Atypical transcriptional regulation and function of a new toxin-antitoxin-like module in *Bradyrhizobium japonicum*

Ph.D. Thesis

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1. Introduction

The new, fast and consistent methods of small genome sequencing accelerated the publication of completely sequenced microbial genomes.. A wide range of predictive bioinformatics approaches, together with the observation of gene fusions in different species, revealed the much greater complexity of prokaryotic organisms, which were previously believed to have simple organization and functions. During the last 30 years, the combination of existing databases, bioinformatics analysis and techniques of molecular biology led to the identification and characterization of a type of genetic modules encoded by a high number of prokaryotic genomes. These are the so-called toxin antitoxin (TA) modules.

TA modules consisting of two partially overlapping genes are ubiquitous among bacteria and archaea (Arcus et al., 2004; Gerdes et al., 2005). Toxin genes encode stable proteins harmful to an essential cell process, and the protein products of the generally upstream located antitoxin genes counteract the toxins by forming a complex with them. TA operons are under the negative control of the toxin-antitoxin complex, which is able to bind to the promoter region and prevent transcription of the module (Gerdes et al. 2005).

Discovered in 1983 by Ogura and co-workers, they were first called "plasmid addiction systems" as they prevented the proliferation of plasmid-free progeny. After their discovery these loci were considered of minor importance, until ten years later, when chromosomal homologs were discovered in *Escherichia coli* (Masuda et al., 1993) renewing interest in them. Chromosomal TA genes are widely distributed and abundant in bacteria that live in changing evironments, and they are thought to control growth and survival in response to starvation or other adverse conditions. The proposed role of TA loci as stress managers under varying environmental stimuli may be of special importance during the adaptation of soil bacteria to oligotrophic conditions. In addition, symbiotic nitrogen-fixing soil bacteria which develop an intimate interaction with leguminous plants have the ability to adapt and function also within the plant host cells during symbiosis.

Accumulation of new interesting data, regarding their roles, and identification of a wide diversity of TA sytems at multiple locations within a genome and in multiple

bacterial species attracted considerable attention as they were reveal to be of high importance to the prokaryotic world.

1.1 Types of TA modules

Two types of TA systems have been identified, depending on the nature of the components. Antitoxins can be proteins or RNAs, while toxins are always proteins. Antitoxins of the type I systems are small RNAs (antisense or adjacent and divergent to the toxin gene) complementary to the toxin mRNA (Fozo et al., 2008, Gerdes and Wagner, 2007). The *hok/sok* system of plasmid R1 is the prototype of the family of antisense RNA-regulated gene systems (Gerdes et al., 1990). The *hok/sok* locus codes for the Hok (*ho*st *k*illing) protein, a membrane-associated toxin that causes irreversible damage of the cell membrane and thus it is lethal to host cells. The *sok* (*s*uppression *o*f *k*illing) gene of this locus encodes an antisense RNA, which is complementary to the *hok* mRNA leader region. The *sok*-RNA is unstable, but constitutively expressed from a relatively strong promoter. By contrast, *hok* mRNA is very stable and constitutively expressed from a relatively weak promoter.

In type II systems, the antitoxin is a small, unstable protein that sequesters the toxin through protein complex formation (Gerdes and Wagner, 2007). The genetic organization of a type II locus is represented in Figure 1. Two adjacent genes form an operon and they partly overlap at the stop/start codons. The encoded proteins (antitoxin and toxin) form a complex and by this way the antitoxin prevents the damaging effect of the toxin, as long as they are co-expressed. The protein complex, and sometimes the antitoxin alone, negatively autoregulates the transcription of the module by binding to its promoter. Under stress conditions (e.g. amino acid starvation, upregulation of specific proteases, presence of antibiotics) (Sat et al., 2001; Hazan et al., 2004,) that inhibit TA expression, the cellular amount of antitoxin decreases faster than that of the toxin because more labile antitoxin is degraded by proteases, allowing the uncomplexed toxin to act freely on various cellular targets.

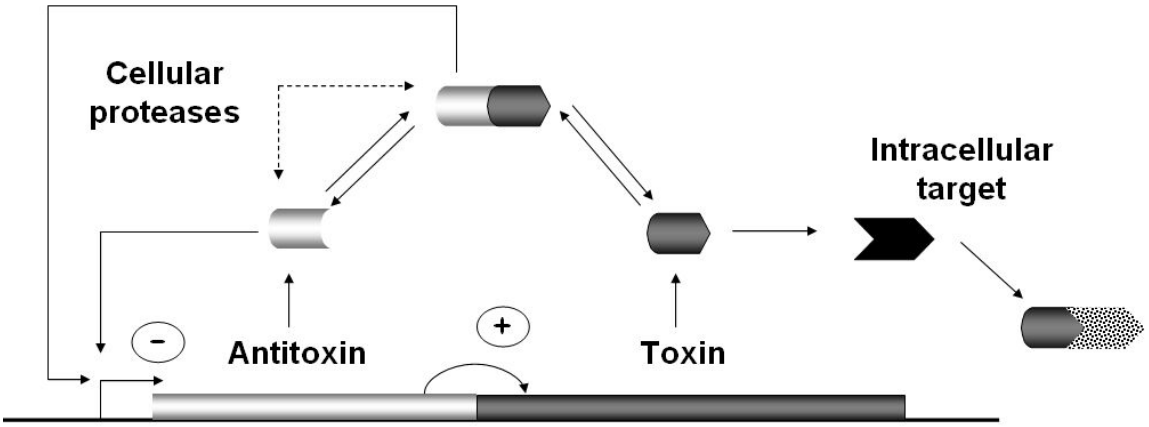


Figure 1. Genetic organization and components of a typical type II TA locus. Toxin gene and protein are shown in dark grey; the antitoxin gene and protein are represented in light grey. Interrupted broken arrows indicate that cellular proteases degrade the antitoxin, either in its free in solution or in complex with the toxin. The arrow in front of the antitoxin and toxin genes indicates a promoter upstream of the TA operon. Arrows pointing at the promoter show that both the antitoxin and the TA complex can bind to the promoter and repress transcription. The curved arrow marks a translational coupling between the toxin and antitoxin gene. The intracellular target is represented with black or patterned symbol. in accordance with its intact or toxin-degraded state (Gerdes et al., 2005).

1.2 Classification of type II TA modules

Gerdes and his coworkers (2005) proposed the arrangement of toxin-antitoxin loci in seven families (Table 1). TA systems have been organized in families on the basis of sequence similarity of the toxins and the nature of their cellular targets. Although ParE toxins have weak but significant similarity with RelE and HigB toxins (Anantharaman and Aravind., 2003), their cellular targets are different: RelE cleaves mRNA, while experimental data indicate that ParE targets DNA gyrase (Jiang et al., 2002). All TA loci belonging to these seven families have the same modular genetic set-up and overall similar regulatory properties, except for the *higBA* family that has a reversed gene order (the *higB* toxin gene is located upstream of *higA* that encodes the antitoxin).

Table 1. The seven typical toxin-antitoxin gene families (Gerdes et al., 2005)

TA family	Toxin	Antitoxin	Target of toxin	Number of loci*	Phyletic distribution
Ccd	CcdB	CcdA	DNA gyrase	5	Gram-negative bacteria
parDE	ParE	ParD	DNA gyrase	59	Gram-negative and Gram-positive bacteria
relBE	RelE	RelB	mRNA	156	Gram-negative and Gram-positive bacteria, Archaea
mazEF	MazF/PemK	MazE/PemI	mRNA	67	Gram-negative and Gram-positive bacteria
phd/doc	Doc	Phd	Translation elongation	25	Gram-negative and Gram-positive bacteria, Archaea
higBA	HigB	HigA	Unknown	74	Gram-negative and Gram-positive bacteria
vapBC/vag	VapC	VapB	RNA**	285	Gram-negative and Gram-positive bacteria, Archaea

* Number of loci found by exhaustive BLAST search in the genomes of 126 prokaryotic organisms

** Arcus et al., 2004 ; Daines et al., 2007

There are additional systems (the *hipBA* type and the restriction-modification enzymes), that are classified sometimes as TA modules, but more frequently they are referred to as modules analogous to TA systems. Although the *hipBA* (high persistence) operon resembles typical organization and autoregulatation of other TA systems, a mutation in *hipA* increases the frequency of persisters that survive penicillin treatment through a molecular mechanism that remains elusive (Gerdes et al., 2005). Restriction-modification enzyme pairs encode a restriction enzyme that cleaves DNA site-specifically and a modification system that modifies the target DNA so that cleavage is prevented. Cells cannot survive with a functioning restriction gene in the absence of the coresponding modification gene (Kobayashi I., 2001).

1.3 Diversity and abundance of TA modules in bacteria

Comprehensive genome analyses have highlighted the vast diversity in the structural composition of TA systems (Anantharaman and Aravind, 2003; Magnuson, 2007). Using sequence profile analysis, Anatharaman and Aravind demonstrated the similarities between the RelE-type of toxins and several families of small, uncharacterized proteins from diverse bacteria and archaea. Gene neighborhood analysis showed that the majority of these proteins were encoded by genes associated with other genes encoding transcription factors that are predicted to function as antitoxins. Although the transcription factors and the toxins of these operons form a compatible functional pair, they can belong to totally unrelated superfamilies of proteins. For example, toxins carrying a PIN domain were identified to be associated with a large array of antitoxins containing one of the DNA binding motifs AbrB, cHTH (helix-turn-helix), Phd/YefM, or MetJ/CopG (Figure 2).

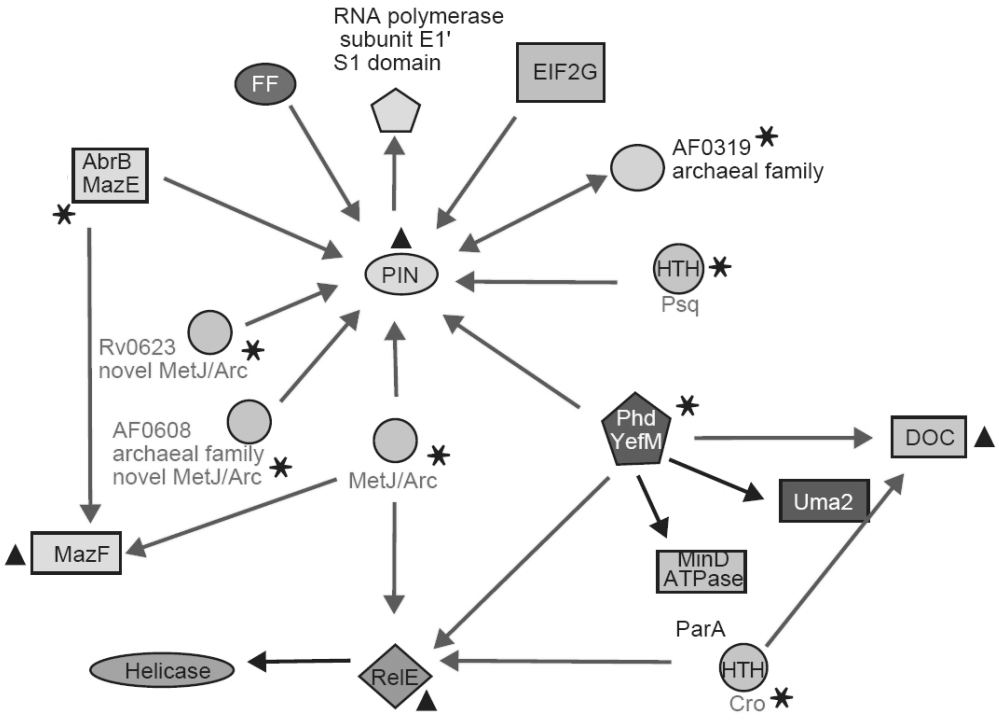
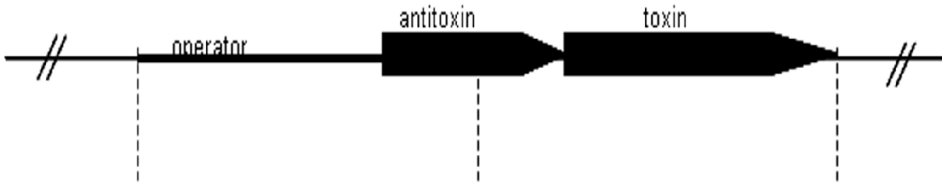


Figure 2. The diversity of TA systems based on the analysis of gene neighborhoods and domain fusions. Although the antitoxins and the toxins of these operons form a compatible functional pair, they can belong to totally unrelated superfamilies of proteins. PIN domains were identified in proteins belonging to unrelated protein families (RNA polymerases, elF2 translation initiation factors) or in toxins associated with antitoxins containing one of the DNA binding motifs AbrB, cHTH (helix-turn-helix), Phd/YefM, or

MetJ/CopG. Triangles indicate toxins and the stars indicate antitoxins/transcription factors (Anantharaman and Aravind, 2003).

Although these toxin-antitoxin modules share many general features, they are a diverse group at the molecular level. Such diversity may indicate long phylogenetic history, positive selection for diversity or multiple origins for the various TA systems. Smith and Magnuson (2004) proposed that toxin-antitoxin systems are composed of two evolutionarily independent modules joined by a transition region. The first module is the operator-repressor represented by the operator sequence of the operon and a DNA binding domain at the N-terminal part of the antitoxin. The second module is formed by the C-terminal part of the antitoxin, required for protein complex formation, and the toxin protein. Recombination events in the transition region between different TA systems may contribute to toxin-antitoxin diversity (Figure 3).



<-----1st Module---><--2nd Module-->

First Parent P operator *repressor-antitoxin toxin*

Recombinant P operator *repressor*-***antitoxin toxin***

Second Parent P operator *repressor-antitoxin toxin*

Figure 3. Modular model for TA systems. Toxin-antitoxin systems are composed of two evolutionarily independent modules: an operator-repressor module (represented by the operator sequence and the N-terminal part of the antitoxin) and an antitoxin-toxin module (represented by the C-terminal part of the antitoxin and the toxin preotein). Recombination between modules may contribute to operon and antitoxin diversity. A schematic of two parental genetic systems (the first in plain text, the second in bold text) and a recombinant structure are shown. The recombination occurs in the transition region between the two modules. Such a recombinant is likely to be functional even if the parental structures are distantly related (Smith and Magnuson., 2004) .

An exhaustive search for the identification of TA systems in prokaryotic genomes revealed that prokaryotic chromosomes often encode multiple copies of TA loci, while others contain no such loci (Figure 4). *Nitrosomonas europeae*, *Mycobacterium tuberculosis*, and *Mycobacterium bovis* contain more than 35 putative TA systems. Others contain no or very few (less than three) putative TA systems, such as *Rickettsia*

prowazeki, *Campylobacter jejuni*, or *Bacillus subtilis*. It was assumed that bacteria living in unstable environments encode toxin-antitoxin modules, while the genomes of obligate intracellular organisms lack TA loci (Pandey and Gerdes, 2005). Support for this notion came from the pattern of TA loci in *Mycobacteria* (*Mycobacterium tuberculosis* H37Rv has 38 loci, whereas *Mycobacterium leprae* has no intact TA loci) and spirochetes (free-living *Leptospira interrogans* has 5 TA loci, while obligate parasitic spirochetes *Treponema pallidum* and *Borrelia burgdorferi* have no TA loci) (Pandey and Gerdes, 2005).

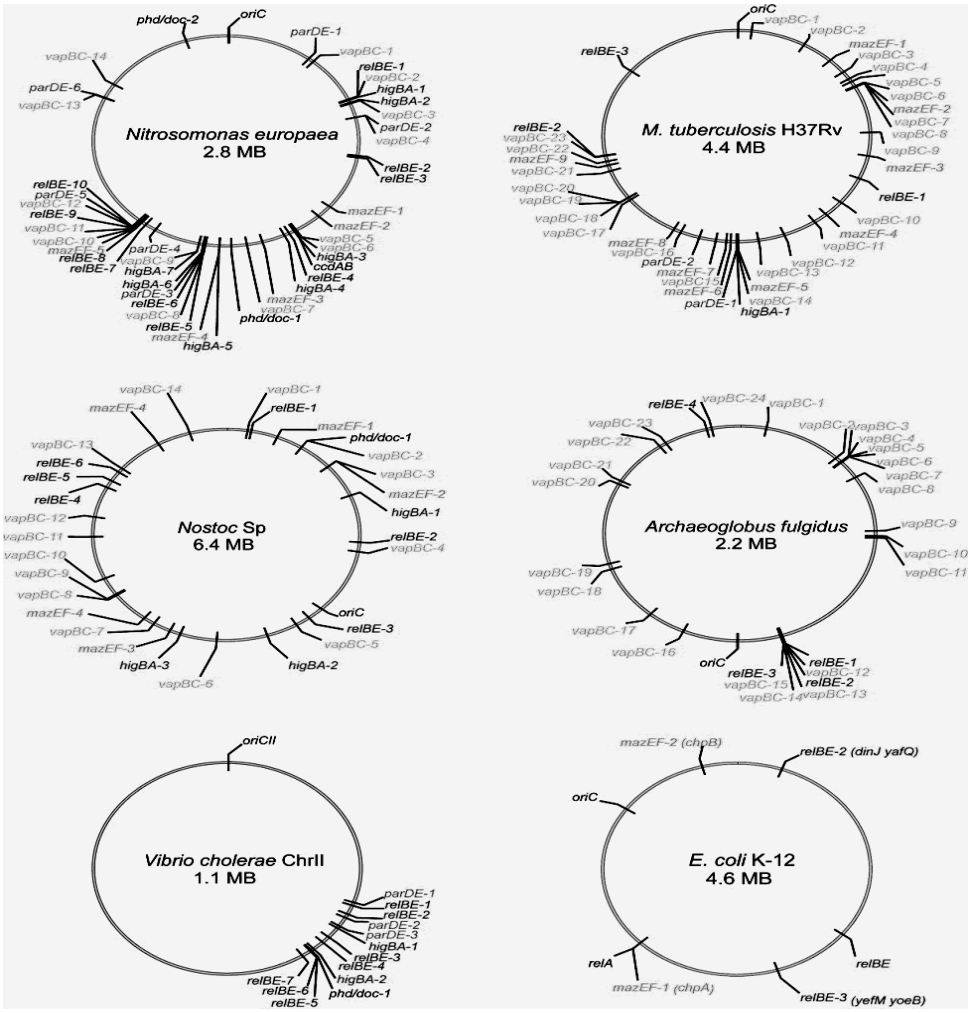


Figure 4. Chromosomal locations of TA loci in different species. The number of chromosomal TA loci in a given species may vary from none to more than 60. *Nitrosomonas europaea* and *Mycobacterium tuberculosis* contain more than 35 putative TA systems. The chromosome of *E. coli* contains five such TA loci (Pandey and Gerdes, 2005).

1.4 Multiple mechanisms of activation and action of TA systems

The action of TA systems is based on the different stabilities of toxin and antitoxin proteins: the unstable antitoxins are degraded faster than the stable toxins (Buts et al., 2005; Gerdes et al., 2005; Hayes, 2003). Under favorable growth conditions, TA loci produce sufficient antitoxin to bind and inactivate the cognate toxin, therefore toxin effects are blocked. However, under unfavorable conditions, the balance between toxin and antitoxin is disturbed, and the free toxin is able to act on various cellular targets. Altered toxin/antitoxin ratio can be induced by stress conditions, such as starvation, antibiotics, DNA damage, and oxidative stress, which can reduce antitoxin synthesis and/or activate antitoxin-degrading proteases (Christensen et al., 2001; Hazan et al., 2004; Sat et al., 2001). In addition, the loss of the toxin-antitoxin genes by segregation, exclusion, mutation or recombination can liberate the toxin and damage the cell.

