosphoenolpyruvate?, oxygen).

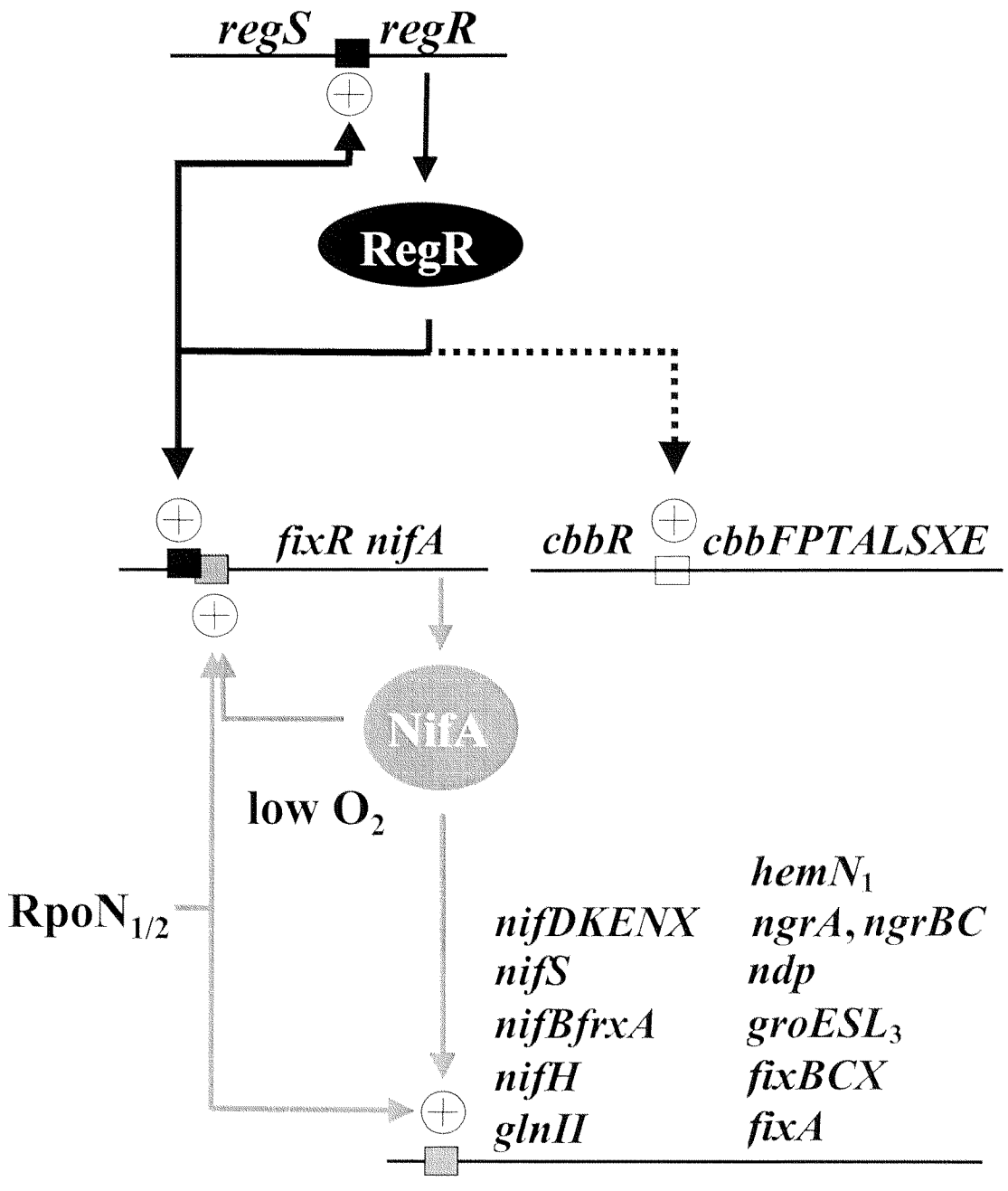


Fig. 8.1. The RegR regulon of *B. japonicum*. RegR activates directly the *fixR-nifA* operon and the *regR* gene by binding to a RegR box upstream of the transcription start sites (solid arrows). Expression of *cbbFPTALSXE* was shown to be dependent on RegR. The dotted arrows indicate that it is still unclear if RegR activates the *cbbFPTALSXE* operon directly or indirectly. An indirect activation may occur via CbbR which is encoded upstream and in the opposite direction of *cbbF*. Under low oxygen conditions, NifA activates in concert with the alternative sigma factors $RpoN_{1/2}$ the genes of the NifA-regulon and the *fixR-nifA* operon. Black and grey boxes indicate promoters activated by RegR and NifA, respectively. The white box indicates the promoters of the *cbbFPTALSXE* operon and the *cbbR* gene.

8.2. Comparison of the two-component regulatory systems RegSR, RegBA and ActSR

The RegSR system exhibits high structural and functional similarity to RegBA and PrrBA of the purple photosynthetic bacteria *R. capsulatus*, *Rhodovulum sulfidophilum*, *Roseobacter denitrificans* and *R. sphaeroides*, and to ActSR from *Sinorhizobium meliloti*. The three homologous systems are presented in Fig. 8.2. RegB and RegS were shown to phosphorylate RegA and RegR, respectively (Inoue *et al.*, 1995; chapter 2), and RegS transferred a phosphoryl label to RegA (chapter 7). It is unknown, if ActS phosphorylates ActR. The *regA* and *regR* genes are subjected to distinct modes of autoregulation. While RegA represses its own transcription, RegR activates its expression (Du *et al.*, 1999; chapter 6). Target genes are known for all three response regulators. The implications have been discussed in chapter 7. Furthermore, it was shown that RegA and ActR activate expression of a *fixR'-'lacZ* fusion in *B. japonicum* and that RegR activated transcription of a RegA target, the *puc* gene *R. capsulatus* (chapter 7). This implies that these response regulators can functionally replace each other.

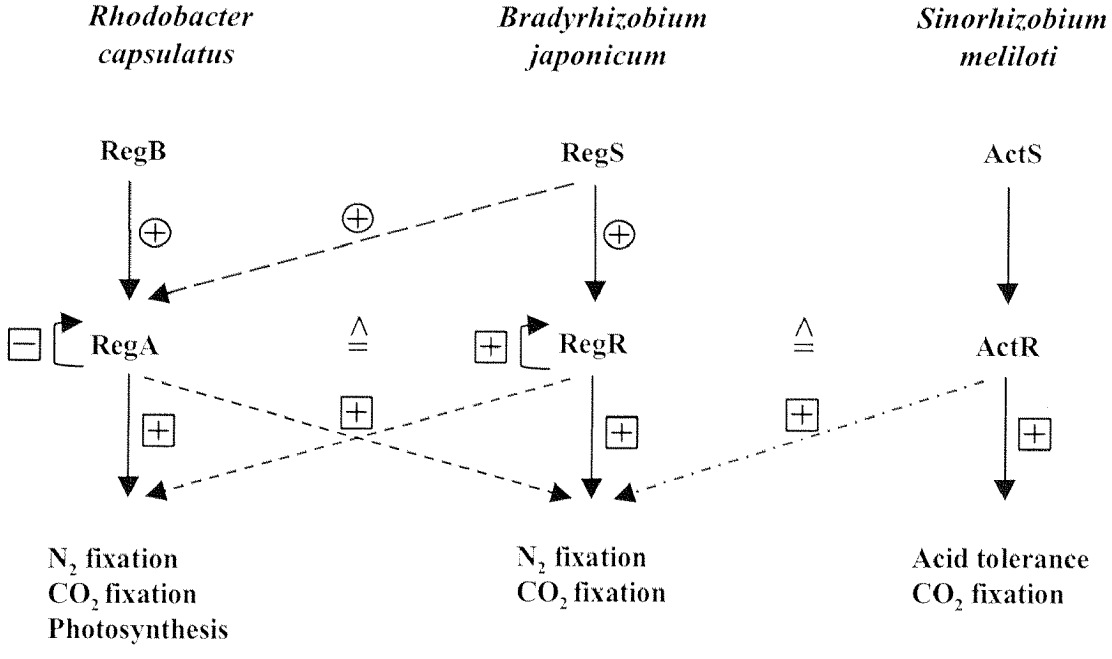


Fig. 8.2. Functional similarity of the three highly homologous two-component regulatory systems RegBA, RegSR and ActSR. Phosphorylation is indicated by an encircled plus sign and transcriptional activation or repression by a boxed plus or minus sign, respectively. Solid arrows refer to genuine functions of the respective regulators in the homologous background whereas dotted lines indicate artificial heterologous functions. The functional similarity of RegA, RegR and ActR is emphasized (≙).

Because, the three two-component regulatory systems control different genes involved in N_2 fixation, CO_2 fixation, photosynthesis or acid tolerance, the question remains why these different regulons are connected via a common regulation. A model explaining why photosynthesis, CO_2 assimilation and N_2 fixation are linked via PrrBA in *R. sphaeroides* was suggested by Joshi & Tabita (1996). They speculated that the large amounts of reductant produced by photochemical reactions and organic carbon oxidation are removed via the nitrogenase system when the Calvin-Benson-Bassham (*cbb*) pathway is blocked. Under these conditions the task of the nitrogenase is not to fix nitrogen for metabolic processes but to remove an excess of reductants. For this purpose, the nitrogenase is also synthesized under normally repressing conditions, i.e. in the presence of ammonia (Joshi & Tabita, 1996). Similarly, the RegBA system was proposed to be involved in the control of the redox balance in *R. capsulatus* (Elsen *et al.*, 2000). Future work will hopefully reveal whether the RegSR system plays a similar role in *B. japonicum* and whether the cellular redox state is sensed via RegSR.

In *R. sphaeroides*, it was shown that the cbb_3-type oxidase and the PrrC protein are necessary for the regulation of photosynthesis genes by the PrrBA-system (Oh & Kaplan, 1999, 2000; Eraso *et al.*, 2000). Kaplan and coworkers postulated that the flow of reductants through the cbb_3-type oxidase (CcoNOQP) regulates the activity of the histidine kinase PrrB via CcoQ and PrrC. However, the cbb_3-type oxidase of *B. japonicum* (FixNOQP), which is highly homologous to CcoNOQP, does not seem to have an influence on RegR-dependent regulation of $fixRp_2$ (H. M. Fischer, unpublished data). In the cyanobacterium *Synechocystis* sp. strain PCC 6803, the redox state of the plastoquinone pool was recently shown to control expression of photosystem II genes via the RppBA two-component system which exhibits some similarity to RegSR (Li & Sherman, 2000). Expression of the genes encoding the photosystem II increased when the plastoquinone pool was oxidized and decreased when the plastoquinone pool was reduced. In a *rppA* deletion mutant lacking the response regulator RppA, expression of the genes encoding the photosystem II reaction center was independent from the redox state of the plastoquinone pool. The authors concluded that RppB can sense changes in the plastoquinone redox poise. The structural similarity of RppB to RegS is 31%. Therefore, it will be interesting to test in *B. japonicum* if changes in the redox state of the quinone pool affects RegSR activity. The two signaling mechanisms, via the cbb_3-type oxidase or via the quinone

pool, are not mutually exclusive because the cbb_3-type oxidase receives the electrons from the reduced quinone pool.

Seite Leer /
Blank leaf

References

Agron, P.G. & Helinski, D.R. (1995) Symbiontic expression of *Rhizobium meliloti* nitrogen fixation genes is regulated by oxygen. In: *Two-Component Signal Transduction* (Hoch, J.A. & Silhavy, T.H. eds) pp. 275-287. American Society for Microbiology, Washington D. C.

Agron, P.G., Ditta, G.S. & Helinski, D.R. (1993) Oxygen regulation of *nifA* transcription *in vitro*. *Proc. Natl. Acad. Sci. USA* **90**, 3506-3510.

Aiba, H., Mizuno, T. & Mizushima, S. (1989) Transfer of phosphoryl group between two regulatory proteins involved in osmoregulatory expression of the *ompF* and *ompC* genes in *Escherichia coli*. *J. Biol. Chem.* **264**, 8563-8567.

Aizawa, S.I., Harwood, C.S. & Kadner, R.J. (2000) Signaling components in bacterial locomotion and sensory reception. *J. Bacteriol.* **182**, 1459-1471.

Alexeyev, M.F. (1995) Three kanamycin resistance gene cassettes with different polylinkers. *Bio Techniques* **18**, 52-56.

Alvarez-Morales, A., Betancourt-Alvarez, M., Kaluza, K. & Hennecke, H. (1986) Activation of the *Bradyrhizobium japonicum nifH* and *nifDK* operons is dependent on promoter-upstream DNA sequences. *Nucleic Acids Res.* **16**, 4025-4039.

Ames, S.K., Frankema, N. & Kenney, L.J. (1999) C-terminal DNA binding stimulates N-terminal phosphorylation of the outer membrane protein regulator OmpR from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **96**, 11792-11797.