The toxins characterized so far include gyrase inhibitors, phosphotransferases, site-specific ribonucleases, ribosome-dependent ribonucleases, and a possible riboexonuclease (Arcus et al., 2004; Jensen and Gerdes, 1995; Pedersen et al., 2003; Zhang et al., 2003 a). In principle, any essential structure or process could be targeted by these toxins. In practice, mRNA-targeting ribonucleases are particularly common and the immediate effects of these RNase toxins seem to be bacteriostatic, rather than bactericidal (Pedersen et al., 2003).

MazF is an endoribonuclease interfering with mRNA function by specifically cleaving cellular mRNA at the ACA triplet sequence, independently of ribosomes. The ACA sequence in the RNA/DNA or RNA/RNA duplex cannot be cleaved, indicating that this sequences within double-stranded duplex structures cannot be digested by MazF. Interestingly, both 16S and 23S rRNAs in the cell are protected from MazF cleavage even if they contain a large number of ACA sequences. The observed resistance of rRNA against MazF cleavage appears to be due to protection by ribosomal proteins. Resistance of some mRNA to the MazF-mediated degradation may also be related to the efficiency of translation initiation. An mRNA having better translation initiation efficiency is likely to be more protected by ribosomes bound to the mRNA (Zhang et al. 2003 b). Pedersen and colleagues reported (2002) that the toxic effect of MazF can be reversed by the action of the antitoxin MazE overexpressed at a later time, and suggested that rather than inducing cell death, MazF induces a state of reversible bacteriostasis. However, it was

found that overexpression of MazE can reverse MazF lethality only over a short period of time. There is a "point of no return" after which the continuous induction of *mazF* causes an irreversible loss of viability (Amitai et al. 2004).

RelE of *E. coli* and its partner antitoxin RelB are encoded by the *relBE* locus. As in the case of MazF, overexpression of RelE inhibited translation by mRNA cleavage. During normal growth, RelB was expressed in excess over RelE. However, during amino acid starvation, the labile RelB antitoxin was degraded by Lon protease, enabling the free RelE endonuclease to cleave ribosome-bound mRNAs between the second and third bases of the A-codons. Such cleavage results in stalling of the ribosomes bound to the damaged mRNAs. *E. coli* transfer-messenger RNA (tmRNA) releases stalled ribosomes from damaged mRNAs, and it tags the new polypeptides from such ribosomes for proteolysis. It was demonstrated that tmRNA stimulated the restarting of translation in RelE-inhibited cells by rescuing ribosomes stalled on RelE cleaved mRNAs. However, this mechanism is activated only in the presence of RelB which neutralizes the RelE toxin or if the tmRNA is overexpressed. Importantly, still no toxin has been reported to be directly bacteriolytic (Christensen and Gerdes, 2003; Christensen et al., 2003).

The largest family of TA modules is formed by the *vapBC* operons, and their functions and mode of action are still under investigation. VapC toxins are homologues of the *pilT* N-terminus domain (PIN domain), which are small proteins with structural homology to the T4 RNase H nuclease domain (Arcus et al., 2005). Multiple sequence alignments have shown that the active site residues are highly conserved within the PIN domains, although their sequence similarity is low. These residues were first predicted *in silico* to have nuclease activity, which was then confirmed *in vitro* (Arcus et al. 2004). Overexpression of VapC of *Mycobacterium smegmatis* determined growth inhibition via disruption of translation, suggesting a ribonuclease function. No effect was obserbed on DNA replication or transcription in the presence of overexpressed VapC toxin (Robson et al., 2009). After the determination of the crystal structure of *Mycobacterium tuberculosis* VapBC-5, it was suggested that VapC-5 could be a 3'-endoribonuclease, an exoribonuclease, or both. In an *in vitro* experiment, VapC-5 clearly showed nuclease activity on double-stranded RNA, but not on DNA (Miallau et al., 2009). Similarly, VapC-1 toxin of *Haemophilus influenzae* is active on free RNA, but does not display general nuclease activity, as it does not degrade double- or single-stranded DNA under

the same conditions (Daines et al. 2007). The sequence specificity and exact mechanism of RNA cleavage by VapC toxins still remains to be elucidated.

1.5 Physiological roles of the toxin-antitoxin modules

RelE and ParE proteins of *E. coli* were the first model proteins to investigate the role of TA systems. These proteins were among the functionally best characterized toxins of the post-segregational killing system (PSK). This is a widespread mechanism that helps low copy number plasmids to be maintained in their bacterial hosts. If the plasmid carrying these TA modules is lost in the progeny, the antidote decays, but the stable toxin persists, killing the cell (Yarmolinsky, M. B., 1995). This eliminates all plasmid-free cells from the population, independently of the manner by which the plasmid was lost, thus ensuring plasmid maintenance (Figure 5).

The biological functions of the chromosomally encoded TA systems have been an area of intense debate for several years. Several current hypotheses have been proposed to explain their function in microbes.

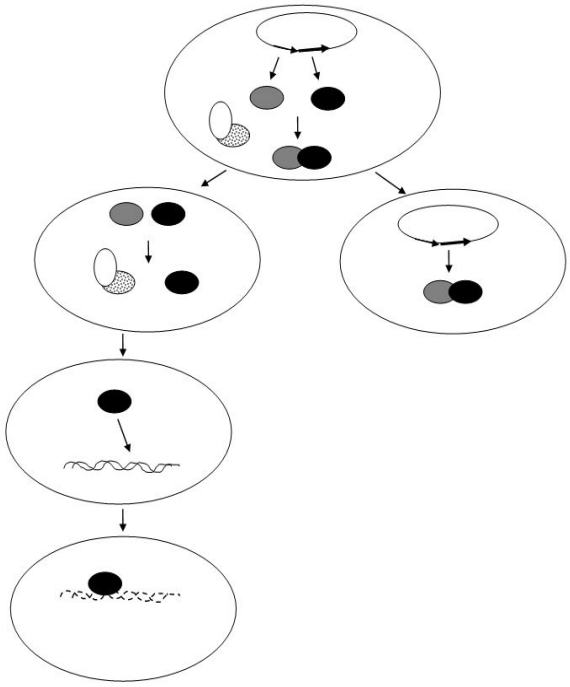


Figure 5. Schematic representation of cell death induced by post-segregational killing. The toxin (black) and antitoxin (grey) proteins form a tight complex that negates the harmful activity of the toxin. The antitoxin is degraded by a protease (white) more rapidly than the toxin, but the latter is continually sequestered by fresh antitoxin. As long as the plasmid is maintained, the cell tolerates the presence of the TA complex (right). If a missegregation event or replication defect produces a plasmid-free cell (left), the degraded antitoxin cannot be replenished, so the liberated toxin attacks an intracellular target to cause death or growth restriction of the plasmid-free cell. The targeting of DNA by the toxin is illustrative only (Hayes, 2003).

Chromosomally located TA modules were considered to have a function similar to that of the plasmid-located TA modules, whose role is to prevent the proliferation of the plasmid free progenies. They were considered to be selfish operons whose functions are related to their own inheritance and those of the neighboring genes (Cooper et al., 2000; Pandey et al., 2005). However, this hypothesis may not be true, since selfish DNA fragments that exploit their hosts are usually difficult to delete from the genomes, while deletions in the chromosomal TA operons was relatively easy to obtain.

It is clear that ectopic overexpression of toxins in the absence of antitoxins prevents bacterial growth. Furthermore, TA-dependent cell death was observed in *E. coli* after the activation of endogenous MazF and YoeB, encoded by two of the five known chromosomal TA loci in this species (Engelberg-Kulka and Hazan, 2003; Kedzierska et al., 2007). Several studies proposed that the MazF toxin induces programmed cell death

in certain cells, allowing a subset of cells to be sacrificed for the benefit of the bacterial population as a whole (Aizenma et al., 1996; Engelberg-Kulka et al., 2004). Activation of these systems was reported under various stressful conditions, such as amino acid starvation (Hazan et al., 2004; Amitai et al., 2004), thymine starvation, DNA damage (Sat et al., 2003), the presence of antibiotics (Sat et al., 2001) or infecting phages (Hazan et al. 2004). However, this hypothesis has been contested by Gerdes and colleagues (Christensen et al., 2003; Gerdes et al., 2005), who argued that several toxins, including MazF, have a bacteriostatic, rather than bactericidal effect that facilitates bacterial adaptation to stress. The part of the population that survives these stress conditions maintains the capacity to resume normal physiology. They reported that transient activation of endogenous TA loci can slow cell metabolism through the inhibition of protein synthesis, but cell growth is restored after the removal of the toxin-inducing stimulus (Christensen and Gerdes, 2003).

When Tsilibaris and co-workers (2007) compared the behavior of *E. coli* wild-type strain and its derivative devoid of all the five TA systems, they were unable to detect TA-dependent programmed death under the conditions previously reported to induce it. Moreover, the presence of the five TA systems advanced neither recovery from the different stresses, nor cell growth under nutrient-limited conditions in competition experiments. Considering these results, it was proposed that in fact chromosomal TA systems may act as antiaddiction modules that protect host bacteria against PSK mediated by their plasmid-encoded counterparts. More recently, the TA system-mediated death hypothesis was revived with new results about how each of the five TA systems of *E. coli* affect bacterial cell death differently in liquid media and during biofilm formation. Of all these systems, only the *mazEF* TA system mediated cell death both in liquid media and during biofilm formation. Another TA system, the *dinJ-YafQ* of *E. coli* was shown to be involved in the death process only during biofilm formation (Kolodin-Gal et al., 2009).

The function and the importance of chromosomally located TA systems are still subjects of intense studies, despite the accumulation of vast information in the last period. At present, all of the hypotheses regarding the function of chromosomal TA systems are greatly disputed and multiple hypotheses may apply to a single TA system.

1.6 Biotechnological and medical applications of TA loci

TA loci were proposed to be useful for several practical purposes in the future.

Plasmid stabilization. TA and PSK loci can efficiently stabilize plasmids. This is useful in large-scale productions that involve recombinant plasmids. Accordingly, the *hok/sok* PSK and *parDE* loci have been used to construct gene cassettes that can be inserted into unstably inherited plasmids and they lead to increased genetic stability of producer strains (Gerdes and Helin, 1988; Pecota et al., 1997).

Positive selection vectors. The *ccdB* and *kid* genes have both been used to construct positive selection vectors. Unique restriction sites for cloning were introduced into toxin genes so that the genes still encoded active toxins. Plasmids harbouring a functional toxin gene cannot be maintained in host cells that are sensitive to the toxin. However, after cloning a DNA restriction fragment into the plasmid-borne toxin gene, the toxin gene is disrupted, so the plasmid can be maintained in the sensitive host cell. These positive selection vectors are useful for constructing libraries that require large numbers of recombinant clones. The *Gateway Technology* of Invitrogen (Carlsbad, California, USA) enables efficient transfer of DNA-fragments between plasmids using a set of recombination sequences. The donor plasmid contains the DNA fragment to be transferred between the recombination sequences while the acceptor plasmid contains the gene coding for the CcdB toxin between the same recombination sequences. Cells that take up unreacted vectors carrying the *ccdB* gene will fail to grow. This allows high-efficiency recovery of the desired clones.

Active biological containment and eukaryotic cell proliferation control. Genetically modified organisms should be maintained in pre-determined and restricted areas. Toxin-encoding genes have been used to construct genetically modified organisms that self-destruct under specific conditions and, therefore, aid containment. The RelE toxin of *relBE* system of *E. coli* was highly toxic in yeast cells offering a potential application as containment control of genetically modified organisms (Kristoffersen et al., 2000). The Kid toxin, of *kis/kid* system, inhibits cell proliferation in yeast, *Xenopus laevis* and human cells, while Kis protects them. These findings allow highly regulatable, selective killing of eukaryotic cells, and could be applied to eliminate cancer cells or specific cell lineages in development (de la Cueva-Mendez et al., 2003).

TA complexes as drug targets. Most free-living pathogenic bacteria encode TA loci, often in multiple copies. Compounds that activate or mimic bacterial toxins could be developed into novel antibiotics that could function through several different mechanisms. One mechanism would be to prevent or reduce the association between members of a given TA pair. A more general mechanism would be based on the manipulation of the signalling pathways that lead to toxin activation.

1.7 Toxin-antitoxin modules in nitrogen fixing symbiotic bacteria

The process of symbiotic nitrogen fixation is restricted to a limited number of bacterial groups, including the genera *Rhizobium, Mesorhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium* and *Frankia*. The members of these genera have the unique ability to induce nitrogen-fixing nodules on the roots or stems of leguminous plants in a host-specific manner. *Sinorhizobium meliloti* nodulates *Medicago, Melilotus* and *Trigonella* species, *Bradyrhizobium japonicum* nodulates *Glycine max* (soybean), *Macroptilium atropurpureum* (siratro) and *Vigna radiata* (mungbean) plants.

The development of symbiotic interactions between soil bacteria and leguminous host plants is governed by signal exchanges between the two partners. The first signals, flavonoid molecules excreted by the plants, induce the expression of bacterial nodulation genes responsible for the synthesis of bacterial signal molecules, the Nod factors (Dénarié et al., 1996). These are lipooligosaccharide molecules with various host specific structural modifications, which induce the formation of new organs, the root nodules on the plants. The invasion of bacteria from the root surface to the inner root tissue takes place through a special tubule, the infection thread. Once inside the nodule, bacteria differentiate to bacteroids and synthesize proteins required for nitrogen fixation. Inside of this nodule structure all the requirements for the nitrogen fixation are fulfilled. They provide nearly anoxic conditions for the oxygen sensitive nitrogenase enzyme (encoded by the bacterial *nif* genes), and carbon supplies (provided by the host plant) for the extremely energy-intensive process of atmospheric nitrogen reduction (Gibson et al., 2008). The fixed nitrogen source can be utilized by the host plant, therefore, legumes are able to tolerate nitrogen-deprived environments. The transition of free-living bacteria to the symbiotic state of bacteroids implies multiple metabolic and morphological changes,

absolutely necessary for the bacteria to survive in a new environment and to perform new tasks (Jones et al., 2007).

The membrane and cell surface chemistry and antigenicity of *Rhizobium* species play an important role in the establishment of successful symbiosis (D'Haeze and Holsters, 2004; Becker et al., 2005). Moreover, when bacteria enter the plant, the environmental conditions change, and so does the cellular membrane. The outer leaflet of the outer membrane in gram-negative bacteria is formed predominantly by lipopolysaccharides, which were shown to be important in the first steps of symbiosis (Lagares et al., 1992; Gordon et al., 2002). The lipid composition of membranes define their stability and integrity, they constitute the permeability barrier, provide the environment for many enzymes and transporter proteins and they influence membrane-related processes, such as protein export and DNA replication (Dowhan et al., 2004). Phosphatidylcholine (PC), the major membrane phospholipid in eukaryotes, is found only in some bacteria including the members of Rhizobia. It was shown that rhizobial PC is required for successful interaction of Rhizobia with their legume host plant during the formation of nitrogen-fixing root nodules (de Rudder et al., 2000).

The phospholipid biosynthesis pathway of Rhizobia follows the same scheme as that of *E. coli* and *Bacillus subtilis,* having glycerol-3-pshosphate as the first intermediate (Figure 6).

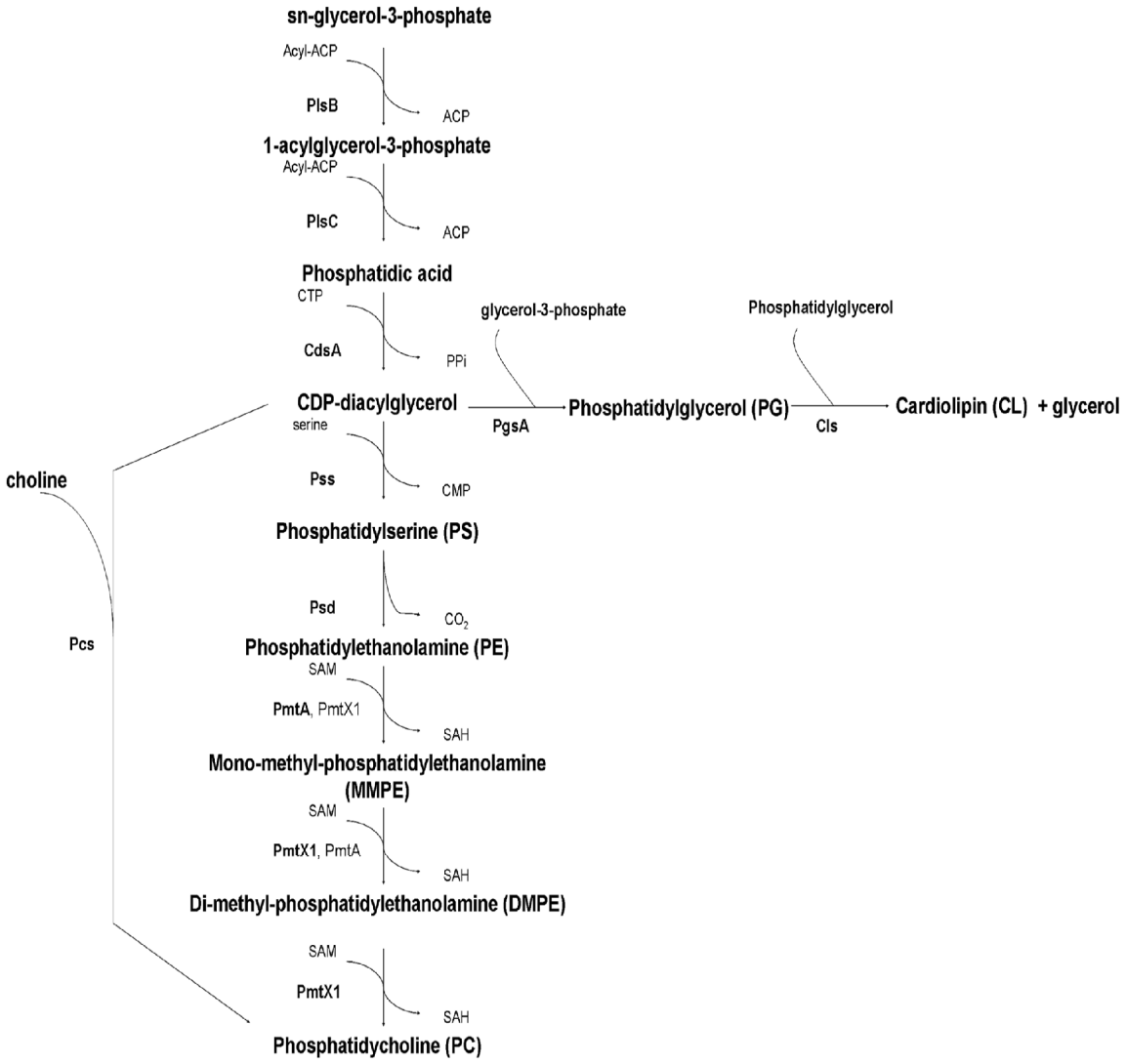


Figure 6. Phospholipid biosynthesis in *Bradyrhizobium japonicum*. Glycerol-3-phosphate acyltransferase (PlsB) catalyzes the addition of the acyl group from acyl carrier protein (ACP) to position 1 of *sn*-glycerol-3-phosphate. The second fatty acid is added by another enzyme, 1-acyl-glycerol- 3-phosphate acyltransferase (PlsC), to form phosphatidic acid. The conversion of phosphatidic acid to CDP-diacylglycerol is catalyzed by CDP-diacylglycerol synthetase (CdsA). CDP-diacylglycerol can be regarded as the central intermediate in bacteria, from which individual phospholipid biosynthesis pathways branch off. Phosphatidylglycerol phosphate synthase (PgsA) transfers *sn*-glycerol-3-phosphate to CDP-diacylglycerol under the release of CMP, thereby producing phosphatidylglycerol (PG). Cardiolipin synthase (Cls) condenses two PG molecules to yield cardiolipin (CL) and free glycerol in a transesterification reaction. The first step in the synthesis of phosphatidylethanolamine (PE) is the condensation of CDP-diacylglycerol with serine to form phosphatidylserine (PS) catalyzed by PS synthase (Pss). The second step in the formation of PE is the decarboxylation of PS, catalyzed by PS decarboxylase (Psd). There are two alternative pathways for phosphatidylcholine (PC) biosynthesis, the CDP-choline and the methylation pathways. In the CDP-choline

pathway, choline is activated to choline phosphate and subsequently to CDP-choline, which condenses with diacylglycerol to obtain PC. In the methylation pathway, PE is *N*-methylated three times by phospholipid *N*-methyltransferase (Pmt) using *S*-adenosylmethionine (SAM) as methyl donor, in order to yield PC via the intermediates monomethylphosphatidylethanolamine (MMPE) and dimethylphosphatidylethanolamine (DMPE).

Previously, the role of the TA locus *ntrPR* of *Sinorhizobium meliloti*, the microsymbiont of alfalfa (*Medicago sativa*) has been investigated (Bodogai et al., 2006; Oláh et al., 2001). The *ntrPR* operon represents a *vapBC*-type TA system, which is the most abundant group of the seven typical TA gene families (Gerdes et al., 2005; Jørgensen et al., 2009). This first example of TA loci in symbiotic bacteria has been shown to have important influence on the plant-bacterium interaction. When the toxin gene *ntrR* was inactivated by Tn5 insertion, the mutant strain developed higher number of root nodules on alfalfa, especially in the presence of combined nitrogen. Moreover, these mutant bacteria induced more efficient nitrogen-fixing nodules, since bacteroids expressed *nif* genes at an elevated level (Oláh et al., 2001).

Based on protein similarities, domain architectures and gene neighborhood analysis we could identify *vapBC*-type TA-like modules in various members of Rhizobia. (Table 2). Most of these bacteria contain several putative TA systems but in *Bradyrhizobium japonicum* USDA110*,* the microsymbiont of soybean, only one possible *vapBC*-type TA-like system was identified. *B. japonicum* belongs to the so-called slow growing Rhizobia and its genome consists of one large chromosome of 9.105 Mbp (Kaneko et al., 2002). Having only one *vapBC-like* representative in its genome, while other *Rhizobium* species posses several, and supposing an involvement of this TA-like system of *B. japonicum* in cellular metabolism similar to that of the *S. meliloti*, made it an interesting candidate for detailed investigation.

Table 2. Toxin antitoxin loci identified in various members of Rhizobia.

Rhizobial species	**Complete *vapBC* loci**	**Chromosome located**	**Plasmid located**
Sinorhizobium meliloti	10	10	-
Rhizobium leguminosarum	11	6	5
Rhizobium etli	4	1	3
***Rhizobium sp* NGR234**	1	-	1
Mezorhizobium loti	5	4	1
Bradyrhizobium japonicum	**1**	**1**	**-**
***Bradyrhizobium sp.* BTAi1**	1	1	-
***Bradyrhizobium sp.* ORS278**	1	1	-

2. Objectives

VapBC-type loci represent an abundant group of TA families, but their presence and copy number vary considerably in different species. It was suggested that bacteria living in changing environments encode TA modules, while the genomes of obligate intracellular organisms lack TA loci. Hence, it was proposed that they are involved in stress response, adjusting metabolic rates under varying environmental stimuli. The metabolism of symbiotic bacteria is greatly influenced by the conditions under which they are able to survive: they can be found in the soil as free-living bacteria, or inside of the nodules developed on the roots of leguminous plant, functioning under microoxic conditions with different metabolic necessities.

Toxin-antitoxin systems of symbiotic bacteria may be important regulators of metabolic adjustments required for survival under variable environmental conditions and/or for the transition of life style from free-living to symbiosis.

Our aim was to identify and structurally and functionally characterize *vapBC*-type TA modules in the symbiotic nitrogen-fixing bacterium *Bradyrhizobium japonicum*, the microsymbiont of soybean (*Glycine max*). Namely, to:

- identify and characterize *vapBC*-type systems in *B. japonicum* by:
  - identification of chromosomally located putative TA operons,
  - determination of operon organization, and sequence similarities to other TA systems,
  - investigation of the regulation of such TA system(s);
- examine the possible function of such TA system in *B. japonicum* by determining:
  - the toxicity of the toxin protein,
  - the involvment of the identified TA operon(s) in cell metabolism.

3. Materials and Methods

3.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this work are described in Table 3. *E. coli* DH5α (Maniatis et al., 1982) and its derivatives were grown at 37°C in Luria-Bertani (LB) medium (Maniatis et al. 1982) supplemented, when appropriate, with 100 µg/ml ampicillin (Amp), 50 µg/ml kanamycin (Km), or 10 µg/ml tetracycline (Tc). *Bradyrhizobium japonicum* USDA110 (wild type, Sp^R) (Regensburger and Hennecke, 1983) was cultivated at 31°C in YEM (yeast-extract-manitol) (Vincent, 1970), PSY (peptone-salt-yeast extract) (Hahn and Hennecke. 1984), or minimal medium (MM) (Guerinot et al., 1990) containing the appropriate antibiotics: 50 µg/ml spectinomycin (Sp), 100 µg/ml kanamycin (Km), 30 µg/ml chloramphenicol (Cm), 50 µg/ml tetracycline (Tc), or 150 µg/ml gentamicin (Gm). A modified MM, in which mannitol was replaced by 0.04% (final concentration) of glycerol as the only carbon source, was used when required. If needed, sodium-dodecyl-sulphate (SDS) was added at a final concentration of 0.01 %.

For the determination of generation times *B. japonicum* wild-type USDA110, the *bat/bto* deletion mutant MP99, the revertant MP119 and complemented MP120 strains were precultured in YEM medium, then diluted to an optical density of 0.1 at 600 nm in YEM, PSY and MM media. Bacterial growth was followed by measuring the optical density at 600 nm. Peptone, when required, was added at a final concentration of 0.1 % (w/v). The experiments were repeated at least six times.

3.2 DNA manipulations

Preparation of plasmid DNA, digestion with restriction enzymes, agarose gel electrophoresis, fragment isolation, cloning procedures, and transformation of *E. coli* cells were performed according to Maniatis et al. (1982). In polymerase chain reactions *Pfu* polymerase (Fermentas) was used to obtain error-free products.

Table 3. Bacterial strains and plasmids used in this study

Strain-Plasmid	Relevant characteristics	Reference/Source
Escherichia coli		
DH5α	*fhuA2* Δ(*argF-lacZ*)U169 *phoA glnV44* Φ80 Δ(*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Maniatis et al. (1982)
Bradyrhizobium japonicum		
USDA110	Sp^r , wild type	Regensburger and Hennecke. (1983)
MP99	Sp^r , USDA110, Δ*bat/bto* (Km^r)	Miclea et al. (2010)
MP119	Sp^r , Km^r, Tet^r, MP99 derivative carrying pMP117	Miclea et al. (2010)
MP120	MP99 derivative carrying the *bat/bto* operon recombined in the chromosome	Miclea et al. (2010)
Plasmids		
pB5	Amp^r, pBluescriptII derivative carrying 1495 bp *Hind*III fragment from Tn5 containing the Km^r cassette	Peter Putnoky (unpublished)
pBluescriptII (SK+)	Amp^r, f1 (+) ori	Alting-Mees and Short. (1989)
pEP82	Tet^r, derivative of pRK290 carrying a promoterless *lacZ* gene	Élő et al. (1998)
pRK 290	Tet^r, broad host range vector	Ditta et al. (1980)
pET-28b	Km^r, f1 (+) ori	Novagen, Madison WI, USA
pRK2013	Km^r, helper plasmid for mobilization of pRK290 derivatives, tra (RK2)	Ditta et al. (1980)
pMP01	pBluescript II derivative, *Bam*HI site deleted from multicloning site	Miclea et al. (2010)
pMP73	Amp^r, 1 kb polymerase chain reaction (PCR) fragment (BAT1-BAT2) in *Eco*RV site of pMP01	Miclea et al. (2010)
pMP74	Amp^r, 1.2 kb PCR fragment (BAT3-BAT4) in *Eco*RV site of pMP01	Miclea et al. (2010)

pMP77	Amp^r, pMP73 derivative carrying the BAT3-BAT4 fragment from pMP74	Miclea et al. (2010)
pMP85	Amp^r, pMP77 derivative carrying a 1.4 kb Km^r cassette in the *Bam*HI site	Miclea et al. (2010)
pMP86	Tet^r, derivative of pRK290 carrying the 3.6 kb *Xho*I–*Spe*I fragment of pMP85	Miclea et al. (2010)
pMP105	Tet^r, derivative of pEP82 carrying the 379 bp *bat/bto* promoter fragment upstream of the *lacZ* gene	Miclea et al. (2010)
pMP109	Amp^r, pBluescript II derivative carrying the 285 bp B-101-F – B-102-R PCR fragment in *Sma*I site	Miclea et al. (2010)
pMP110	Amp^r, pBluescript II derivative carrying the 426 bp B-103-F – B-104-R PCR fragment in *Sma*I site	Miclea et al. (2010)
pMP111	Amp^r, pBluescript II derivative carrying the 711 bp B-101-F – B-104-R PCR fragment in *Sma*I site	Miclea et al. (2010)
pMP112	Km^r, derivative of pET-28b carrying the *Nco*I-*Sal*I fragment of pMP109	Miclea et al. (2010)
pMP113	Km^r, derivative of pET-28b carrying the *Nco*I-*Sal*I fragment of pMP110	Miclea et al. (2010)
pMP114	Km^r, derivative of pET-28b carrying the *Nco*I-*Sal*I fragment of pMP111	Miclea et al. (2010)
pMP117	Tet^r, derivative of pRK290 carrying 959 bp BAT-PR-F – B-104-R PCR fragment	Miclea et al. (2010)
pMP118	Tet^r, derivative of pRK290 carrying 2.9 kb BAT1 – BAT2 PCR fragment	Miclea et al. (2010)

3.3 Construction of strains and plasmids

3.3.1 Construction of a *bat/bto* deletion derivative of *B. japonicum*

For the deletion of the *bat/bto* operon PCR primers were designed to amplify a 1 kb upstream region and a 1.2 kb downstream region of the operon. The 5' ends of two of the primers contain a *Bam*HI restriction site (Table 4). The primers BAT1 and BAT2 were used for the amplification of the upstream region, while BAT3 and BAT4 were used for the amplification of the downstream region. The PCR products were cloned separately in a pBluescript II (SK+) (Alting-Mees and Short, 1989) derivative (*Bam*HI site was removed from multicloning site) to obtain pMP73 with the upstream region and

pMP74 with the downstream region of the *bat/bto* operon. A *SmaI-Bam*HI fragment of pMP74, containing the downstream region was cloned in pMP73, digested with the same enzymes. In the resulting plasmid MP77 the upstream and downstream regions were joined at the *Bam*HI restriction site. This site was used to introduce a kanamycin resistance cassette between the two regions, resulting in pMP85. The construct containing the upstream and downstream regions with the kanamycin resistance cassette was recovered by *Xho*I-*Spe*I cleavage and was cloned in the wide host-range plasmid pRK290 (Ditta et al., 1980) at the *Eco*RI restriction site, yielding pMP86. This plasmid was conjugally transferred from *E. coli* to *B. japonicum* wild-type strain by triparental mating, using the helper plasmid pRK2013 (Ditta et al. 1980). The *bat/bto* operon was replaced by the kanamycin resistance cassette by homologous double recombination between the upstream and downstream regions present both on the chromosome and the plasmid pMP86.

Southern hybridization was performed to confirm the replacement of *bat/bto* genes with the kanamycin resistance cassette. A 1351 bp *Hind*III-*Sal*I fragment carrying the kanamycin resistance cassette was hybridized as a probe to the *Bam*HI-digested total DNA of the mutant and wild-type strains to prove the presence of the selectable marker in the mutant. Furthermore, the presence of the resistance cassette and the absence of the *bat/bto* module were also checked by PCR. Primer pairs were designed, in each case one of the primers was specific for the neighboring genomic region (BAT-UP-F and BTO-DW-R for the upstream and downstream regions, respectively), whereas the other one was specific for the kanamycin cassette (KM-R and KM-F) (Table 4). Using the corresponding primer pairs (BAT-UP-F and KM-R, KM-F and BTO-DW-R), amplified fragments of correct sizes were detected from the total DNA of the mutant strain demonstrating that the resistance cassette replaced specifically the *bat/bto* module. Finally, by sequencing of the upstream and downstream regions amplified from the total DNA of the mutant strain by using BAT-1 BAT-2, and BAT-3 BAT-4, respectively, no further DNA sequence changes were revealed in the neighboring regions.

Table 4. Oligonucleotide sequences

Primer	Sequence (5' to 3')
BAT1	CGTGGTCGTAGAGCTTCTTGA
BAT2[a]	GGATCCTGCCAGCAAGAACCAATA
BAT3[b]	GGATCCCCCTATCTCAACCGCAC
BAT4	GCATCACGGACCGTTTCAA
BAT-UP-F	ATGGCCGAGGAAGGGATAG
BTO-DW-R	ACCATGCACCTTCCTGATTT
KM-R	GGCTTCCCAACCTTACCAG
KM-F	TCGGCATCCAGGAAACCA
BAT-PR-F	TCAAACAGACGCATCCCCCGG
BAT1.2	TCACAACACTGCACCCGAC
BAT2.1	GCCGAATGCAGCGAAATCC
B101-F[c]	CTTGCTGCCATGGCCATCA
B102-R	AGCAGGTTCACGGCATCT
B103-F[d]	CGACGAGATGCCATGGACCT
B104-R	AGATAGGGCTATGGGTCGCT
HIS1	GAGATGGTCGAGAAGATCC
HIS2	ATCGTAACGCAAGCTAATC
PTA1	CTTCGTCCAGTTCACCTAT
PTA2	TTAATCCTTGCGATACACC
PTX1	GGTCTACGATCTCGTGTT
PTX2	AGAGCACCTCGACATTGC

[a] The primer contains a *Bam*HI site at the 5' end (underlined).

[b] The primer contains a *Bam*HI site at the 5' end (underlined).

[c] The primer contains an *Nco*I site in the sequence (underlined).

[d] The primer contains an *Nco*I site in the sequence (underlined).

3.3.2 Construction of the complemented strain MP120

In order to confirm that the pleiotropic effects observed in the phenotype of MP99 are all due to the deletion of the *bat/bto* operon, a complemented strain MP120 was constructed. Primers BAT-PR-F and B-104-R (Table 4) were used to amplify a region

consisting of the coding sequence of *bat/bto* operon and 248 bp upstream of the translation start site of the operon. The amplicon was cloned into the *Eco*RI restriction site of plasmid pRK290 (resulting in the plasmid pMP117), and introduced into the *B. japonicum* MP99 mutant strain by triparental mating yielding the complemented strain MP120.

3.3.3 Construction of the revertant strain MP119

To avoid any possible unwanted effect of multiple copies present in the *bat/bto* complemented strain, in another experiment a revertant strain (MP119) was constructed. A DNA fragment containing the *bat/bto* genes and a 1 kb upstream and 1.2 kb downstream region of the operon was amplified by PCR using the primers BAT1 and BAT4. This fragment was cloned in the *Eco*RI restriction site of plasmid pRK290. The resulting plasmid pMP118 was conjugally transferred from *E. coli* to the MP99 strain by triparental mating, and the *bat/bto* region was inserted into the chromosome by double homologous recombination. The presence of the wild-type *bat/bto* genes was confirmed by Southern hybridization using as probe a PCR fragment amplified by the BAT1.2 and BAT2.1 primers (Table 4). This fragment covers 677 bp region of the *bat/bto* operon.

3.3.4 Plasmid containing the *bat/bto* promoter region

To characterize the expression of *bat/bto* operon, a 379 bp fragment consisting of the 233 bp intergenic region, 144 bp of the *glpD* and 2 bp of the *bat/bto* coding regions, respectively, was amplified with the primers BAT1 and BAT2, then digested with *Bam*HI-*Sal*I restriction enzymes. The promoter-containing product was treated with Klenow polymerase and inserted in the *Bgl*II site of plasmid pEP82 (Élő et al., 1998) upstream of the promoterless *lacZ* gene. The resulting pMP105plasmid was introduced into wild-type, mutant and complemented *B. japonicum* strains by conjugation.

3.3.5 Plasmids carrying *bat*, *bto* or *bat/bto* operon under the Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter of pET-28b

To investigate the effect of the ectopic expression of *bat/bto* module in *E. coli* cells, plasmids containing *bat*, *bto* or *bat/bto* genes were constructed by cloning the appropriate PCR products first in pBluescriptII digested with *Sma*I. The following plasmids were obtained: pMP108 carrying the *bat* gene amplified by the primers B101-F and B102-R, pMP110 carrying the *bto* gene amplified by the primers B103-F and B104-

R (Table 4), and pMP111 carrying the entire *bat/bto* operon amplified by the primers B101-F and B104-R (Table 4).

The pBluescriptII derivatives containing the cloned fragments cleaved at *Nco*I site of the fragment and *Sal*I site present in the multicloning site of the vector, and the inserts were re-cloned in the *Nco*I-*Sal*I digested pET-28b vector. The plasmids carrying the *bat* (pMP112), *bto* (pMP113), or *bat/bto* (pMP114) genes under the inducible promoter were transformed in *E. coli* BL21-Gold (DE3) competent cells. Thus, the *Nco*I restriction site of pET-28b and the translational start codon ATG were restored in the plasmids due to the presence of an *Nco*I site in the sequence of forward primers (Table 4, underlined).

3.4 Viability tests

E. coli BL21-Gold (DE3) cells with plasmids pMP112, pMP113, pMP114 or the vector pET-28b were grown overnight in LB medium supplemented with kanamycin. The cultures were diluted in the same medium to obtain equal optical densities (OD_{600}=0.1), and were induced with 1mM IPTG. Cultures were grown at 37^{o}C and samples were removed to determine optical density and viable cell number. The experiment were repeated at least three times.

3.5 Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from cells at exponential phase using the RNA Easy Mini Kit from Qiagen. Reverse transcription was performed from identical amounts (1µg) of RNA obtained from wild-type, MP99 and MP120 strains using the "Transcription First Strand cDNA Synthesis Kit" (Roche Applied Science). RT-PCR was performed with identical volumes of cDNA samples as templates using primers specific for the following genes: *hisS* (HIS1 and HIS2), *pmtA* (PTA1 and PTA2) and *pmtX1* (PTX1 and PTX2) (Table 4). Controls without the reverse transcriptase enzyme or without the template cDNA were also performed. All experiments were repeated three times.

3.6 Confocal laser scanning microscopy (CLSM)

The differences in shape and size of the mutant and wild-type bacteria were demonstrated by CLSM. Aliquots were removed from early exponential phase cultures of *B. japonicum* wild-type and MP99 strains grown in YEM medium. The bacteria were centrifuged, resuspended and fixed with a PBS (phosphate buffered saline)-formaldehyde solution for 1 hour. Samples were laid on microslides and stained with a 10mg/ml acridine-orange solution.