Anthamatten, D. & Hennecke, H. (1991) The regulatory status of the *fixL*-like and *fixJ*-like genes in *Bradyrhizobium japonicum* may be different from that in *Rhizobium meliloti*. *Mol. Gen. Genet.* **225**, 38-48.

Anthamatten, D., Scherb, B. & Hennecke, H. (1992) Characterization of a *fixLJ*-regulated *Bradyrhizobium japonicum* gene sharing similarity with the *Escherichia coli fnr* and *Rhizobium meliloti fixK* genes. *J. Bacteriol.* **174**, 2111-2120.

Appleby, C.A. & Bergersen, F.J. (1980) Preparation and experimental use of leghemoglobin. In: *Methods for Evaluating Biological Nitrogen Fixation* (Bergersen, F.J. ed.) pp. 315-335. John Wiley & Sons Ltd., Chichester, UK.

Arthur, M., Molinas, C. & Courvalin, P. (1992) The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J. Bacteriol.* **174**, 2582-2591.

Baikalov, I., Schröder, I., Kaczor-Grzeskowiak, M., Grzeskowiak, K., Gunsalus, R.P. & Dickerson, R.E. (1996) Structure of the *Escherichia coli* response regulator NarL. *Biochemistry* **35**, 11053-11061.

Ban, C. & Yang, W. (1998) Crystal structure and ATPase activity of MutL: implications for DNA repair and mutagenesis. *Cell* **95**, 541-552.

Barrios, H., Fischer, H.M., Hennecke, H. & Morett, E. (1995) Overlapping promoters for two different RNA polymerase holoenzymes control *Bradyrhizobium japonicum nifA* expression. *J. Bacteriol.* **177**, 1760-1765.

Barrios, H., Grande, R., Olvera, L. & Morett, E. (1998) *In vivo* genomic footprinting analysis reveals that the complex *Bradyrhizobium japonicum fixR-nifA* promoter region is differently occupied by two distinct RNA polymerase holoenzymes. *Proc. Natl. Acad. Sci. USA* **95**, 1014-1019.

Bassler, B.L. & Silverman, M.R. (1995) Intercellular communication in marine *Vibrio* species: density-dependent regulation of the expression of bioluminoscence. In: *Two-Component Signal Transduction* (Hoch, J.A. & Silhavy, T.H. eds) pp. 431-445. American Society for Microbiology, Washington D. C.

Bauer, C.E., Elsen, S. & Bird, T.H. (1999) Mechanisms for redox control of gene expression. *Annu. Rev. Microbiol.* **53**, 495-523.

Bauer, E. (2000) Charakterisierung des Regulationssystems RegSR von *Bradyrhizobium japonicum* und seiner Rolle in der globalen Regulation von Genen für Symbiose, N_2- und CO_2-Fixierung. Ph.D. thesis, dissertation No. 13519, ETH Zürich.

Bauer, E., Kaspar, T., Fischer, H.M., & Hennecke, H. (1998) Expression of the *fixR-nifA* operon in *Bradyrhizobium japonicum* depends on a new response regulator, RegR. *J. Bacteriol.* **180**, 3853-3863.

Beck, C. (1998) Die Analyse des "housekeeping"-Promotors von *Bradyrhizobium japonicum* als Basis für die Entwicklung eines sequenzspezifischen *in vitro*-Transkriptionssystems. Ph.D. thesis, dissertation No. 13519, ETH Zürich.

Beck, C., Marty, R., Kläusli, S., Hennecke, H. & Göttfert, M. (1997) Dissection of the transcription machinery for housekeeping genes of *Bradyrhizobium japonicum*. *J. Bacteriol.* **179**, 364-369.

Beutel, B.A. & Gold, L. (1992) *In vitro* evolution of intrinsically bent DNA. *J. Mol. Biol.* **228**, 803-812.

Bilwes, A.M., Alex, L.A., Crane, B.R. & Simon, M.I. (1999) Structure of CheA, a signal-transducing histidine kinase. *Cell* **96**, 131-141.

Birck, C., Mourey, L., Gouet, P., Fabry, B., Schumacher, J., Rousseau, P., Kahn, D. & Samama, J.P. (1999) Conformational changes induced by phosphorylation of the FixJ receiver domain. *Structure* **7**, 1505-1515.

Bird, T.H., Du, S. & Bauer, C.E. (1999) Autophosphorylation, phosphotransfer, and DNA-binding properties of the RegB/RegA two-component regulatory system in *Rhodobacter capsulatus*. *J. Biol. Chem.* **274**, 16343-16348.

Bitte, L. & Kabat, D. (1974) Isotopic labeling and analysis of phosphoproteins from mammalian ribosomes. *Methods Enzymol.* **30**, 563-590.

Bott, M., Meyer, M. & Dimroth, P. (1995) Regulation of anaerobic citrate metabolism in *Klebsiella pneumoniae*. *Mol. Microbiol.* **18**, 533-546.

Bourret, R.B., Davagnino, J. & Simon, M.I. (1993) The carboxy-terminal portion of the CheA kinase mediates regulation of autophosphorylation by transducer and CheW. *J. Bacteriol.* **175**, 2097-2101.

Bowman, W.C., Du, S., Bauer, C.E. & Kranz, R.G. (1999) *In vitro* transcription and repression of photosynthesis gene transcription in *Rhodobacter capsulatus*. *Mol. Microbiol.* **33**, 429-437.

Bradford, M.M. (1976) A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Buggy, J.J., Sganga, M.W. & Bauer, C.E. (1994) Characterization of a light-responding trans-activator responsible for differentially controlling reaction center and light-harvesting-I gene expression in *Rhodobacter capsulatus*. *J. Bacteriol.* **176**, 6936-6943.

Cho, H.S., Lee, S.Y., Yan, D., Pan, X., Parkinson, J.S., Kustu, S., Wemmer, D.E. & Pelton, J.G. (2000) NMR structure of activated CheY. *J. Mol. Biol.* **297**, 542-551.

Cohen-Bazire, G., Sistrom, W.R. & Stanier, R.Y. (1957) Kinetic studies of pigment synthesis by non-sulfur purple bacteria. *J. Cell. Comp. Physiol.* **49**, 25-68.

Da Re, S., Bertagnoli, S., Fourment, J., Reyrat, J.M. & Kahn, D. (1994) Intramolecular signal transduction within the FixJ transcriptional activator: *in vitro* evidence for the inhibitory effect of the phosphorylatable regulatory domain. *Nucleic Acids Res.* **22**, 1555-1561.