Detection was performed using Olympus Fluoview FV1000 CLSM (Olympus Life). Microscope configuration was the following: objective: 60 x UPLSAPO (1.35, oil); excitation: 488 nm; detection range: 500-600 nm; scanning dimensions: 512 x 512 pixel; sampling speed: 10 μs/pixel; confocal aperture: 110 μm; zoom: 15 x; line averaging: 2 x.

3.7 Atomic force microscopy (AFM)

Bacteria were grown at 31°C in YEM medium. Aliquots were removed from the cultures at early logarithmic phase and washed with PBS. To immobilize the cells, muscovite mica surface was coated with a 0.1% (w/v) solution of poly-L-lysine (Mw >300000) for at least 2 hours. Bacterial adhesion was carried out at room temperature by depositing 20 μl of bacterial suspension onto mica surface, buffered at pH 7, and was incubated for 20 minutes. The sample holder containing the bacteria was filled with distilled water during the experiments. To study the effect of the ionic detergent sodium-dodecyl-sulphate (SDS) on *B. japonicum* wild-type and MP99 mutant cells, we supplemented early logarithmic phase cultures (YEM medium) with 0.01% SDS and incubated further for 1 and 2 hours. Aliquots were removed from the cultures, deposited on micro slides and thoroughly dried. Samples were scanned with AFM in tapping mode.

AFM measurements were carried out with an Asylum MFP-3D head and controller (Asylum Research) in AC mode. The driver program MFP-3D Xop was written in IGOR Pro software (version 5.05b, Wavemetrics). Silicon nitride cantilevers (BioLever Mini BL-AC40TS, Olympus Optical Co.) with a nominal spring constant of 90 pN/nm were used. Typically 512 × 512 pixel scans were taken at 0.3-1.0 line/second scan

rate under water. Both trace and retrace images were measured and compared for accuracy, no difference was found between them. A number of at least 40 cells were subjected to the measurements.

To characterize the changes in the mechanical properties of membranes, the elastic (Young's) modulus was determined by force measurements carried out in liquid with the previously mentioned cantilevers in triggered mode, with the z piezo working in a closed loop. Calibration of the spring constant of each individual cantilever was performed by thermal fluctuation technique (Hutter and Bechhofer, 1993). Force curves were recorded at a constant speed of 600 nms^{-1}. Usually 5-10 force measurements were carried out at the same point, and then averaged. As a reference, force curves on flat mica surface in water were also recorded. The measurements were made with a 10 nm trigger, which corresponds to a maximum loading force less than 1 nN. All experiments were repeated at least four times.

To calculate the Young's modulus, the approaching part of the force curves was used in order to avoid the effect of adhesive forces between the tip and the cellular membrane. Calculations were done based on the theory of Sneddon, further developed for AFM (Dimitriadis et al., 2002; Mathur et al., 2001; Vinckier and Semenza, 1998). As the model refers to small indentation values, the bottom part of the curves was used in calculations. In these force measurements, two different effects were recorded simultaneously: the cantilevers own deflection, and the indentation of the sample. As the cantilever's own deflection was subtracted from the curves, forces on hard mica surface were also recorded and used as reference. Calculations were performed in Matlab (The MathWorks Inc.). The force as a function of indentation Δz, for a conical tip with opening angle α is described by the equation:

$$F(\Delta z) = \frac{2E^*}{\pi(tg(\alpha))}\Delta z^2$$

where E* is the relative Young's modulus:

$$\frac{1}{E^*} \approx \frac{1-\mu_m^2}{E_m}$$

E_m is the elastic modulus and μ_m is the Poisson ratio of the cell (Vinckier and Semenza, 1998). By fitting the indentation – force curves with a second order polynomial

function, the coefficient of the polynomial will provide the E_m of the measured sample (Bálint et al., 2007).

3.8 Detection of polysaccharides

To determine the exopolysaccharide production of *B. japonicum* wild-type and MP99 strains, aliquots were removed from the cultures grown in YEM medium (which allows the production of exopolysaccharides) at the same optical density (OD_{600} 0.6) and were pelleted by centrifugation. Samples were collected from the layer above the pellet and assayed for carbohydrate content by the phenol-sulfuric acid test (Dubois et al, 1956).

For the isolation of total polysaccharides, bacterial cultures of *B. japonicum* wild-type, MP99 and MP120 strains were grown in liquid PSY medium. 200 µl cultures of exponential phase were pelleted by centrifugation, washed and extracted with hot phenol-water method (Kiss et al. 1997). The aqueous phases were pooled and exhaustively dialyzed (membrane cutoff 6000-8000 kDa) against water. This crude polysaccharide solution was lyophilized and then dissolved in 100 µl of water.

Total polysaccharide extracts were analyzed by DOC-polyacrylamide gel elecrophoresis using 18% polyacrylamide gels with deoxycholic acid as detergent (Krauss et al., 1988). Equal amounts of polysaccharide extracts were loaded on gels, except when otherwise described. Sample buffer, gel electrophoresis buffer and silver staining specific to lipopolysaccharides were used as described by Krauss et al. (1988). Experiments were performed three times.

3.9 Lipid extraction

Fifty ml of cells grown in PSY medium were harvested at OD_{600}=0.8, washed twice with PBS and the wet bacterial cell paste was subjected to lipid extraction procedure according to a modified Bligh and Dyer method (Kates, 1986). The final dried lipid extract was immediately dissolved in 300 µl of chloroform : methanol=2 : 1 and stored at -20 ºC.

3.10 Fatty acid analysis of total lipid extracts

Fifty µl of lipid samples were dried in ampoulles and transmethylated with 2 ml of 5% (v/v) acetyl chloride (in methanol) at 80 ºC for 2 h. The resulting fatty acid methyl esters (FAME) were extracted with hexane, the solvent was evaporated, the residue dissolved in 50 µl of benzene and analyzed using a gas chromatography-mass spectrometry (GC-MS) system (Shimadzu GC-MS-QP2010) equipped with a BPX70 capillary column (10 m x 0.1 mm x 0.2 µm). A 1 µl aliquot was injected onto the column, maintained at 150°C for 2 min, programmed to heating at 6°C /min from 150 to 215°C, then at 20°C/min to 235°C, and then maintained isothermally for 2 min. Fatty acid composition is given as wt % of total. The measurements were made in triplicate.

3.11 Separation of individual phospholipids

Individual lipid classes were separated by thin-layer chromatography on silica gel G plates (Merck 5721). First, a developing solvent of aceton : petroleum ether (30-50 ºC, 1 : 3) was used to run to the top of the plate, which was then dried. Second, a chloroform : methanol : acetic acid (65 : 25 : 10) solvent was run for 2/3 of the plate. Lipids were visualized by spraying the plate with 0.05% 8-anilinonaphtalene-1-sulfonate in methanol, and identified using authentic standards. Depending on the relative fluorescence 0.5-5 µg C24:0 fatty acid was applied on the spots as internal standards. The spots were scraped off the plate, transmethylated and FAME was analyzed as above. All measurements were done in triplicate.

3.12 β-galactosidase measurements

B. japonicum strains, grown at 31^{o}C in PSY medium containing the appropriate antibiotics, were diluted to OD_{600}=0.1 and further cultivated. The *β*-galactosidase activity was determined as described by Oláh et al. (2001). Glycerol was added to the medium when required at a final concentration of 0.04%. Measurements were repeated five times.

3.13 Symbiotic interaction with soybean host plant

Soybean seeds (*Glycine max var.* Pannonia Kincse) were surface-sterilized, germinated for 3 days in darkness at room temperature and then placed in pots containing clay beads (Lodeiro and Favelukes, 1999). One ml of *B. japonicum* wild-type or MP99 mutant cultures in the exponential growth phase (OD_{600}= 0.6) were used for inoculation of each seedling. For each bacterial strain, 30 soybean plants were analyzed. Plants were watered daily with Fahraeus nitrogen-free nutrient solution (Fahreus G., 1957). After 60 days, plants were harvested, the number of nodules and the dry weight of shoots were determined. To analyze nodule occupancy, 40 nodules were randomly collected from plants inoculated either by the wild-type or by the MP99 mutant strain, surface-sterilized by repeated washing with 96% ethanol and finally with sterile distilled water. The nodules were homogenized, suspended in 0.9% (w/v) NaCl and 40-80 µl of the suspension was placed on YEM agar plates. Two hundred colonies from each strain were checked for kanamycin resistance to select for the MP99 mutant, and for spectinomycin resistance to select for the wild-type strain.

3.14 Databases and software

The complete genomic sequence of *Bradyrhizobium japonicum* was available online at www.kazusa.or.jp/rhizobase/. Sequence analyses were done using the Bioedit program, ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Larkin et al., 2007) and Promscan (http://molbiol-tools.ca/promscan/) (Studholme and Dixon, 2003).

4. Results

4.1 Identification and structural characterization of the bsl2435/bll2434 operon, a putative toxin-antitoxin locus in the genome of *Bradyrhizobium japonicum* USDA110

The complete nucleotide sequence of *B. japonicum* strain USDA110 was published in 2002 (Kaneko et al.). The genome of *B. japonicum* is a single circular 9,105,828 bp chromosome containing 8317 potential protein-coding genes, one set of rRNA genes and 44 tRNA genes.

Similarity search using the deduced protein sequence of a previously identified rhizobial toxin NtrR (Bodogai et al. 2006) as a query, and gene neighborhood analysis pointed out a single candidate for *vapBC*-type system in the *B. japonicum* genome. The chromosomal operon bsl2435/bll2434 designated as *bat/bto* operon (*B*radyrhizobium antitoxin/*B*radyrhizobium toxin), encodes a protein pair (Figure 7). The *bat/bto* locus consists of a 285 bp upstream open reading frame (ORF) *bat* that encodes a putative protein of 94 amino acids and a 426 bp downstream ORF *bto* that encodes a putative protein of 141 amino acids. The translational start codon for *bto* overlaps with the last base of the translational stop codon of *bat*, which is a strong indication of translational coupling. The Bto protein, representing the putative toxin component contains a PIN domain, which has been identified in many bacteria, *Archaea*, and eukaryots (Makarova et al., 1999). The Bat protein represents the putative antitoxin component and according to its annotation contains a PhdYefM-like domain between amino acids 14 and 86. The best-characterized member of this protein family is Phd of bacteriophage P1, the antidote partner of Doc (Lehnherr et al. 19,93).

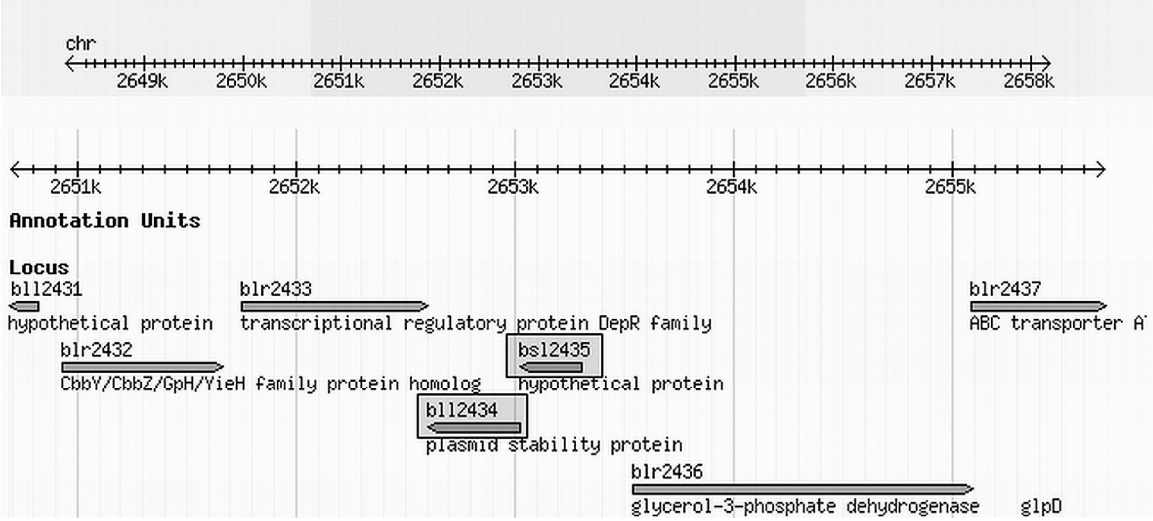


Figure 7. Chromosomal location of *bat/bto* operon (bsl2435/bll2434) of *B. japonicum* (www.kazusa.or.jp/rhizobase). The *bat/bto* operon is located upstream of the *glpD* gene (blr2436) coding for the aerobic glycerol-3-phosphate dehydrogenase and downstream of a *glpD*–repressor gene (blr2433).

A bioinformatic analysis of nearly a hundred PIN domain sequences identified a set of 4 conserved acidic residues and a fifth conserved position where there is a serine or threonine residue. This has led to a suggested nuclease function (Clissold and Ponting. 2000). The T4 RNase H family of nucleases, which includes the exonuclease domain of Taq polymerase and the flap endonuclease, share the same conserved quartet of amino acid residues. The Bto protein (encoded by the bll2434 gene) contains 3 conserved residues (Asp-6, Glu-43, Asp-105) and a substitution at position 123, where the acidic aspartic acid is replaced by the neutral asparagine residue (Figure 8). The conserved residues of previously identified toxin NtrR of *S. meliloti* also matched those of the Bto protein of *B. japonicum* (Figure 8). A fifth invariant residue, threonine at position 121 as well as alanine at position 112, thought to be involved in metal-dependent nuclease activity are also conserved in these PIN–containing proteins.

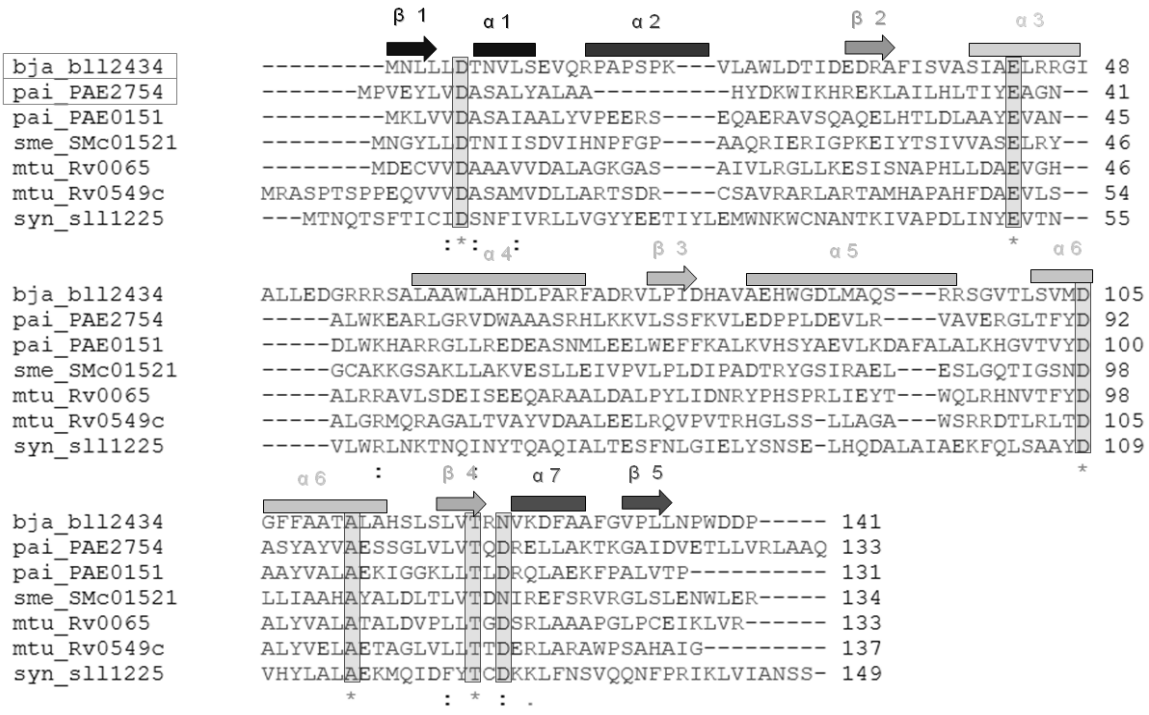


Figure 8. Sequence and structural alignments of PIN domain proteins. The selected protein sequences were aligned using the ClustalW program. Six positions are highlighted identifying the conserved residues: Asp-6, Glu-43, Asp-105, Ala-112, and Thr-121 (the position number of the residues refer to the position in the Bto protein). The Bto (encoded by bll2434) and NtrR (encoded by SMc01521) proteins contains a substitution at position 123, where acidic aspartic acid is replaced by asparagine residue. Secondary structure elements (β-sheets and α-helices) of Bto are shown above the alignment. Sequences are named according to their genome and open reading frame labels: bja-*B. japonicum*; pai-*Pyrobaculum aerophilum*; sme-*S. meliloti*; mtu-*Mycobacterium tuberculosis*; syn-*Synechocystis sp*.

As the primary structure of the Bto protein showed similarities to those of known toxins we were interested in determining other possible structural similarities. Using the crystallographically determined structure of the archaeal PIN-domain-containing protein PAE2754, we predicted the structure of Bto protein using Swiss Model, an automated comparative protein modeling server from Expasy (http://www.expasy.ch/). This Bto protein model consists of a central 5-stranded parallel beta sheet with 4 helices packed on one side of the sheet and 3 alpha helices on its other side (Figure 9). The secondary structure elements of the Bto protein are similar to those of the archaeal PIN domain protein, which has the same arrangement of 5-stranded parallel beta sheet and 7 alpha helices (Arcus et al., 2004). The PAE2754 protein was demonstrated to have Mg^{2+} dependent nuclease activity given by the 4 highly conserved acidic residues, which form

a negatively charged pocket in the center of the molecule. This acidic pocket is the active site for nuclease activity of different enzymes containing PIN domain (Makarova et al., 1999).

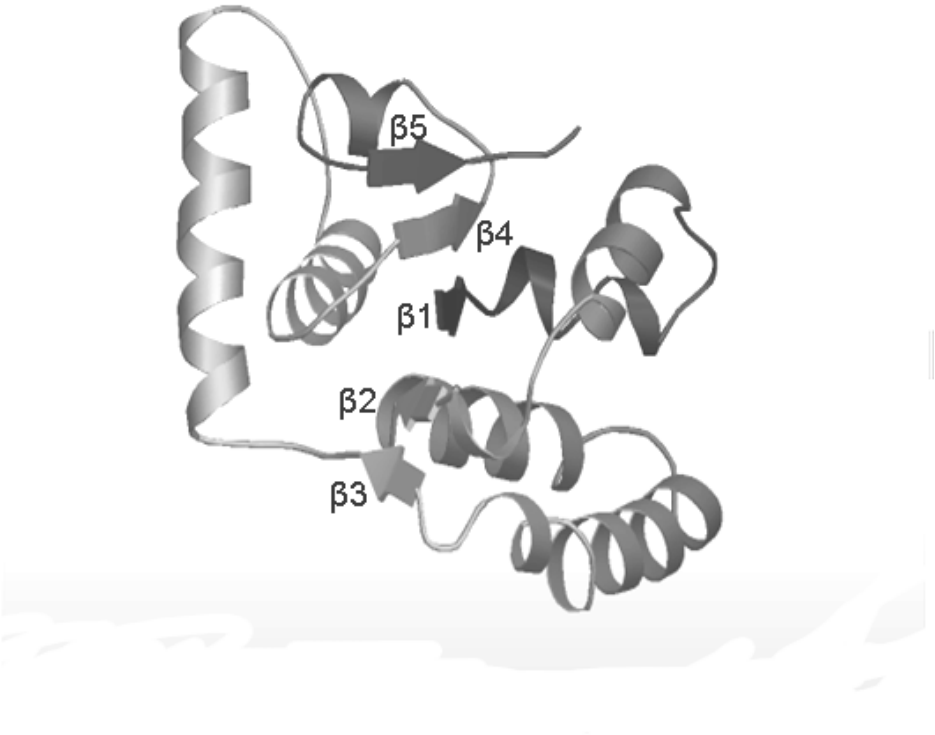


Figure 9. Predicted structure and topology of the Bto monomer. Beta sheets are labeled sequentially, starting with the N-terminal part of the protein as in Figure 8. The Bto protein model consists of a central 5 parallel beta sheet with 7 alpha helices packed on both sides of the parallel beta sheets. Protein structure was predicted by Swiss Model (http://www.expasy.ch/) using the automated mode.

Further sequence analysis revealed that the *bat/bto* operon may also be the result of a recombinatorial event as was proposed by the model of Smith and Magnuson (2004). Our results showed that the Bat protein is likely composed of two different modules. The N-terminal part of the Bat protein exhibited a higher degree of identity with the Phd module of *phd/doc* TA system (Figure 10), whereas the C-terminal part showed similarity to the AbrB domain of the NtrP antitoxin, a member of the *vapBC*-type *ntrPR* system in *S. meliloti* (Bodogai et al., 2006).

```
Bat          MADHNTAPDTLPADDTWTLANAKARLSQVIDRAQTGPQI-ITRHGKPNAVIVSAEEWARK 59
Phd of Ø P1  ------------MQSINFRTARGNLSEVLNNVEAGEEVEITRRGREPAVIVSKATFE-- 45
                         :: .: .*:..**:*:::.::* :: ***:*:  *****   :

Bat          TARKGTLAEFLLASPLRGADLALERMHDAPRDEMP- 94
Phd of Ø P1  AYKKAALDAEFAS--------LFDTLDSTNKELVNR 73
             : :*.:*   : :          :: :..: :: :

Bat          MADHNTAPDTLPADDTWTLANAKARLSQVIDRAQTGPQIITRHGKPNAVIVSAEEWARKT 60
NtrP         MP--VPLPSSRPKEVKLFRNNRSQAVRIPAEFELPGDRVLIRR-EGTRLII--EPIARPA 55
             *.   . *.: * : .    * .  :    :   .* ::: *: : . :*:  *  ** :

Bat          ARKGTLAEFLLASPLRGADLALERMHDAPRDEMP-- 94
NtrP         DIVELLAEWKKEAPL-GPEDRFPDVEDIPARPEKIF 90
                  ***:   :** *.:  :  :.* *
```

Figure 10. Alignment of the deduced amino acid sequence of the Bat protein with antitoxins Phd of phage P1 and NtrP of *S. meliloti*. The N terminal parts of Phd and Bat show 23% identity in 59 amino acids, but their C terminal parts are different (5.7% identity in 35 amino acids). By contrast, Bat and NtrP exhibit homology at their C terminal parts (24% identity in 34 amino acids), whereas the N terminal regions are different (16.7% identity in 60 amino acids). A recombination junction proposed for Phd antitoxin (Smith and Magnuson, 2004) is also present in Bat (underlined) and is located within the transition region.

According to its structural organization and sequence similarities to TA protein domains, the *bat/bto* operon belongs to the *vapBC* family of bacterial TA modules and was choosen for further functional characterization.

4.2 Activity of the *bat/bto* promoter

The negative autoregulation of TA operons by either TA complexes or free antitoxins is a common property of the characterized TA systems (Gerdes, 2000; Gerdes et al., 2005; Oláh et al., 2001). As it was demonstrated previously, the expression of a TA system, determined by a promoter-*lacZ* fusion, was at a very low level in the wild-type strain. By contrast, the promoter activity increased several times in the mutant background lacking the toxin-antitoxin complex. To examine the regulation of the *bat/bto* operon, the plasmid pMP105, carrying the promoter region on a 379 bp fragment upstream of a promoterless *lacZ* gene, was introduced into wild-type, *bat/bto* mutant and *bat/bto* complemented *B. japonicum* strains, and β-galactosidase activities were determined. The enzyme activity determined in the mutant strain was considerably lower (5.2±0.5 Miller units) than that in wild-type background (114.8±3.9 Miller units) (Figure

11A, 2 and 1, respectively) or in the complemented strain (117.3±6.1 Miller units) (Figure 11A, 3). Thia indicated that the regulation of the *bat/bto* operon does not exhibit the characteristics of typical TA modules.

Examination of the chromosomal neighborhood of the *bat/bto* operon revealed that it is located upstream of the *glpD* and downstream of the *glpR* genes (Figure 7). The well-characterized *glpD* of *E. coli* encodes a membrane-associated aerobic glycerol-3-phosphate dehydrogenase. GlpD converts glycerol-3-phosphate (G3P), the key substrate in the phospholipid biosynthesis, to dihydroxi-acetone-phosphate (DHAP), an intermediate of pyruvate, which serves as carbon and energy source in the citric acid cycle (Figure 12). The GlpD enzyme has a central role mediating the glycolytic and the phospholipid biosynthetic pathways, therefore the level of *glpD* expression is important for cell metabolism. The expression of *glpD* is negatively regulated by the repressor protein GlpR, which binds to multiple operator sequences in the *glpD* promoter (Yang and Larson, 1996; Yang and Larson, 1998; Ye and Larson, 1988). The presence of the inducer glycerol-3-phosphate (G3P) reduces the binding activity of GlpR to the operators and thereby releases repression of the *glp* genes. The putative promoter region of the *bat/bto* operon in *B. japonicum* partially overlaps the putative promoter region of *glpD* (Figure 11B), which is transcribed in opposite direction from the complementary strand. Using the PromScan software, we could identify two putative GlpR binding regions in this intergenic sequence (Figure 11B). Further examination revealed the presence of repeated sequences similar to the characteristic GlpR binding sites identified by computer-assisted analysis of Rhizobia (Danilova et al., 2003). The consensus sequence determined from data of three other *Rhizobium* species contains 3-4 direct repeats of –TTTCGTT- separated by 3-4 nucleotides (Danilova et al. 2003). The putative GlpR binding sites in *Bradyrhizobium* contain 2 repeats without spaceing. One of them partially overlaps with a direct repeat sequence which, similarly to other TA modules (Bodogai et al., 2006; Magnuson and Yarmolinsky, 1998; Marianovsky et al., 2001), may represent potential binding site for the toxin-antitoxin complex. The other putative GlpR binding site is located just upstream of the *glpD* gene. We supposed that a potential competition of the toxin-antitoxin complex and GlpR for the overlapping binding sites in the promoter region of *bat/bto* may influence the regulation of the *bat/bto* system of *B. japonicum*.

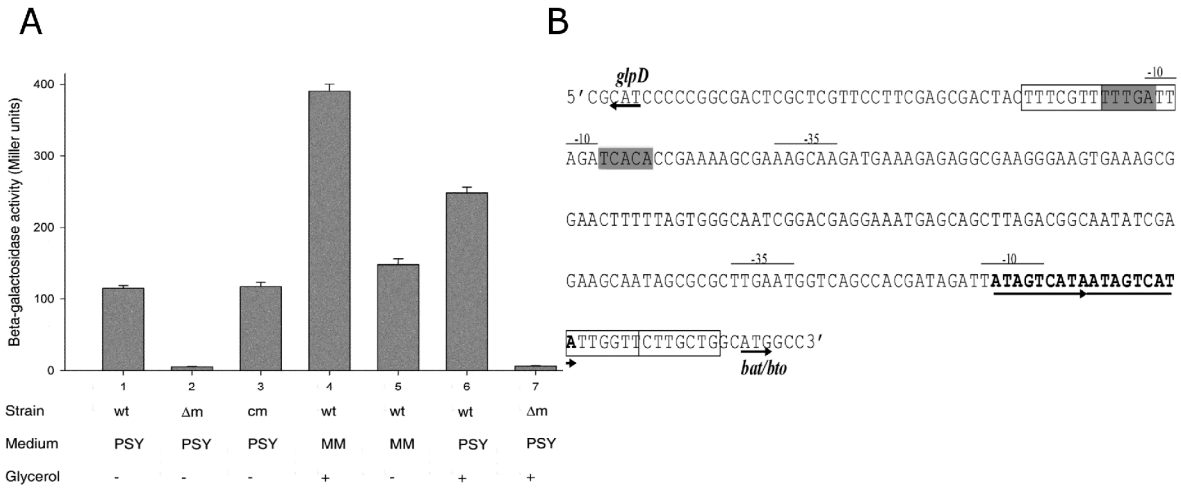


Figure 11. Activity of the *bat/bto* promoter. A: β-galactosidase activity expressed from plasmid pMP105 carrying a 379 bp segment consisting of the 233 bp intergenic region, 144 bp of the *glpD* and 2 bp of the *bat/bto* coding regions, respectively. Enzyme activities of *B. japonicum* wild-type (wt; 1, 4, 5, 6), MP99 deletion mutant (Δm; 2, 7) and MP120 complemented (cm; 3) strains carrying pMP105 were determined. Bacteria were grown in PSY (1, 2, 3, 6, 7) or in MM (4, 5) media at 31°C to OD_{600}= 0.5. Cultures were induced by the addition of 0.04% glycerol as indicated. The measurements were repeated five times.. B: The intergenic region of *glpD* and the *bat/bto* module with repeated sequences (**ATAGTCATA**) representing potential binding sites of the Bat/Bto complex (underlined with arrows). Putative -10 and -35 regions in the promoter of *glpD* and *bat/bto* are superlined. A TGn extension of the -10 motif in the *glpD* promoter region may account for the lower conservation of the -35 region. A possible catabolite repression protein (CRP) binding site TTTGA-N_5-TCACA upstream of *glpD* is shaded. Translation start sites for *glpD* and *bat/bto* are shown by short arrows. Putative GlpR binding sites exhibiting similarity to the sequence unit –TTTCGTT- characteristic for α-proteobacteria (Danilova et al., 2003) are boxed.

To examine this assumption, β-galactosidase activity expressed from plasmid pMP105 was determined in wild-type bacteria grown in minimal medium containing the inducer glycerol, the precursor of G3P. The activity was considerably higher than that obtained in minimal medium without glycerol (Fig. 11A, 4 and 5), demonstrating that the expression of the TA module was positively influenced, probably by the inhibition of GlpR binding. Since *bat/bto* mutant cells were not able to grow in MM, we could not perform similar experiments with the MP99 strain. Wild-type bacteria grown in PSY medium with glycerol also exhibited a higher activity level than in PSY medium without glycerol (Fig. 11A, 6 and 1, respectively). In contrast to other TA systems, however, the very low expression levels observed in the deletion mutant MP99, compared to those of

the wild-type and complemented strains in PSY medium without glycerol indicated that the toxin-antitoxin complex may influence its expression positively.

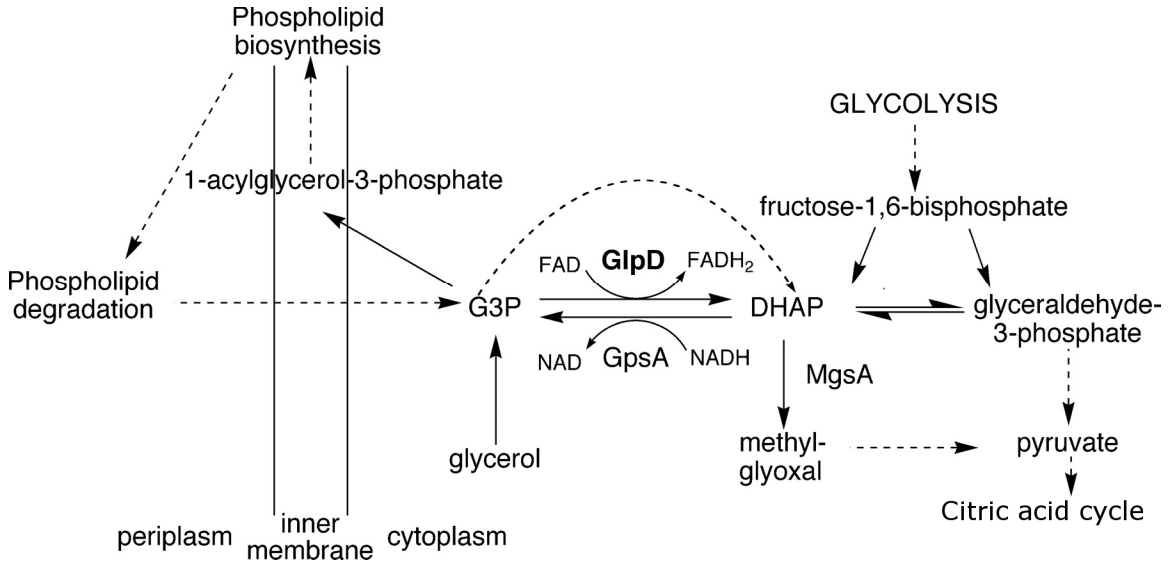


Figure 12. Central role of aerobic glycerol-3-phosphate dehydrogenase (GlpD) in cell metabolism.The GlpD catalyzes the conversion of the precursor of phospholipids biosynthesis (glycerol-3-phosphate G3P) into a substrate for energy generation (di-hydroxi-acetone-phosphate DHAP) (Spoering et al., 2006)

4.3 The effect of ectopic expression of Bat and Bto proteins on the viability of *E. coli*

To test the presumed toxic properties of the Bto protein we examined the viability of *E. coli* strains carrying the *bto* gene and, as a control, either the *bat* gene or the *bat/bto* operon cloned in the pET-28b vector. Gene expression was induced by adding IPTG to the cells, and then the growth of the cultures and the number of viable cells was determined. Surprisingly, *E. coli* cells expressing the *bto* toxin (from plasmid pMP113) showed similar growth properties after induction as the control cells carrying the empty vector (Figure 13A). But the strains expressing the cloned antitoxin (pMP112) or the *bat/bto* operon (pMP114) grew very poorly (Figure 13A). In accordance with the growth properties, the number of viable cells containing either the plasmid pET-28b or pMP113 continuously increased, whereas the living cell number of the strains with plasmids pMP112 or pMP114 drastically decreased by the third hour after induction (Figure 13B). These data revealed that the *bat/bto* system may be composed of a functionally inactive

toxin component and an antitoxin that has an unusual activity. The more pronounced decrease of the number of viable cells in the presence of both components suggests a role for the toxin via complex formation with its antitoxin partner.

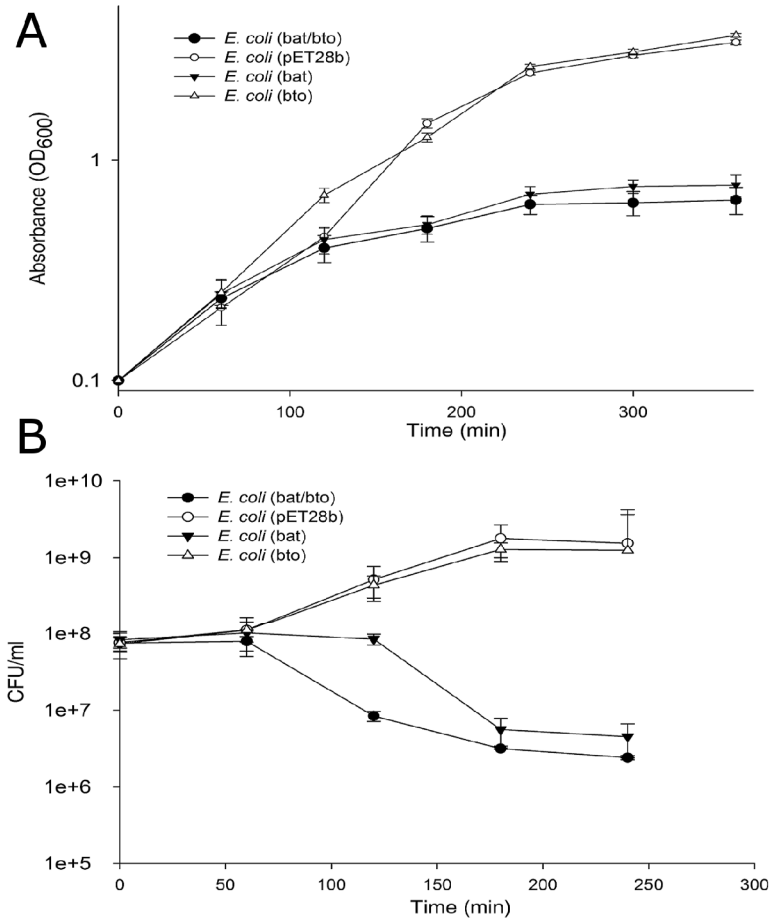


Figure 13. Ectopic expression of *bat. bto* and *bat/bto* genes. Growth (A) and viability (B) of *E. coli* carrying the vector plasmid (pET-28b: open circles), the cloned *bat* antitoxin (pMP112: filled triangles), the *bto* toxin (pMP113: open triangles) or both genes (pMP114: filled circles) expressed from the IPTG-inducible promoter of plasmid pET-28b. *E. coli* cells grown in LB medium were induced by 1 mM IPTG at OD_{600}=0.1. Growth of the culture was followed by measuring optical density at 600 nm. Samples were taken at the indicated time, diluted and plated on LB medium to determine the number of viable cells. Standard deviation was calculated from the data of three experiments.

4.4 Altered division rate of the *bat/bto* mutant strain is dependent on the nitrogen and carbon sources of the growth medium

Deletion of the *bat/bto* operon of *B. japonicum* resulted in a considerably decreased generation time of the mutant bacteria, as compared to that of the wild-type strain, in YEM medium (Figure 14A). Rapid growth stopped when the optical density of the MP99 culture reached 0.8 at 600 nm. Under these conditions, more than 80% of the cells sustained their viability for more than 6 days. The growth of bacteria could be restored by transferring the cells into fresh medium where higher optical densities (OD_{600}=1.2) were obtained (Figure 15A). In PSY medium the generation time of MP99 was even shorter (about 2 hours) and the culture could reach a much higher optical density (Figure 14A).

To determine which component of the PSY medium was responsible for the improved growth rate, the effect of two components, peptone and biotin was tested by adding them separately to YEM medium. The presence of biotin did not improve bacterial growth in YEM. Moreover, the growth of *B. japonicum* strains was not influenced when biotin was absent in PSY medium (data not shown). The addition of peptone, a rich source of complex nitrogen and carbon, however, promoted a better growth of MP99 in YEM medium (data not shown), suggesting a role of complex nitrogen source in the fast growth. This was confirmed when minimal medium (MM), containing ammonium sulphate as nitrogen source, was used. The *bat/bto* mutant was unable to grow in MM, whereas the growth of the wild-type bacterium was only delayed (Figure 14C). When peptone was added to MM the growth of MP99 was similar to that in YEM (Figure 14D). Considering the role of the complex nitrogen in the growth of the *B. japonicum bat/bto* mutant, we also tested another type of medium that contains complex nitrogen source. The TA medium has in its composition tryptone, another type of protein derivate, which is a specially formulated enzymatic digest of casein, similar to peptone. It is used to support higher growth rates in media prepared for molecular biology. The growth rates of the *B. japonicum* strains were much more influenced by this type of complex nitrogen. The *B. japonicum* wild-type bacterium was unable to grow in this type of medium, while the growth of the *bat/bto* mutant was exuberant reaching a very high optical density (Figure 15 B).

To examine whether the altered division rate of the mutant strain was due to the deletion of the *bat/bto* module, MP99 was complemented in *trans* with a plasmid

containing the *bat/bto* coding region and the putative promoter. The growth of the complemented strain MP120 was similar to that of the wild-type strain, both in YEM and MM media (Figure 14E and 14F, respectively), suggesting that the altered generation rate of the MP99 mutant strain was due to the absence of the *bat/bto* operon. The revertant MP119 strain (obtained by restoring of the *bat/bto* operon in the MP99 mutant strain) exhibited similar growth pattern as the wild-type and MP120 complemented strains (data not shown).