Da Re, S., Schumacher, J., Rousseau, P., Fourment, J., Ebel, C. & Kahn, D. (1999) Phosphorylation-induced dimerization of the FixJ receiver domain. *Mol. Microbiol.* **34**, 504-511.

Ditta, G., Stanfield, S., Corbin, D. & Helinski, D.R. (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc. Natl. Acad. Sci. USA* **77**, 7347-7351.

Dixon, R. (1998) The oxygen-responsive NifL-NifA complex: a novel two-component regulatory system controlling nitrogenase synthesis in γ-Proteobacteria. *Arch. Microbiol.* **169**, 371-380

Du, S., Bird, T.H. & Bauer, C.E. (1998) DNA binding characteristics of RegA. *J. Biol. Chem.* **273**, 18509-18513.

Du, S., Kouadio, J.L. & Bauer, C.E. (1999) Regulated expression of a highly conserved regulatory gene cluster is necessary for controlling photosynthesis gene expression in response to anaerobiosis in *Rhodobacter capsulatus*. *J. Bacteriol.* **181**, 4334-4341.

Dubbs, J.M., Bird, T.H., Bauer, C.E. & Tabita, F.R. (2000) Interaction of CbbR and RegA* transcription regulators with the *Rhodobacter sphaeroides cbb*$_I$ promoter-operator region. *J. Biol. Chem.* **275**, 19224-19230.

Dutta, R., Qin, L. & Inouye, M. (1999) Histidine kinases: diversity of domain organization. *Mol. Microbiol.* **34**, 633-640.

Egger, L.A., Park, H. & Inouye, M. (1997) Signal transduction via the histidyl-aspartyl phosphorelay. *Genes Cells* **167**, 167-184.

Elsen, S., Dischert, W., Colbeau, A. & Bauer, C.E. (2000) Expression of uptake hydrogenase and molybdenum nitrogenase in *Rhodobacter capsulatus* is coregulated by the RegB-RegA two-component regulatory system. *J. Bacteriol.* **182**, 2831-2837.

Emmerich, R., Panglungtshang, K., Strehler, P., Hennecke, H. & Fischer, H.M. (1999) Phosphorylation, dephosphorylation and DNA-binding of the *Bradyrhizobium japonicum* RegSR two-component regulatory proteins. *Eur. J. Biochem.* **263**, 455-463.

Eraso, J.M. & Kaplan, S. (1994) *prrA*, a putative response regulator involved in oxygen regulation of photosynthesis gene expression in *Rhodobacter sphaeroides*. *J. Bacteriol.* **176**, 32-43.

Eraso, J.M. & Kaplan, S. (1995) Oxygen-insensitive synthesis of the photosynthetic membranes of *Rhodobacter sphaeroides*: a mutant histidine kinase. *J. Bacteriol.* **177**, 2695-2706.

Eraso, J.M. & Kaplan, S. (2000) From redox flow to gene regulation: role of the PrrC protein of *Rhodobacter sphaeroides* 2.4.1. *Biochemistry* **39**, 2052-2062.

Fenner, B.J., Tiwari, R.P., Reeve, W.G., Dilworth, M.J. & Glenn, A.R. (2000) ActR is a global genetic regulator in *Sinorhizobium meliloti*. In: *Nitrogen fixation: from molecules to crop productivity* (Pedrosa, F.O., Hungria, M., Yates, G. & Newton, W.E. eds) pp 89-90, Kluwer Academic Publishers, London.

Fiedler, U. & Weiss, V. (1995) A common switch in activation of the response regulators NtrC and PhoB: phosphorylation induces dimerization of the receiver modules. *EMBO J.* **14**, 3696-3705.

Fischer, H.M. & Hennecke, H. (1987) Direct response of *Bradyrhizobium japonicum nifA*-mediated *nif* gene regulation to cellular oxygen status. *Mol. Gen. Genet.* **209**, 621-626.

Fischer, H.M. (1994) Genetic regulation of nitrogen fixation in rhizobia. *Microbiol. Rev.* **58**, 352-386.

Fischer, H.M. (1996) Environmental regulation of rhizobial symbiotic nitrogen fixation genes. *Trends Microbiol.* **4**, 317-320.

Fischer, H.M., Alvarez-Morales, A. & Hennecke, H. (1986) The pleiotropic nature of symbiotic regulatory mutants: *Bradyrhizobium japonicum nifA* gene is involved in control of *nif* gene expression and formation of determinate symbiosis. *EMBO J.* **5**, 1165-1173.

Fischer, H.M., Babst, M., Kaspar, T., Acuña, G., Arigoni, F. & Hennecke. H. (1993) One member of a *groESL*-like chaperonin multigene family of *Bradyrhizobium japonicum* is co-regulated with symbiotic nitrogen fixation genes. *EMBO J.* **12**, 2901-2912.

Fischer, H.M., Fritsche, S., Herzog, B. & Hennecke, H. (1989) Critical spacing between two essential cysteine residues in the interdomain linker of the *Bradyrhizobium japonicum* NifA protein. *FEBS Lett.* **255**, 167-71.

Forst, S., Comeau, D., Norioka, S. & Inouye, M. (1987) Localization and membrane topology of EnvZ, a protein involved in osmoregulation of OmpF and OmpC in *Escherichia coli*. *J. Biol. Chem.* **262**, 16433-16438.

Fujitaki, J.M. & Smith, R.A. (1984) Techniques in the detection and characterization of phosphoramidate-containing proteins. *Methods Enzymol.* **107**, 23-36.

Galinier, A., Garnerone, A.M., Reyrat, J.M., Kahn, D., Batut, J. & Boistard, P. (1994) Phosphorylation of the *Rhizobium meliloti* FixJ protein induces its binding to a compound regulatory region at the *fixK* promoter. *J. Biol. Chem.* **269**, 23784-23789.

Gibson, J.L. & Tabita, F.R. (1993) Nucleotide sequence and functional analysis of *cbbR*, a positive regulator of the Calvin cycle operons of *Rhodobacter sphaeroides*. *J. Bacteriol.* **175**, 5778-5784.

Gilles-Gonzalez, M.A., Ditta, G.S. & Helinski, D.R. (1991) A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti*. *Nature* **350**, 170-172.

Gilles-Gonzalez, M.A., Gonzalez, G. & Perutz M.F. (1995) Kinase activity of oxygen sensor FixL depends on the spin state of its heme iron. *Biochemistry* **34**, 232-236.

Gong, W., Hao, B. & Chan, M.K. (2000) New mechanistic insights from structural studies of the oxygen-sensing domain of *Bradyrhizobium japonicum* FixL. *Biochemistry* **39**, 3955-3962.

Gong, W., Hao, B., Mansy, S.S., Gonzalez, G., Gilles-Gonzalez, M.A. & Chan, M.K. (1998) Structure of a biological oxygen sensor: a new mechanism for heme-driven signal transduction. *Proc. Natl. Acad. Sci. USA* **95**, 15177-15182.

Göttfert, M., Hitz, S. & Hennecke, H. (1990) Identification of *nodS* and *nodU*, two inducible genes inserted between the *Bradyrhizobium japonicum nodYABC* and *nodIJ* genes. *Mol. Plant-Microbe Interact.* **3**, 308-316.

Grzeszik, C., Jeffke, T., Schaferjohann, J., Kusian, B. & Bowien, B. (2000) Phosphoenolpyruvate is a signal metabolite in transcriptional control of the *cbb* CO_2 fixation operons in *Ralstonia eutropha*. *J. Mol. Microbiol. Biotechnol.* **2**, 311-320.

Hahn, M. & Hennecke, H. (1984) Localized mutagenesis in *Rhizobium japonicum*. *Mol. Gen. Genet.* **193**, 46-52.

Hoch, J.A. & Silhavy, T.H. (1995) *Two-Component Signal Transduction*. American Society for Microbiology, Washington D. C.

Hoch, J.A. (1995) Control of cellular development in sporulating bacteria by the phosphorelay two-component signal transduction system. In: *Two-Component Signal Transduction* (Hoch, J.A. & Silhavy, T.H. eds) pp. 129-144. American Society for Microbiology, Washington D. C.

Hokin, L.E., Sastry, P.S., Galsworthy, P.R. & Yoda, A. (1965) Evidence that a phosphorylated intermediate in a brain transport adenosine triphosphatase is an acyl phosphate. *Proc. Natl. Acad. Sci. USA* **54**, 177-184.

Hultquist, D.E. (1968) The preparation and characterization of phosphorylated derivatives of histidine. *Biochim. Biophys. Acta* **153**, 329-340.

Inoue, K., Kouadio, J.L.K., Mosley, C.S. & Bauer, C.E. (1995) Isolation and *in vitro* phosphorylation of sensory transduction components controlling anaerobic induction of light harvesting and reaction center gene expression in *Rhodobacter capsulatus*. *Biochemistry* **34**, 391-396.

Iuchi, S. & Lin, E.C.C. (1990) Purification and phosphorylation of the Arc regulatory components of *Escherichia coli*. *J. Bacteriol.* **174**, 5617-5623.

Iuchi, S. & Lin, E.C.C. (1995) Signal transduction in the Arc system for control of operons encoding aerobic respiratory enzymes. In: *Two-Component Signal Transduction* (Hoch, J.A. & Silhavy, T.H. eds) pp. 223-231. American Society for Microbiology, Washington D. C.

Jeffke, T., Gropp, N.H., Kaiser, C., Grzeszik, C., Kusian, B. & Bowien, B. (1999) Mutational analysis of the *cbb* operon (CO_2 assimilation) promoter of *Ralstonia eutropha*. *J. Bacteriol.* **181**, 4374-4380.

Joshi, H.M. & Tabita, F.R. (1996) A global two-component signal transduction system that integrates the control of photosynthesis, carbon dioxide assimilation, and nitrogen fixation. *Proc. Natl. Acad. Sci. USA* **93**, 14515-14520.

Kahn, D. & Ditta, G. (1991) Modular structure of FixJ: homology of the transcriptional activator domain with the –35 binding domain of sigma factors. *Mol. Microbiol.* **5**, 987-997.

Karls, R.K., Wolf, J.R. & Donohue, T.J. (1999) Activation of the *cycA* P2 promoter for the *Rhodobacter sphaeroides* cytochrome c_2 gene by the photosynthesis response regulator. *Mol. Microbiol.* **34**, 822-835.

Kaspar, S., Perozzo, R., Reinelt, S., Meyer, M., Pfister, K., Scapozza, L. & Bott, M. (1999) The periplasmic domain of the histidine autokinase CitA functions as a highly specific citrate receptor. *Mol. Micobiol.* **33**, 858-872.

Kaspar, T. (1997) Reinigung und Charakterisierung des postulierten Regulatorproteins für die aerobe *fixR-nifA*-Expression in *Bradyrhizobium japonicum*. Ph.D. thesis, dissertation No. 12173, ETH Zürich.

Kato, M., Mizuno, T., Shimizu, T. & Hakoshima, T. (1997) Insights into multistep phosphorelay from the crystal structure of the C-terminal HPt domain of ArcB. *Cell* **88**, 717-723.

Keener, J. & Kustu, S. (1988) Protein kinase and phosphoprotein phosphatase activities of nitrogen regulatory proteins NTRB and NTRC of enteric bacteria: roles of the conserved amino-terminal domain of NTRC. *Proc. Natl. Acad. Sci. USA* **85**, 4976-4980.

Kirndörfer, M., Jäger, A. & Klug, G. (1998) Integration host factor affects the oxygen-regulated expression of photosynthesis genes in *Rhodobacter capsulatus*. *Mol. Gen. Genet.* **258**, 297-305.

Kramer, G. & Weiss, V. (1999) Functional dissection of the transmitter module of the histidine kinase NtrB in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **96**, 604-609.

Kullik, I. (1992) Untersuchungen zur Funktion und Regulation der beiden *rpoN* Gene von *Bradyrhizobium japonicum*. Ph.D. thesis, dissertation No. 9895, ETH Zürich.

Kullik, I., Fritsche, S., Knobel, H., Sanjuan, J., Hennecke, H. & Fischer, H.M. (1991) *Bradyrhizobium japonicum* has two differentially regulated, functional homologs of the σ^{54} gene (*rpoN*). *J. Bacteriol.* **173**, 1125-1138.

Kullik, I., Hennecke, H. & Fischer, H.M. (1989) Inhibition of *Bradyrhizobium japonicum nifA*-dependent gene activation by oxygen occurs at the NifA protein level and is irreversible. *Arch. Microbiol.* **151**, 191-197.

Kusian, B. & Bowien, B. (1995) Operator binding of the CbbR protein, which activates the duplicate *cbb* CO_2 assimilation operons of *Alcaligenes eutrophus*. *J. Bacteriol.* **177**, 6568-6574.