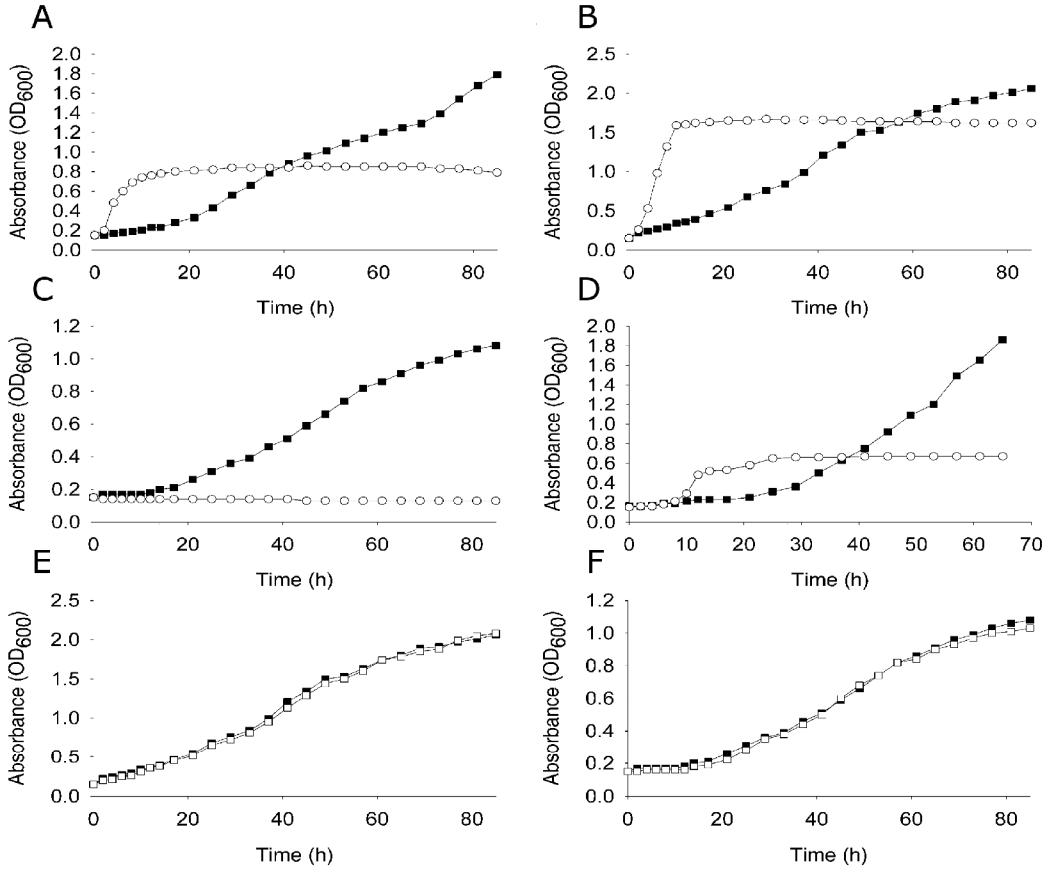


Figure 14. Comparison of the growth of *B. japonicum* strains in different media. A, B, C, D: Growth curves of *B. japonicum* wild-type (filled squares) and *bat/bto* mutant MP99 (open circles) strains in YEM (A), PSY (B) and MM media without (C) or with (D) the addition of peptone. E, F: Comparison of the growth of *B. japonicum* wild-type and MP120 complemented strains in YEM (E) and MM (F) media. Cultures were grown at 31°C in liquid medium containing the appropriate antibiotics. At the beginning of the experiments starter cultures were diluted in the corresponding media to obtain an optical density of 0.15. Bacterial growth was followed by measuring the optical density at 600 nm.

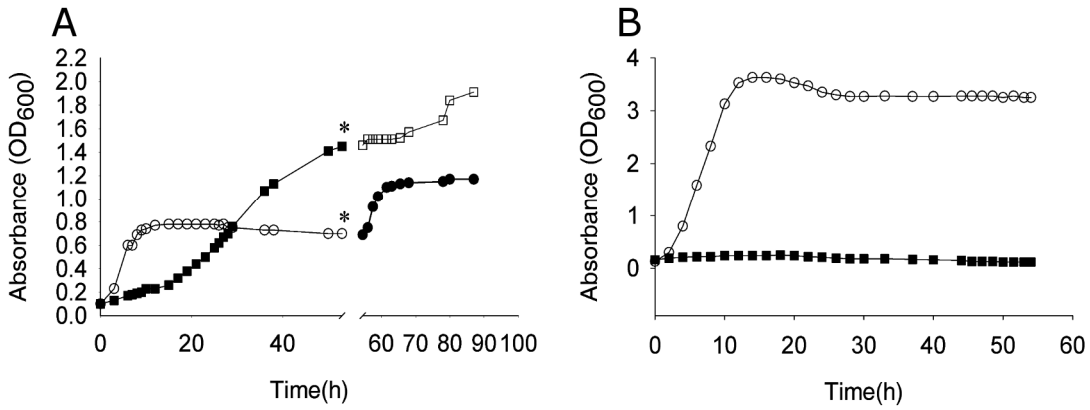


Figure 15. Growth of *B. japonicum* strains in different conditions. A, B: Growth curves of *B. japonicum* wild-type (filled squares) and *bat/bto* mutant MP99 (open circles) strains in YEM (A) and TA media (B). A: *- after 52 hours of growth, the cultures were briefly centrifuged at 4000rpm and resuspended in identical amount of fresh YEM medium. After resuspension (*), wild-type is represented by empty squares and MP99 mutant by filled circles (A). At the beginning of the experiments starter cultures were diluted in the corresponding media to obtain an optical density of 0.15. Cultures were grown at 31°C in liquid medium containing the appropriate antibiotics. The growth of bacteria was followed by measuring the optical density at 600 nm.

4.5 Cell size and mechanical parameters are affected by the *bat/bto* mutation

The faster dividing MP99 cells also exhibited considerably altered morphological properties. The differences in shape and size of the mutant and wild-type bacteria were demonstrated by the help of laser scanning microscope (LSM) and atomic force microscope (AFM) (Figures 16 and 17). The LSM images of acridin orange-stained fixed bacteria showed that the mutant cells are smaller in size and have different shape compared to the wild-type cells (Figure 16).

Indeed, the three-dimensional surface profiles of living cells obtained by AFM demonstrated that wild-type bacteria had elongated cylindrical shape (2-2.5 μm long and 0.5-0.6 μm wide), whereas the mutant cells were more compact and round shaped with decreased length (1-1.5 μm) and increased width (1 - 1.4 μm). The height profile shown in Figure 17B was determined between the markers indicated in Figure 17A.

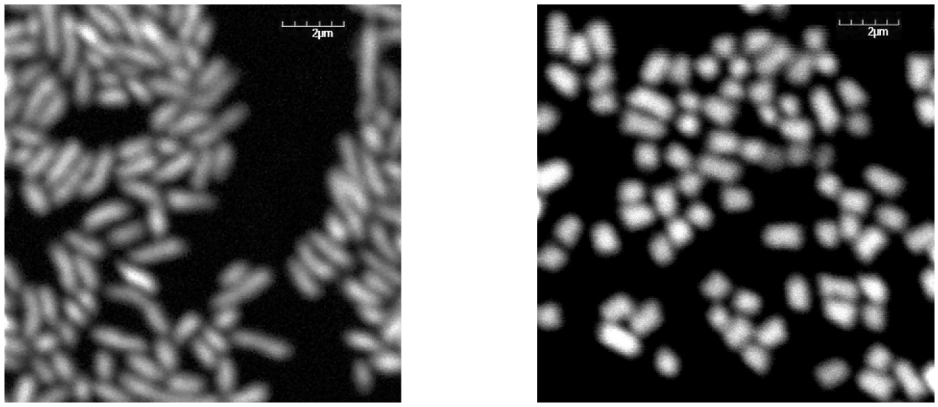


Figure 16. Laser scanning microscope images of *B. japonicum* wild-type (left) and *bat/bto* mutant MP99 (right) cells. Aliquots were removed from early logarithmic phase cultures grown in YEM medium. Bacteria were fixed with a phosphate buffered saline-formaldehyde solution for 1 hour. Samples were laid on microslides and stained with a 10 mg/ml acridineorange solution.

To investigate the mechanical properties of the mutant cells, local force measurements were performed by recording the indentation of the AFM cantilever in function of the vertical displacement of the z piezo. As the distance and indentation curves demonstrated, higher distance (Figure 18A) and indentation (Figure 18B) values were obtained with the mutant cells compared to the wild-type strain, indicating a softer cell surface. This was confirmed by the calculated elastic modulus which was about one order of magnitude lower for the mutant strain (E_m = 0.6±0.24 Mpa) than that of the wild-type (E_m = 4.5±0.92 Mpa) (Figure 18).

In order to explain the observed morphological changes, the amount and composition of several cell membrane components of the mutant and wild-type strains were investigated in further experiments.

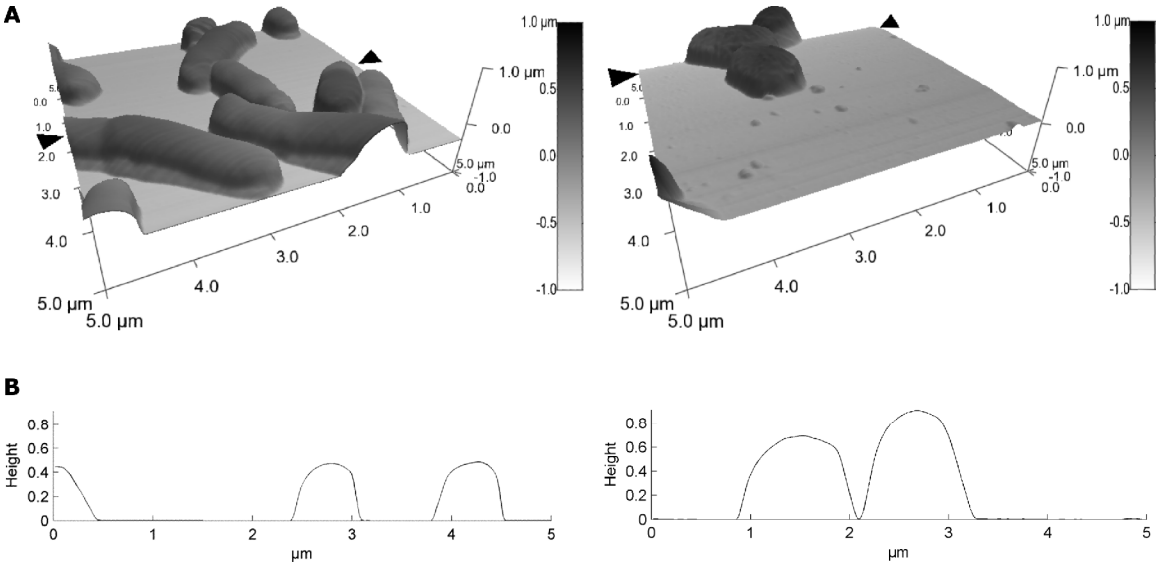


Figure 17. Cell shape and size properties of *B. japonicum* strains determined by AFM. A: Three-dimensional AFM profiles of *B. japonicum* wild-type (left) and *bat/bto* mutant MP99 (right) cells constructed from 5x5 µm scan images. Both trace and retrace images were measured at 0.3-1.0 line/sec scan rate. B. Height profiles of AFM images were drawn between the markers (black triangels). Images were made after immobilization of bacterial suspensions, grown at 31^{o}C in YEM medium, on a poly-L-lysine precoated mica surface.

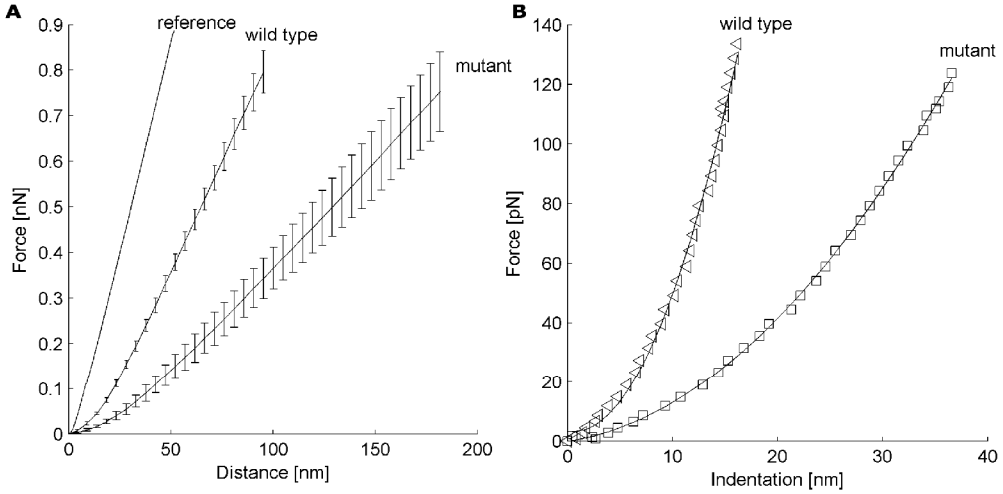


Figure 18. Elastic properties of *B. japonicum* wild-type and *bat/bto* mutant bacteria measured by AFM. A: force-distance curves with standard deviation error bars. B: force-indentation curves calculated by subtracting the reference from the curves of wild-type and mutant cells. Lines are the fitted second order polynomials. Immobilized living bacterial cells were prepared as in Figure 17.

4.6 The *bat/bto* mutant membrane is more resistant to SDS treatment

In a preliminary experiment we assayed the growth of *B. japonicum* wild-type and MP99 mutant strains under different stress conditions (in the presence of SDS, at high concentrations of salt, in the presence of hydrogen peroxide). High concentrations of salt, or presence of hydrogen peroxide did not promote any difference in the response of *B. japonicum* strains, but we observed that SDS completly inhibited the growth of the wild-type strain, while the growth of the mutant was not affected at the same concentration of SDS in the YEM medium. We analyzed the cell morphology of both strains after SDS treatment with the help of AFM. We observed that even at SDS concentration as low as 0.01%, wild type cells lysed, whereas there was no visible effect on the *bat/bto* mutant cells even after 2 hours (Figure 19).

4.7 The *bat/bto* mutation results in differences in the pattern of polysaccharides and phospholipids

B. japonicum wild-type bacteria produce large amounts of polysaccharides during culturing in liquid or on solid media (Minamisawa,. 1989). We observed that the MP99 strain formed less mucous colonies than the wild-type strain. We determined the amount of produced exopolysaccharides of wild-type and mutant bacteria by the phenol-sulfuric acid method. The *bat/bto* mutant strain exhibited a significant, four-fold decrease in exopolysaccharide production (4.8±0.8 μg glucose equivalent/mg protein·ml) compared to that of the wild-type bacteria (19.7±2.3 μg glucose equivalent/mg protein·ml).

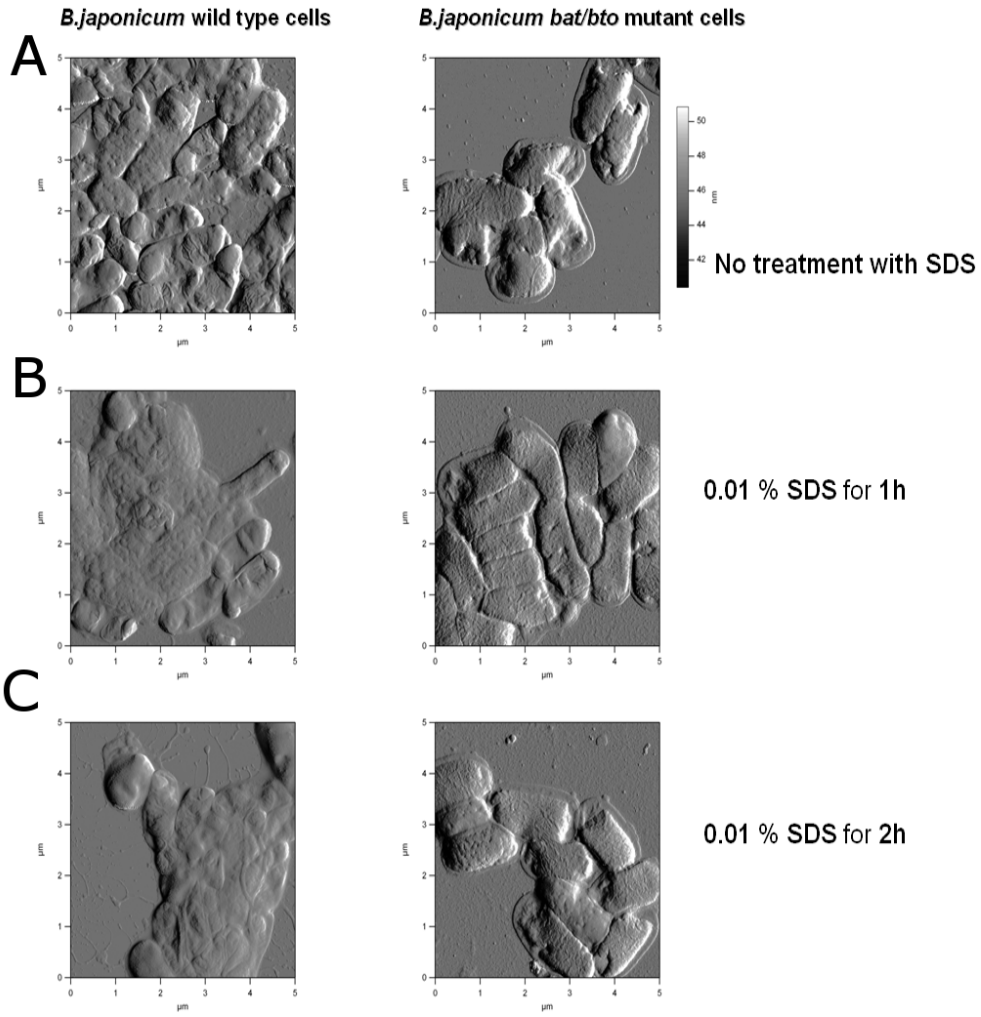


Figure 19. AFM images of SDS effect on *B.japonicum* strains. A, B, C: AFM amplitude images of *B. japonicum* wild-type (left) and *bat/bto* mutant MP99 (right) cells constructed from 10x10 µm scans. Cells were grown in YEM medium at 31°C and at early exponential phase they were treated with 0.01% SDS for one hour (B) or two hours (C). Cells without SDS treatment were also investigated (A). Aliquots were removed from cultures, deposited on micro slides and thoroughly dried. Samples were scanned with AFM in tapping mode.

Lipopolysaccharides (LPSs), greatly contributing to the structural and physiological integrity of the bacterial cell, were analyzed in further experiments. *Rhizobium* LPSs also play an important role in the symbiotic infection process. *Rhizobium* mutants producing defective LPSs that lack the O-chain polysaccharide were shown to be defective in nodulation (Stacey et al., 1991). The differences in LPS patterns between the wild-type and MP99 strains were remarkable (Figure 20). The heterogeneous banding region of wild-type LPSs at high molecular weight (HMW) (Figure 20, lane 1)

consisted of LPS molecules which were separated according to the number of O-antigen repeating units or differences in the electric charges of these units (Carrion et al., 1990). The low molecular weight (LMW) bands (Figure 20, lanes 2 and 4) represented incomplete LPS of the mutant strain, which either lacked the entire O-antigen repeating unit or contained only 1-2 repeating units. The mutant also produced lower amounts of complete LPS molecules, with altered mobility as compared to the wild-type strain (Figure 20, lane 4). In the complemented strain MP120 which carried the re-introduced *bat/bto* operon, the heterogenous banding region characteristic for the wild-type lipopolysaccharide pattern was restored (Figure 20, lane 3).