Levit, M.N., Liu, Y. & Stock, J.B. (1998) Stimulus response coupling in bacterial chemotaxis: receptor dimers in signalling arrays. *Mol. Microbiol.* **30**, 459-466.

Lewis, R.J., Brannigan, J.A., Muchová, K., Barák, I. & Wilkinson, A.J. (1999) Phosphorylated aspartate in the structure of a response regulator protein. *J. Mol. Biol.* **294**, 9-15.

Lewis, R.J., Muchová, K., Brannigan, J.A., Barák, I., Leonard, G. & Wilkinson A.J. (2000) Domain swapping in the sporulation response regulator Spo0A. *J. Mol. Biol.* **297**, 757-770.

Li, H. & Sherman, L.A. (2000) A redox-responsive regulator of photosynthesis gene expression in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J. Bacteriol.* **182**, 4268-4277.

Lois, A.F., Weinstein, M., Ditta, G.S. & Helinski, D.R. (1993) Autophosphorylation and phosphatase activities of the oxygen-sensing protein FixL of *Rhizobium meliloti* are coordinately regulated by oxygen. *J. Biol. Chem.* **268**, 4370-4375.

Loomis, W.F., Shaulsky, G. & Wang, N. (1997) Histidine kinases in signal transduction pathways in eukaryotes. *J. Cell. Sci.* **110**, 1141-1145.

Martensen, T.M. (1984) Chemical properties, isolation and analysis of O-phosphates in proteins. *Methods Enzymol.* **107**, 3-23.

Martinez-Hackert, E. & Stock, A.M. (1997) The DNA-binding domain of OmpR: crystal structures of a winged helix transcription factor. *Structure* **15**, 109-124.

Masuda, S., Matsumoto, Y., Nagashima, K.V.P., Shimada, K., Inoue, K., Bauer, C.E. & Matsuura, K. (1999) Structural and functional analysis of photosynthetic regulatory genes *regA* and *regB* from *Rhodovulum sulfidophilum*, *Roseobacter denitificans*, and *Rhodobacter capsulatus*. *J. Bacteriol.* **181**, 4205-4215.

Miller, J.H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mizuno, T. (1999) Compilation of all genes encoding two-component phosphotransfer signal transducers in the genome of *Escherichia coli*. *DNA Research* **4**, 161-168.

Morett, E., Fischer, H.M. & Hennecke, H. (1991) Influence of oxygen on DNA binding, positive control, and stability of the *Bradyrhizobium japonicum* NifA regulatory protein. *J. Bacteriol.* **173**, 3478-3487.

Mosley, C.S., Suzuki, J.Y. & Bauer, C.E. (1994) Identification and molecular genetic characterization of a sensor kinase responsible for coordinately regulating light harvesting and reaction center gene expression in response to anaerobiosis. *J. Bacteriol.* **176**, 7566-7573.

Msadek, T., Kunst, F. & Rapoport, G. (1995)) A signal transduction network in *Bacillus subtilis* includes the DegS/DegU and ComP/ComA two-component systems. In: *Two-Component Signal Transduction* (Hoch, J.A. & Silhavy, T.H. eds) pp. 447-471. American Society for Microbiology, Washington D. C.

Nakashima, K., Sugiura, A., Kanamaru, K. & Mizuno, T. (1993) Signal transduction between the two regulatory components involved in the regulation of the *kdpABC* operon in *Escherichia coli*: phosphorylation-dependent functioning of the positive regulator, KdpE. *Mol. Microbiol.* **7**, 109-116.

Nakashima, K., Sugiura, A., Momoi, H. & Mizuno, T. (1992) Phosphotransfer signal transduction between two regulatory factors involved in the osmoregulated *kdp* operon in *Escherichia coli*. *Mol. Microbiol.* **6**, 1777-1784.

Narberhaus, F., Krummenacher, P., Fischer, H.M. & Hennecke, H. (1997) Three disparately regulated genes for σ^{32}-like transcription factors in *Bradyrhizobium japonicum*. *Mol. Microbiol.* **24**, 93-104.

Nellen-Anthamatten, D., Rossi, P., Preisig, O., Kullik, I., Babst, M., Fischer, H.M. & Hennecke, H. (1998) *Bradyrhizobium japonicum* $FixK_2$, a crucial distributor in the FixLJ-dependent regulatory cascade for control of genes inducible by low oxygen levels. *J. Bacteriol.* **180**, 5251-5255.

Newman, J.D., Falkowski, M.J., Schilke, B.A., Anthony, L.C. & Donohue, T.J. (1999) The *Rhodobacter sphaeroides* ECF sigma factor, σ^E, and the target promoters *cycA* P3 and *rpoE* P1. *J. Mol. Biol.* **294**, 307-320.

Norrander, J., Kempe, T. & Messing, J. (1983) Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26**, 101-106.

O'Gara, J.P., Eraso, J.M. & Kaplan, S. (1998) A redox-responsive pathway for aerobic regulation of photosynthesis gene expression in *Rhodobacter sphaeroides* 2.4.1. *J. Bacteriol.* **180**, 4044-4050.

Oh, J.I. & Kaplan, S. (1999) The cbb_3 terminal oxidase of *Rhodobacter sphaeroides* 2.4.1.: structural and functional implications for the regulation of spectral complex formation. *Biochemistry* **38**, 2688-2696.

Oh, J.I. & Kaplan, S. (2000) Redox signaling: globalization of gene expression. *EMBO J.* **19**, 4237-4247.

Parkinson, J.S. & Kofoid, E.C. (1992) Communication modules in bacterial signaling proteins. *Annu. Rev. Genet.* **26**, 71-112.

Pelton, J.G., Kustu, S. & Wemmer, D.E. (1999) Solution structure of the DNA-binding domain of NtrC with three alanine substitutions. *J. Mol. Biol.* **292**, 1095-1110.

Phillips-Jones, M.K. & Hunter, C.N. (1994) Cloning and nucleotide sequence of *regA*, a putative response regulator gene of *Rhodobacter sphaeroides*. *FEMS Microbiol. Lett.* **116**, 269-276.

Popham, D.L., Szeto, D., Keener, J. & Kustu, S. (1989) Function of a bacterial activator protein that binds to transcriptional enhancers. *Science* **243**, 629-635.

Porter, S.C., North, A.K. & Kustu, S. (1995) Mechanism of transcriptional activation by NtrC. In: *Two-Component Signal Transduction* (Hoch, J.A. & Silhavy, T.H. eds) pp. 147-158. American Society for Microbiology, Washington D. C.