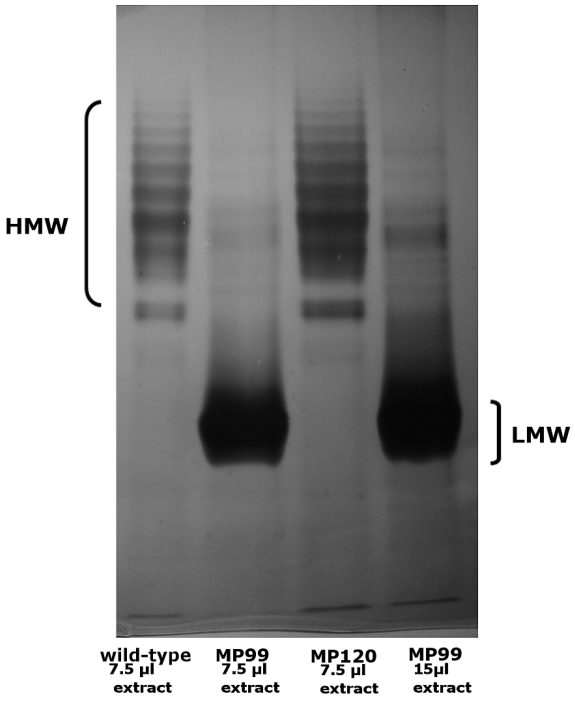


Figure 20. Sodium deoxycholate-polyacrylamide gel electrophoresis (DOC-PAGE) of polysaccharide extracts from *B. japonicum* wild-type, mutant MP99 and complemented MP120 strains, silver stained for lipopolysaccharides (LPS). Two types of LPS patterns (HMW and LMW) of different molecular weight are shown in the figure. Polysaccharides were extracted from cultures grown in PSY medium at 31^{o}C to OD_{600} = 0.6. DOC-PAGE was performed on 18% polyacrylamide gels.

The considerable change in the shape of the cells and in the softness of the mutant membrane observed by AFM force measurements suggested an alteration of the membrane composition of the *bat/bto* mutant. To determine the phospholipid (PL) composition of membranes of wild-type and MP99 strains, thin layer chromatography

coupled with gas chromatography/mass spectrometry (GC/MS) determination of fatty acids was used (Figure 21). The main phospholipids of the wild-type cells were phosphatidylethanolamine (PE), phosphatidylcholine (PC), cardiolipin (CL), phosphatidylglycerol (PG), the intermediate lipid mono-methyl-phospatidylethanolamine (MMPE) and lysophosphatidyl ethanolamine (LPE) (Figure 21 and Table 5).

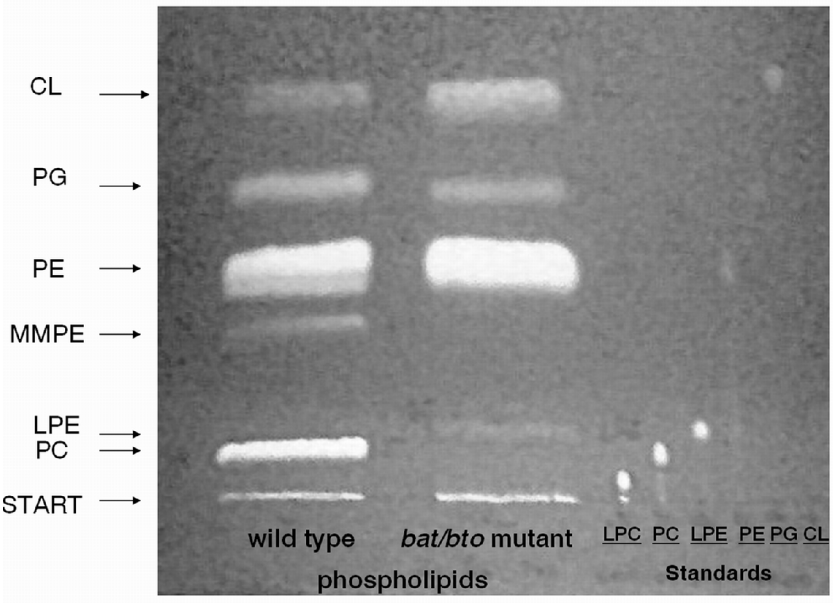


Figure 21. Thin layer chromatography of phospholipid samples. Separation was performed on silica gel G plates. Samples were visualized by spraying with 0.05% 8-anilinonaphtalene-1-sulfonate. The identified phospholipids are: LPE: lysophosphatidylethanolamine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; CL: cardiolipin; PC: phosphatidylcholine; MMPE: mono-methyl-phosphatidylethanolamine; LPC: lyso phosphatidylcholine.

There were striking differences in the PL composition of the two strains (Figure 21), of which the most remarkable was the lack of PC in the mutant. The main phospholipid in both cell types was PE, which increased in the mutant in parallel with the disappearance of PC. Cardiolipin (CL) was present in higher amounts in the mutant, at the expense of its precursor, PG (Figure 21 and Table 5).

As the physico-chemical properties of phospholipids are determined by both the head group and the alkyl chain regions, the fatty acid composition of total phospholipids was also investigated from both wild-type and MP99 strains (Figure 22A and B).

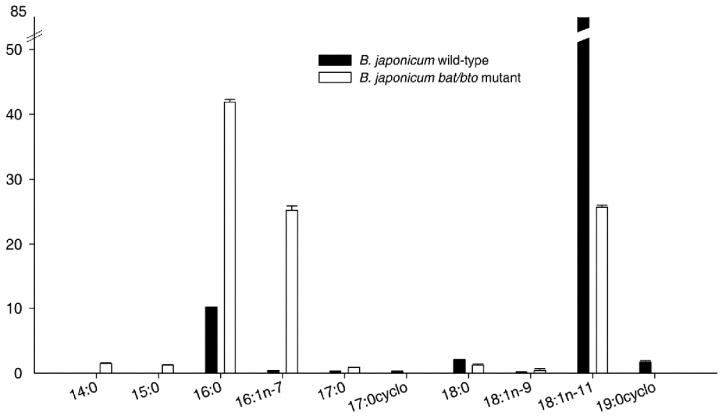
Table 5. Phospholipid composition of *B. japonicum* strains

Phospholipids	*B. japonicum*	
	wild type %	*bat/bto* mutant %
LPE	**-**	**2.4±0.1**
PE	**51.2±1.4**	**75.4±8.9**
MMPE	**3.4±0.0**	**-**
PC	**24.5±1.1**	**-**
PG	**13.7±1.9**	**7.9±0.4**
CL	**7.2±0.5**	**14.3±1.5**

In the wild-type bacteria *cis*-vaccenic acid (18:1) was the major fatty acid making up about 80% of total fatty acids, the amount of which decreased to less than one third (25%) in the mutant strain. As the amount of palmitoleic acid (16:1) increased more than 50 times (0.4% in the wild-type vs. 25.2% in the mutant cells), it was obvious that fatty acid elongation was blocked in the mutant strain. However, there was a more overall modification in fatty acid synthesis as palmitic acid (16:0) content increased from 14% in the wild-type to 42% in the mutant cells. This resulted in a considerable decrease in the ratio of unsaturated fatty acids in the mutant (50.9%) compared to a much higher value in wild-type cells (83.3%) suggesting a fundamental alteration in the lipid chain order and the polymorphic characteristics of membrane lipids.

To explain the significant changes observed in phospholipid composition, the expression of several genes involved in PC biosynthesis was investigated.

A



B

Fatty acids %	Bacterial strain: *B. japonicum* wild type strain	Bacterial strain: *B. japonicum bat/bto* mutant strain
14:0	-	1.5±0.1
15:0	-	1.2±0.1
16:0	10.2±0.1	41.9±0.4
16:1 n-7	0.4±0.0	25.2±0.7
17:0	0.3±0.0	0.9±0.0
17:0 cyclo	0.3±0.0	2.0±0.0
18:0	2.1±0.0	1.2±0.2
18:1 n-9	0.1±0.1	0.4±0.3
18:1 n-11	84.8±0.0	25.7±0.3
19:0 cyclo	1.7±0.2	-

Figure 22. Fatty acid composition of total lipid extracts (A and B). The amounts of fatty acids, as the percentage of total lipids, are indicated with SD values. All experiments were done in triplicate.

4.8 Transcripts for the two phospholipid N-methyltransferases PmtA and PmtX1 are not detectable in the *bat/bto* mutant cells

The absence of PC in the membranes of the MP99 mutant cells was an intriguing observation. Moreover, MMPE, the first intermediate during the conversion of PE to PC, was also absent in the mutant membranes.

PC biosynthesis is accomplished by three successive methylation steps of PE in many prokaryotes. An alternative direct pathway, which requires the presence of free choline and of PC synthase, was documented in several bacteria (Martínez-Morales et al. 2003) (Figure 23).

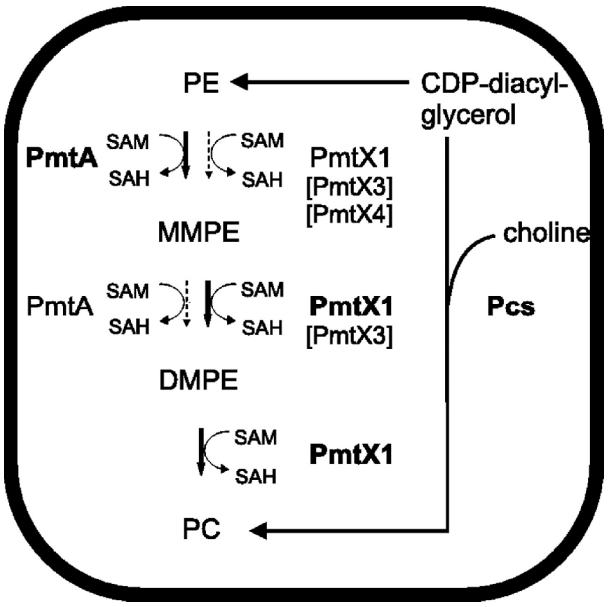


Figure 23. Model of phosphatidylcholine (PC) biosynthesis in *B. japonicum*. In the methylation pathway, PC is formed by three successive methylations of phosphatidylethanolamine (PE) via the intermediates monomethylphosphatidylethanolamine (MMPE) and dimethylphosphatidylethanolamine (DMPE), using the methyl donor *S*-adenosyl-L-methionine (SAM). In the CDP-choline pathway, PC is obtained from the condensation of CDP-diacylglycerol and free choline, with CDP-choline as intermediate. Thick arrows and bold letters indicate the predominant reaction(s) performed by each enzyme. SAH: *S* –adenosylhomocysteine (Hacker et al. 2008).

In *B. japonicum* the first methylation step is performed predominantly by the enzyme PmtA, whereas PmtX1 catalyzes mainly the second and the third subsequent methylation steps (Hacker et al. 2008) (Figure 23). With the use of RT-PCR we checked the level of mRNA from these two genes. We observed that both the *pmtA* and *pmtX1* transcripts were detectable in the wild-type cells (Figure 24, lanes 5 and 8), but they were not detected in the mutant strain (Figure 24, lanes 6 and 9) or in the mutant strain carrying the vector plasmid pRK290 (data not shown). In the complemented strain MP120 *pmtA* and *pmtX1* mRNA levels were restored (Figure 24, lanes 7 and 10). Therefore, the lack of the enzymes catalyzing the methylation reactions explained the absence of PC in the *bat/bto* mutant bacteria. As a positive control, the expression of histidyl-tRNA synthetase (*hisS*) was detected in all strains (Figure 24, lanes 2, 3 and 4.).

Figure 24. Transcript levels of *pmtA*, *pmtX1* and *hisS* genes. RNA was isolated from cultures of *B. japonicum* strains grown in PSY medium at 31°C. Reverse transcription was performed from identical amounts of RNA obtained from *B. japonicum* wild-type (lanes 2, 5 and 8), MP99 *bat/bto* mutant (lanes 3, 6 and 9) and MP120 complemented strains (lanes 4, 7 and 10). Amplification reactions were done using gene-specific primers for *hisS* (lanes 2, 3 and 4), *pmtA* (lanes 5, 6 and 7) and *pmtX1* (lanes 8, 9 and 10). Lane 1: DNA ladder.

4.9 Symbiotic phenotype of the *bat/bto* mutant

We have shown previously that a mutation in the toxin component of the *ntrPR* module of *S. meliloti* results in increased nodulation and more efficient nitrogen fixation capacity in symbiosis with the host plant alfalfa (Oláh et al. 2001). Since both the Bto and NtrR toxins are PIN domain homologues, we examined whether the Bto toxin may have a similar effect on symbiotic properties. Soybean plants were inoculated with the mutant MP99 and with the wild-type strain, and nodule formation and plant biomass production were determined after 60 days. Deletion of the *bat/bto* region resulted in decreased symbiotic capacity. The mutant strain produced about 60% less nodules on soybean (51.7±6 nodules/plant formed by the wild-type bacteria compared to 22.5±4 nodules/plant elicited by the mutant MP99 strain). The average dry weight of plants inoculated by the mutant strain was about 40% lower than that of the wild-type-inoculated plants (0.57±0.13 g/plant and 0.95±0.12 g/plant, respectively). The average dry weight of uninoculated plants was 0.38±0.07 g/plant.

Bacteria were recovered from 40 randomly collected nodules of both types and 200 colonies from each inoculation were tested for kanamycin and spectinomycin resistance, the markers of the mutant and wild-type strains, respectively. One hundred percent of the colonies obtained from the MP99 mutant-elicited nodules were kanamycin-resistant and

exhibited the compact morphology of the MP99 mutant colonies (low amount of polysaccharide production). Their growth on YEM agar plates was also similar to that of the MP99 strain. Randomly selected colonies were further tested in YEM liquid medium and their generation time was identical to that of the mutant strain (data not shown). The nodules elicited by the wild-type strain contained only kanamycin-sensitive bacteria.

5. Discussion

The *bat/bto* operon identified and experimentally characterized in this work is the first TA-like module of the soybean microsymbiont *Bradyrhizobium japonicum*. The *bat/bto* operon of *B. japonicum* is structurally similar to bacterial TA modules but it does not exhibit the characteristic autoregulatory circuit of such modules identified in various bacterial species. Due to the site of integration in the chromosome, the regulation of the *bat/bto* expression seems to be more complex involving several factors. We demonstrated that the deletion of the TA module caused remarkable physiological alterations resulting in morphological and structural changes. The observed effects define new functions for a TA system.

5.1 Structure and organization of the *bat/bto* operon

TA loci have been grouped into seven major families based on their module organization and domain structure (Gerdes et al., 2005; Jørgensen et al., 2009). Members of the *vapBC* family are composed of a toxin that carries a PIN domain, first described as the PilT N-terminal domain in several bacteria (Makarova et al., 1999; Wall and Kaiser, 1999; Wolfgang et al., 2000), and an antitoxin that containes one of the DNA binding motifs AbrB, cHTH, Phd/YefM, or MetJ/CopG (Anantharaman and Aravind, 2003). The *bat/bto* module of *B. japonicum* is composed of two members arranged into an operon. The size and organization of the genes in the *bat/bto* operon are similar to those of other known TA systems. However, the structure of the operon shows some particularities.

Data obtained on various TA systems proved that the N- and C-terminal parts of antitoxin and toxin proteins play different roles. The N termini of the antitoxins are involved in repressor activity (*via* binding to operator regions of their promoter) while the C termini are involved in the neutralization of the toxin by protein-protein complex formation. The N-terminal (repressor) region is functionally independent of the C-terminal antitoxin region. Both the N- and C-terminal parts of the toxins are responsible for the protein complex formation with the antitoxin (Bernard and Couturier, 1991; Ruiz-Echevarria et al., 1995; Zhang et al., 2003b). Smith and Magnuson (2004) proposed that

the toxin-antitoxin operons are composites of two functional modules, an operator-repressor and an antitoxin-toxin module, joined together by a transition region (Figures 2 and 10). As further sequence analysis revealed, the *bat/bto* module may also be a composite of two different modules. Bto is a PIN domain-containing protein whose partner is generally a SpoVT/AbrB-domain containing protein (Anantharaman. and Aravind, 2003). Our data demonstrated the Bat protein is a mosaic protein since the C-terminal region is similar to the SpoVT/AbrB domain (constituting the partner for the PIN domain-containing Bto protein in the antitoxin-toxin module), while the N-terminal part shows similarity to the Phd protein, a member of the Phd/Doc family of TA systems (representing together with the operator sequence of the operon's promoter the operator-repressor module). The transition region between these two modules is similar to that of the Phd/Doc module. Recombination in the repressor-antitoxin transition region preserves the independent module arrangement (operator-repressor associations as well as the antitoxin-toxin association) producing a functional recombinant with new possibilities of action. The *bat/bto* operon seems to be the product of such recombination event between the *vapBC* (SpoVT/AbrB domains) and *phd/doc* families of TA systems (Figure 25).

Therefore, this *vapBC*-type TA module of *B. japonicum* is composed of a mixture of Phd and SpoVT/AbrB-type antitoxins and a PIN domain-type toxin. Other cases of such recombinant systems between members of different TA families were also reported previously (Hayes, 1998; Smith and Magnuson., 2004; Schmidt et al., 2007) supporting the proposed evolutionary origin of new TA systems by gene shuffling between a few major classes of toxins and antitoxins.

The conservation of characteristic residues, the similarities in the predicted secondary and tertiary structures of the Bto protein, as well as the structural organization classifies the *bat/bto* module as a toxin-antitoxin system.

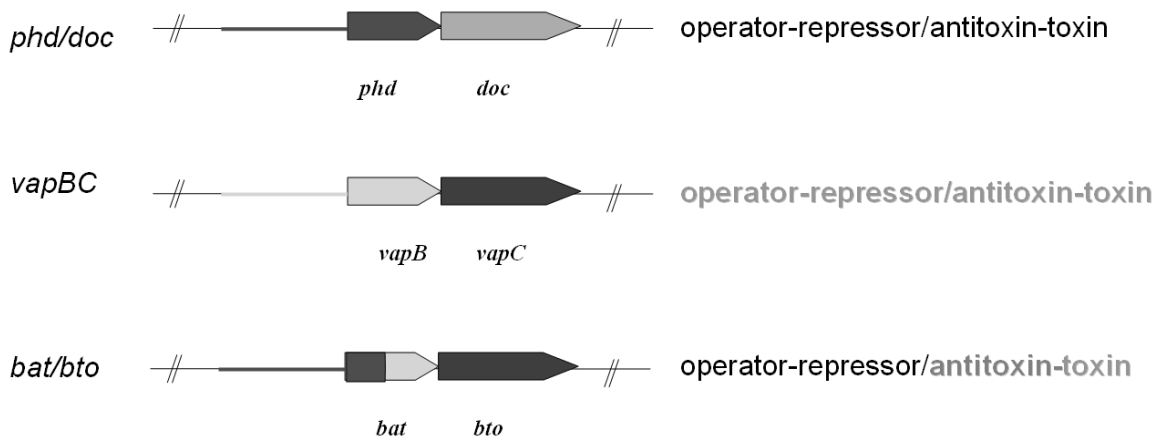


Figure 25. Modular model for the *bat/bto* TA system. It was proposed that TA systems are composites of two separable modules: an operator-repressor module (operator region of the promoter and the N-terminal part of the antitoxin) and an antitoxin-toxin module (C-terminal part of the antitoxin and the toxin) (Smith and Magnuson, 2004). Recombination between modules contributes to operon and antitoxin diversity. The *bat/bto* operon of *B. japonicum* is the product of such recombination event between two different TA systems the *phd/doc* and *vapBC*. The operator-repressor module of *bat/bto* seems to belong to the *phd/doc* family while the antitoxin-toxin module to the *vapBC* family.