Prodromou, C., Roe, S.M., O'Brien, R., Ladbury, J.E., Piper, P.W. & Pearl, L.H. (1997) Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* **90**, 65-75.

Qian, Y. & Tabita, F.R. (1996) A global signal transduction system regulates aerobic and anaerobic CO_2 fixation in *Rhodobacter sphaeroides*. *J. Bacteriol.* **178**, 12-18.

Qin, L., Dutta, R., Kurokawa, H., Ikura, M. & Inouye, M. (2000) A monomeric histidine kinase derived from EnvZ, an *Escherichia coli* osmosensor. *Mol. Microbiol.* **36**, 24-32.

Regensburger, B. & Hennecke, H. (1983) RNA polymerase from *Rhizobium japonicum*. *Arch. Microbiol.* **135**, 103-109.

Reyrat, J.-M., David, M., Batut, J. & Boistard, P. (1994) FixL of *Rhizobium meliloti* enhances the transcriptional activity of a mutant FixD54N protein by phosphorylation of an alternate residue. *J. Bacteriol.* **176**, 1969-1976.

Richardson, D.J. (2000) Bacterial respiration: a fexible process for a changing environment. *Microbiology* **146**, 551-571.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor NY.

Sanger, F., Nicklen, S. & Coulson, A.R. (1977) Sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Santero, E., Hoover, T.R., North, A.K., Berger, D.K., Porter, S.C. & Kustu, S. (1992) Role of integration host factor in stimulating transcription from the σ^{54}-dependent *nifH* promoter. *J. Mol. Biol.* **227**, 6026-20.

Schröder, I., Wolin, C.D., Cavicchioli, R. & Gunsalus, R.P. (1994) Phosphorylation and dephosphorylation of the NarQ, NarX, and NarL proteins of the nitrate-dependent two-component regulatory system of *Escherichia coli*. *J. Bacteriol.* **176**, 4985-4992.

Sganga, M.W. & Bauer, C.E. (1992) Regulatory factors controlling photosynthetic reaction center and light-harvesting gene expression in *Rhodobacter capsulatus*. *Cell* **68**, 945-954.

Simon, R., Priefer, U. & Pühler, A. (1983) Vector plasmids for *in vivo* and *in vitro* manipulation of gram-negative bacteria. In: Pühler, A. (ed.) Molecular genetics of the bacteria-plant interaction, Springer-Verlag, Heidelberg, Germany, pp 98-106.

Smith, D.E. & Fisher, P.A. (1984) Identification, developmental regulation, and response to heat shock of 2 antigenically related forms of a major nuclear envelope protein in *Drosophila* embryos: Application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. *J. Cell Biol.* **99**, 20-28.

Solà, M., Gomis-Rüth, F.X., Serrano, L., González, A. & Coll, M. (1999) Three-dimensional crystal structure of the transcription factor PhoB receiver domain. *J. Mol. Biol.* **285**, 675-678.

Stewart, V. & Rabin, R.S. (1995) Dual sensors and dual response regulators interact to control nitrate and nitrite-responsive gene expression in *Escherichia coli*. In: *Two-Component Signal Transduction* (Hoch, J.A. & Silhavy, T.H. eds) pp. 233-252. American Society for Microbiology, Washington D. C.

Stock, A.M., Mottonen, J.M., Rasmussen, B.F., West, A.H., Stock, J.B., Ringe, D. & Petsko, G.A. (1993) Structure of the Mg^{2+}-bound form of CheY and mechanisms of phosphoryl transfer in bacterial chemotaxis. *Biochemistry* **32**, 13375-13380.

Stock, A.M., Mottonen, J.M., Stock, J.B. & Schutt, C.E. (1989) Three-dimensional structure of CheY, the response regulator of bacterial chemotaxis. *Nature* **337**, 745-749.

Stock, J.B., Surette, M.G., Levit, M. & Park, P. (1995) Two-component signal transduction systems: structure-function relationships and mechanisms of catalysis. In: *Two-Component Signal Transduction* (Hoch, J.A. & Silhavy, T.H. eds) pp. 25-51. American Society for Microbiology, Washington D. C.

Studier, F.W., Rosenberg, A.H., Dunn, J.J. & Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60-89.

Swanson, R.V., Alex, L.A. & Simon, M.I. (1994) Histidine and aspartate phosphorylation: two-component systems and the limits of homology. *Trends Biochem. Sci.* **19**, 485-490.

Tanaka, T., Saha, S.K., Tomomori, C., Ishima, R., Liu, D., Tong, K.I., Park, H., Dutta, R., Qin, L., Swindells, M.B., Yamazaki, T., Ono, A.M., Kainosho, M., Inouye, M. & Ikura, M. (1998) NMR structure of the histidine kinase domain of the *E. coli* osmosensor EnvZ. *Nature* **396**, 88-92.

Thöny, B., Anthamatten, D. & Hennecke, H. (1989) Dual control of the *Bradyrhizobium japonicum* symbiotic nitrogen fixation regulatory operon *fixRnifA*: analysis of *cis*- and *trans*-acting elements. *J. Bacteriol.* **171**, 4162-4169.

Thöny, B., Fischer, H.M., Anthamatten, D., Bruderer, T. & Hennecke, H. (1987) The symbiotic nitrogen fixation regulatory operon (*fixR-nifA*) of *Bradyrhizobium japonicum* is expressed aerobically and is subject to a novel, *nifA*-independent type of activation. *Nucleic Acids Res.* **15**, 8479-8499.

Tiwari, R.P., Reeve, W.G., Dilworth, M.J. & Glenn, A.R. (1996) Acid tolerance in *Rhizobium meliloti* strain WSM419 involves a two-component sensor-regulator system. *Microbiology* **142**, 1693-1704.

Tomomori, C., Tanaka, T., Dutta, R., Park, H., Saha, S.K., Zhu, Y., Ishima, R., Liu, D., Tong, K.I., Kurokawa, H., Qian, H., Inouye, M. & Ikura, M. (1999) Solution structure of the homodimeric core domain of *Escherichia coli* histidine kinase EnvZ. *Nat. Struct. Biol.* **6**, 729-734.

Tuerk, C. & Gold, L. (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science.* **249**, 505-510.

Varughese, K.I., Madhusudan, Zhou, X.Z., Whiteley, J.M. & Hoch, J.A. (1998) Formation of a novel four-helix bundle and molecular sites by dimerization of a response regulator phosphotransferase. *Mol. Cell* **2**, 485-493.

Vescovi, E.G., Ayala, Y.M., Di Cera, E. & Groisman E.A. (1997) Characterization of the bacterial sensor protein PhoQ. *J. Biol. Chem.* **272**, 1440-1443.

Vichivanives, P., Bird, T.H., Bauer, C.E. & Tabita, R.F. (2000) Multiple regulators and their interactions *in vivo* and *in vitro* with the *cbb* regulons of *Rhodobacter capsulatus*. *J. Mol. Biol.* **300**, 1079-1099.

Volz, K. & Matsumura, P. (1991) Crystal structure of *Escherichia coli* CheY refined at 1.7-Å resolution. *J. Biol. Chem.* **266**, 15511-15519.

Volz, K. (1995) Structural and functional conservation in response regulators. In: *Two-Component Signal Transduction* (Hoch, J.A. & Silhavy, T.H. eds) pp. 53-64. American Society for Microbiology, Washington D. C.

Walker, M.S. & DeMoss, J.A. (1993) Phosphorylation and dephosphorylation catalyzed *in vitro* by purified Ccmponents of the nitrate sensing system, NarX and NarL*. *J. Biol. Chem.* **268**, 8391-8393.

Weiss, D.S., Batut, J., Klose, K.E., Keener, J. & Kustu, S. (1991) The phosphorylated form of the enhancer-binding protein NtrC has an ATPase activity that is essential for activation of transcription. *Cell* **67**, 155-167.

Weiss, V. & Magasanik, B. (1988) Phosphorylation of nitrogen regulator I (NR_I) of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **85**, 8919-8923.

Weiss, V., Claverie-Martin, F. & Magasanik, B. (1992) Phosphorylation of nitrogen regulator I of *Escherichia coli* induces strong cooperative binding to DNA essential for activation of transcription. *Proc. Natl. Acad. Sci. USA* **89**, 5088-5092.

Wigley, D.B., Davies, G.J., Dodson, E.J., Maxwell, A. & Dodson, G. (1991) Crystal structure of the DNA gyrase B protein. *Nature* **351**, 624-629.

Wyman, C., Rombel, I., North, A.K., Bustamante, C. & Kustu, S. (1997) Unusual oligomerization required for activity of NtrC, a bacterial enhancer-binding protein. *Science* **275**, 1658-1661.

Yan, D. & Kustu, S. (1999) "Switch I" mutant forms of the bacterial enhancer-binding protein NtrC that perturb the response to DNA. *Proc. Natl. Acad. Sci. USA* **96**, 13142-13146.

Yan, D., Cho, H.S., Hastings, C.A., Igo, M.M., Lee, S.Y., Pelton, J.G., Stewart, V., Wemmer, D.E. & Kustu, S. (2000) Beryllofluoride mimics phosphorylation of NtrC and other bacterial response regulators. *Proc. Natl. Acad. Sci. USA* **96**, 14789-14794.

Yang, Y. & Inouye, M. (1991) Intermolecular complementation between two defective mutant signal-transducing receptors of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **88**, 11057-11061.

Yen, H.C. & Marrs, B.L. (1976) Map of genes for carotenoid and bacteriochlorophyll biosynthesis in *Rhodopseudomonas capsulata*. *J. Bacteriol.* **126**, 619-629.

Young, D.A., Bauer, C.E., Williams, J.C. & Marrs, B.L. (1989) Genetic evidence for superoperonal organization of the genes for photosynthesis pigments and pigment-binding proteins in *Rhodobacter capsulatus*. *Mol. Gen. Genet.* **218**, 1-12.

Zhou, H., Lowry, D.F., Swanson, R.V., Simon, M.I. & Dahlquist, F.W. (1995) NMR studies of the phosphotransfer domain of the histidine kinase CheA from *Escherichia coli*: assignments, secondary structure, general fold, and backbone dynamics. *Biochemistry* **34**, 1358-1370.

Curriculum vitae

of Ralf Emmerich

Born on March 2nd, 1971 in Friedrichshafen, Germany

1977-1981	Primary school in Eriskirch, Germany
1981-1990	Secondary education at the Montfort-Gymnasium Tettnang, Germany; Abitur
1990-1991	Military service
1991-1997	Studies of Biochemistry at the Universität Bayreuth, Germany and the Université Paris VII (Denis Diderot), France (ERASMUS-scholarship); Diploma in Biochemistry
1997-present	Research assistant at the Institute of Microbiology, Swiss Federal Institute of Technology (ETH), Zürich; Ph.D. Thesis

Publications

Bauer, E., Kaspar, T., Panglungtshang, K., **Emmerich, R.,** Fischer, H.M. & Hennecke, H. (1998) A two-component regulatory system ensures *nifA* expression in *Bradyrhizobium japonicum*. In: *Biological nitrogen fixation for the 21st century* (Elmerich, C., Kondorosi, A. & Newton, W.E., eds), pp. 135-136. Kluwer Academic Publishers, London.

Emmerich, R., Panglungtshang, K., Strehler, P., Hennecke, H. & Fischer, H.M. (1999) Phosphorylation, dephosphorylation and DNA-binding of the *Bradyrhizobium japonicum* RegSR two-component regulatory proteins. *Eur. J. Biochem.* 263, 455-463.

Fischer, H.M., Bauer, E., **Emmerich, R.,** Minder, A., Narberhaus, F., Schaeren, S., Zingg, D. & Hennecke, H. (1999) Integration of regulatory networks for different physiological functions in *Bradyrhizobium japonicum*. In: *Highlights of nitrogen fixation research* (Martínez, M. & Hernández, G., eds), pp. 189-193. Kluwer Academic Publishers, London.

Emmerich, R., Strehler, P., Bauer, E., Fischer, H.M. & Hennecke, H. (1999) Functional analysis of the *Bradyrhizobium japonicum* RegSR two-component regulatory system. In: *Nitrogen fixation: from molecules to crop productivity.* (Pedrosa, F.O., Hungria, M., Yates, G. & Newton, W.E., eds), pp. 89-90. Kluwer Academic Publishers, London.

Emmerich, R., Hennecke, H. & Fischer, H.M. (2000) Evidence for a functional similarity between the two-component regulatory systems RegSR, ActSR, and RegBA (PrrBA) in α-Proteobacteria. *Arch. Microbiol.*, in press.

Emmerich, R., Strehler, P., Hennecke, H. & Fischer, H.M. (2000) An imperfect inverted repeat is critical for DNA-binding of the response regulator RegR of *Bradyrhizobium japonicum*. *Nucleic Acids Res.*, in press.