5.2 Regulation of the *bat/bto* operon

Although the size and organization of the genes and the deduced amino acid sequences of the proteins encoded by the *bat/bto* module classified it as a TA system, the expression of the module did not exhibit the typical negative autoregulatory properties of most TA modules. Typically a TA system carried by a plasmid vector has a very low level of expression in the wild-type strain where its transcription is repressed by the binding of the toxin-antitoxin complex to the promoter, but has an increased expression level in the mutant background where the toxin-antitoxin complex is absent. By contrast, the expression level of *bat/bto* was higher in the wild-type background than in the deletion mutant strain, suggesting a positive regulatory role for the toxin-antitoxin complex. Moreover, microarray data (Pessi et al., 2007) revealed that the genes corresponding to *bat* and *bto* genes are constitutively expressed in wild-type *B. japonicum* under all conditions tested (free living, symbiotic and microoxic conditions). This indicates that there is no repression of *bat/bto* in wild-type cells, but the Bat/Bto complex is present and seems to be involved in positive regulation. The putative binding

site of the Bat/Bto complex follows the -10 motif in the promoter region (Figure 11B). Therefore, direct autoactivation seems unlikely since competition between the complex and the RNA polymerase for the same DNA region is presumed. Further experiments are required to clarify this mechanism.

Several examples have already demonstrated a more complex regulation of TA systems than a simple negative autoregulation. Two modules with unusual regulation were identified in *Mycobacterium tuberculosis*, where RelB and RelF antitoxins acted as transcriptional activators on their respective promoters (Korch et al., 2009). However, together with their toxin pairs, these proteins repressed the expression to basal levels. The absence of a typical autorepression was also demonstrated in *Staphylococcus aureus*. The promoter of the *mazEF* module in this bacterium was downregulated by *sigB* encoding an alternative sigma factor, and was activated by the SarA transcriptional regulator (Donegan and Cheung, 2009). The expression of *mazEF* module of *E. coli* was shown to be positively regulated by the factor for inversion stimulation (FIS) (Marianovsky et al., 2001). FIS is a nucleoid-associated protein which, by binding as homodimer to the DNA region upstream of the promoters, causes DNA bending, thus increasing the binding efficiency of RNA polymerase. In the *bat/bto* promoter, 92 bp upstream of the start codon we identified a putative FIS-binding site (GGACGAGGAAATGAGC), which may also contribute to the regulation of *bat/bto* expression.

In addition to the lack of negative autoregulation of *bat/bto*, the partial overlapping of the potential binding sites of GlpR protein and Bat/Bto complex suggested the involvement of the downstream-located GlpR protein in *bat/bto* regulation. Indeed, in the presence of the inducer glycerol, which diminishes the binding efficiency of GlpR, an increased promoter activity of the *bat/bto -lacZ* fusion was observed in the wild-type cells grown either in rich or minimal medium. In the genome of three *Bradyrhizobium* strains (*B. japonicum* USDA 110, *B. sp*. BTAi1 and *B. sp*. ORS278) one of the multiple *glpD* copies is located downstream of *glpR*. Putative GlpR binding sites could be localized only in the promoter region of this copy. By computer-assisted analyses the presence of 3-4 direct repeats of the consensus –TTTCGTT- separated by 3-4 nucleotides was described in three other *Rhizobium* species (Danilova et al. 2003). Although, none of the *glpD* promoters in different *Bradyrhizobium* strains carry these sequences, the *glpD* promoters located upstream of the *glpR* genes are characterized by the presence of T-rich

sequences interrupted by 2-3 G/C nucleotides. The lack of negative autoregulation, the involvement of GlpR in the regulation of *bat/bto* expression and the presence of a multitude of overlapping binding sites for different regulators are clear indicators of a complex regulation for *bat/bto* operon.

5.3 Toxicity of the Bat/Bto proteins

Our observation that the ectopic expression of Bto toxin had no influence on the viability of *E. coli* suggested that the Bto protein has lost its function characteristic for the toxin components of TA modules. It is conceivable that the loss of toxicity is due to the replacement of one of the essential acidic residues with an asparagine near to the C-terminal end. The structural analysis of PIN-domain proteins revealed that the quartet of acidic amino acids constitute the active site that binds Mg^{2+} or Mn^{2+} ions and facilitates the cleavage of single-stranded RNA (Bunker et al 2008). More surprisingly, the ectopic expression of either the Bat antitoxin or the complete module resulted in a remarkable loss of cell viability. Whether these unconventional properties of the *bat/bto* system are due to the binding of antitoxin to some yet unknown regulatory regions, or to possible interactions with TA modules of *E. coli*, remain to be further examined.

By analyzing nearly 400 natural isolates of *E. coli*, Mine and coworkers (2009) proved that 29% of the ccd_{O157} variants were composed of an active antitoxin and an inactive toxin. Moreover, CcdB toxin proteins were more diversified and lost their function more frequently than the antitoxins. Therefore, the analysis of the genome context of a TA module is important, because TA-like genes could be in close proximity to genes coding for proteins that give clues to their biological function in the organism in which they are present. For example, in archaea and cyanobacteria where a large number of PIN-domain containing TA operons are present, these operons are associated with two-component response regulators, NADH dehydrogenase operons and cytochrome c-type biogenesis proteins. In these cases, the PIN-domain TA operons appear to be among cassettes of genes which leads to an expansion of cellular metabolic functions (Arcus et al, 2005). It is also important to differentiate between their function at the time of incorporation and their present biological function, which might be only distantly related. One hypothesis is that chromosomal TA systems, acquired by horizontal gene transfer,

confer no selective advantages to the host, and represent just genomic material that will be lost, although at a very slow rate, due to their addictive proprieties. The most accepted hypothesis is that once resident within a bacterial genome, TA operons might become integrated in regulatory networks, performing new cellular roles. The identification of a novel *E. coli* TA system, the *prlF-yhaV* module composed of RelE-like toxin and a swapped-hairpin antitoxin, showed that these proteins only appear to associate during the folding of the PrlF antitoxin which has an additional function not connected to its antitoxin activity (Schmidt et al., 2007). The *spoVT* gene product of *B. subtilis*, which shares the same protein domain with several *vapBC*-type antitoxins, was shown to be capable of regulating the expression of several genes, either positively or negatively (Bagyan et al., 1996). These examples suggest a wide range of possible independent functions for the antitoxin, and may give an explanation for the unexpected effect of the Bat antitoxin on the viability of *E. coli* cells.

Comparing the intergenic regions between the *glpD* and *glpR* genes of three *Bradyrhizobium* species (*B. japonicum* USDA110, *B. sp*. BTAi1 and *B. sp*. ORS278) we observed a considerable DNA sequence similarity (80%), similar length and the presence of identical putative binding sites for GlpR. We suppose that the *bat/bto* operon was acquired by horizontal gene transfer and was inserted between the *glpD* and the *glpR* genes of *B. japonicum* USDA110 resulting in overlapping regulatory regions. The possible lack of stronger selective pressure may have resulted in the inactivation of the toxin function, whereas the antitoxin (and its complex with the cognate toxin) became part of a metabolic process such as the competition with the GlpR protein.

5.4 Physiological role of the *bat/bto* operon in *B. japonicum*

Deletion of the *bat/bto* operon resulted in alterations in several metabolic pathways leading to characteristic phenotypic changes. The electrophoretic pattern of cell membrane lipopolysaccharides revealed the accumulation of incomplete LPS molecules. Comparison of the phospholipids and fatty acid compositions in the wild-type and mutant strains showed substantial alterations. PC was completely absent in the mutant strain, whereas the amounts of PE and CL increased substantially. Only a minority of all bacterial species produces PC and there is growing evidence that the

fitness of those bacteria that produce PC depends on this particular phospholipid. PC was shown to be critical for the survival of *S. meliloti* (de Rudder et al. 2000) and for the proper recovery of *P. aeruginosa* and *L. pneumophila* from cryo-preserved stocks (Wilderman et al., 2002, Conover et al., 2008). Decreased amount of PC is known to be compensated by elevated PE level (Minder et al., 2001) but this does not explain the reduced amount of PG and the increase of CL in the mutant membrane. It is known that PE and CL are both non-bilayer forming lipids. In the cells the balance between bilayer and non-bilayer lipids is tightly regulated and has been shown to be essential for viability (Rietveld et al. 1993). The regulation of the tendency to form the hexagonal phase can be achieved by alterations in membrane fatty acid composition that influence phospholipid shape (Horváth et al. 1986). The cone-shaped geometry of PE with relatively small head group size coupled with the relatively large area occupied by the unsaturated 18:1 acyl chains promotes the formation of non-bilayer, hexagonal structures. This might explain why the content of unsaturated fatty acid species (18:1) decreased in the mutant strain, which had a dramatically increased PE content. Taken together, it is highly conceivable that the above restructuring of lipids is aimed at re-establishing lateral packing order, bilayer stability, membrane permeability and, ultimately, membrane functionality in the mutant strain (Vigh et al., 2005).

In prokaryotes one of the biosynthetic pathways yielding PC is the enzymatic methylation of PE catalyzed by phospholipid *N*-methyltransferase (Pmt) in three consecutive steps using *S*-adenosylmethionine as methyl donor. Alternatively, PC can be synthesized directly from free choline by phosphatidylcholine synthase (Pcs) (Figure 23). Pcs is also present and functional in *B. japonicum*, although no uptake system exists for choline in this species (Boncompagni et al., 1999; Hacker et al., 2008). Therefore, block of the methylation pathway results in the absence of PC in the MP99 mutant strain. We have shown that the methylation steps are defective, since the transcripts of the genes coding for PmtA and PmtX1 (Hacker et al. 2008) were not detected in the mutant cells. We suppose that the absence of Bat/Bto affects indirectly these transcript levels either via the activation of a degrading factor, or by influencing an unknown positive regulatory factor involved in the transcription of these genes.

The profound alterations in the lipid composition of the mutant membranes may result in changes of cell division rate, cell shape and membrane rigidity. The distribution

of phospholipids in membranes is an important factor in the process of division-site selection. At the mid-cell domain optimal lipid composition is required for the Z-ring positioning. In *E. coli* cardiolipin-enriched domains were detected at the cell poles and at the division septum, suggesting their involvement in cell division (Kates, 1986; Mileykovskaya and Dowhan, 2005). Membrane synthesis is also required for cell expansion and might be regulated spatially and temporally in the same manner as cell wall synthesis. The increased amounts of CL, PE, and the incomplete LPS molecules in the mutant strain may therefore influence cell division rate and cell shape by altering the formation of division site selection and the elongation phase between two divisions. We assume that the incomplete LPS and the altered membrane composition may also be responsible for the lower symbiotic efficiency of MP99 strain. PC reduction in *B. japonicum* is known to produce a symbiotic defect, although vegetative growth is unaffected (Minder et al., 2001). Earlier data proved that exo- and lipopolysaccharide molecules acting as signals to avoid plant defense reactions may have an important role in plant-bacterium interactions (Stacey et al., 1991).

Glycerol-3-phosphate dehydrogenase encoded by the *glpD* gene, has a central function at the crossroads of the glycolytic pathway and the phospholipid biosynthetic pathway (Figure 12). GlpD converts G3P, the key precursor of phospholipid biosynthesis, to dihydroxiacetone-phosphate, an intermediate of pyruvate in the glycolytic pathway. Our data suggest that in *B. japonicum* the addition of glycerol resulted in a higher expression of the *bat/bto* genes, probably due to the absence of competing GlpR at the overlapping binding site. Further experiments are required to clarify whether the Bat/Bto proteins may also influence directly or indirectly the regulation of *glpD,* thereby affecting the amount of available substrate for phospholipids biosynthesis and/or the glycolytic pathway.

Chromosomally located TA systems of *E. coli* were shown to act as bacterial metabolic stress managers (Buts et al., 2005; Gerdes et al., 2005), associated with the modulation of the global level of translation under various stress conditions and nutrient limitation. In contrast to these data, deletion of the *B. japonicum bat/bto* operon resulted in alterations of several metabolic pathways and defective symbiotic performance probably due to the changes in lipopolysaccharide and phospholipid composition of the cellular membrane. In conclusion, the *bat/bto* operon seems to be involved in the

maintenance of the normal physiological state of the cell, modulating the metabolic rates to a level that may support a better adaptation to changing environmental conditions.

6. References

Aizenman, E., Engelberg-Kulka, H., and Glaser, G. 1996. An *Escherichia coli* chromosomal "addiction module" regulated by 3',5'-bispyrophosphate: A model for programmed bacterial cell death. Proc. Natl. Acad. Sci. USA, 93:6059-6063.

Alting-Mees, M. A., and Short, J.M. 1989. pBluescript II: gene mapping vectors. Nucleic Acids Res. 17:9494.

Amitai, S., Yassin, Y., and Engelberg-Kulka, H. 2004. MazF-mediated cell death in *Escherichia coli*: A point of no return. J. Bacteriol. 186:8295-8300.

Anantharaman, V., and Aravind, L. 2003. New connections in the prokaryotic toxin-antitoxin network: relationship with the eukaryotic nonsense-mediated RNA decay system. Genome Biol. 4:R81.

Arcus, V. L., Backbro, K., Roos, A., Daniel, E. L., and Baker, E. N. 2004. Distant structural homology leads to the functional characterization of an archaeal PIN domain as an exonuclease. J. Biol. Chem. 279:16471–16478.

Arcus, V. L., Rainey, P. B., and Turner, S. J. 2005. The PIN-domain toxin-antitoxin array in mycobacteria. Trends in Microbiology. 13: 360-365.

Bagyan, I., Hobot, J., and Cutting, S. 1996. A compartmentalized regulator of developmental gene expression in *Bacillus subtilis*. J Bacteriol. 178: 4500-7.

Bálint, Z., Krizbai, I. A., Wilhelm, I., Farkas, A. E., Pardutz, A., Szegletes, Z., and Varo, G. 2007. Changes induced by hyperosmotic mannitol in cerebral endothelial cells: an atomic force microscopic study. Eur. Biophys. J. 36:113-120.

Becker, A., Fraysse, N., and Sharypova, L. 2005. Recent advances in studies on structure and symbiosis-related function of rhizobial K-antigens and lipopolysaccharides. Mol. Plant-Microbe Interact. 18:899–905.

Bernard, P., and Couturier, M. 1991. The 41 carboxy-terminal residues of the miniF plasmid CcdA protein are sufficient to antagonize the killer activity of the CcdB protein. Mol. Gen. Genet. 226:297-304.

Bodogai, M., Ferenczi, S., Bashtovyy, D., Miclea, P., Papp, P., and Dusha, I. 2006. The *ntrPR* operon of *Sinorhizobium meliloti* is organized and functions as a toxin-antitoxin module. Mol. Plant-Microbe Interact. 19:811-822.

Boncompagni, E., Osteras, M., Poggi, M. C., and Le Rudulier, D. 1999. Occurrence of choline and glycine betaine uptake and metabolism in the family *Rhizobiaceae* and their roles in osmoprotection. Appl. Environ. Microbiol. 65:2072-2077.

Brooun, A., Liu, S., and Lewis, K. 2000. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. Antimicrob. Agents Chemother. 44:640-646.

Bunker, R. D., McKenzie, J. L., Baker, E. N., and Arcus, V. L. 2008. Crystal structure of PAE0151 from *Pyrobaculum aerophilum*, a PIN-domain (VapC) protein from a toxin-antitoxin operon. Proteins 72:510-518.

Buts, L., Lah, J., Dao-Thi, M. H., Wyns, L., and Loris, R. 2005. Toxin-antitoxin modules as bacterial metabolic stress managers. Trends Biochem. Sci. 30:672-679.

Campbell, G.R.O., Reuhs, B.L., and Walker, G.C. 2002. Chronic intracellular infection of alfalfa nodules by *Sinorhizobium meliloti* requires correct lipopolysaccharide core. Proc. Natl. Acad. Sci. U S A. 99:3938–3943.

Carrion, M., Bhat, U. R., Rheus, B., and Carlson, R. W. 1990. Isolation and characteriyation of the lipopolysaccharides from *Bradyrhizobium japonicum*. J. Bacteriol. 172:1725-1731.

Christensen, S.K. and Gerdes, K. 2003. RelE toxins from bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA. Mol. Microbiol. 48: 1389-1400.

Christensen, S. K., Mikkelsen, M., Pedersen, K., and Gerdes, K. 2001. RelE, a global inhibitor of translation, is activated during nutritional stress. Proc. Natl. Acad. Sci. U.S.A. 98:14328-14333.

Christensen, S.K., Pedersen, K., Hansen, F.G. and Gerdes, K. 2003. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. J. Mol. Biol. 332: 809-819.

Clissold, P. M., and Ponting, C. P. 2000. PIN domains in nonsense-mediated mRNA decay and RNAi. Current Biology. 10:R888-890.

Conover, G.M., Martinez-Morales, F., Heidtman, M.I., Luo, Z.Q., Tang, M., and Chen, C. 2008. Phosphatidylcholine synthesis is required for optimal function of *Legionella pneumophila* virulence determinants. Cell Microbiol 10: 514–528.

Cooper, T.F. and Heinemann, J.A. 2000. Postsegregational killing does not increase plasmid stability but acts to mediate the exclusion of competing plasmids. Proc. Natl. Acad. Sci. USA. 97:12643-12648.

Daines D.A., Wu M.H., and Yuan S.Y. 2007. VapC-1 of nontypeable *Haemophilus influenzae* is a ribonuclease. J. Bacteriol. 189:5041–5048

Danilova, L. V., Gelfand, M. S., Lyubetsky, V. A., and Laikova, O. N. 2003. Computer-assisted analysis of regulation of the glycerol-3-phosphate metabolism in genomes of Proteobacteria. Mol. Biol. 37:716-722.

de la Cueva-Mendez, G. 2003. Distressing bacteria: Structure of a prokaryotic detox program. Mol. Cell. 11:848–850.

Dénarié, J., Debellé, F., and Promé, J.-C. 1996. Rhizobium lipochitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. Annu. Rev. Biochem. 65:503-535.

de Rudder, K.E., López-Lara, I.M., and Geiger, O. 2000. Inactivation of the gene for phospholipid N-methyltransferase in *Sinorhizobium meliloti*: phosphatidylcholine is required for normal growth. Mol. Microbiol. 37:763-72.

D'Haeze, W., and Holsters, M. 2004. Surface polysaccharides enable bacteria to evade plant immunity. Trends in Microbiology. 12:555-561.

Dimitriadis, E. K., Horkay, F., Maresca, J., Kachar, B., and Chadwick, R.S.. 2002. Determination of elastic moduli of thin layers of soft material using the atomic force microscope. Biophys. J. 82:2798–2810.

Ditta, G., Stanfield, S., Corbin, D., and 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. U.S.A. 77:7347–7351.

Donegan, N. P., and Cheung, A. L. 2009. Regulation of the *mazEF* toxin-antitoxin module in *Staphylococcus aureus* and its impact on *sigB* expression. J. Bacteriol. 191:2795-2805.

Dowhan, W., Mileykovskaya. E., and Bogdanov, M. 2004. Diversity and versatility of lipid–protein interactions revealed by molecular genetic approaches. Biochimica et Biophysica Acta (BBA) – Biomembranes.1666:19-39

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350–356.

Élő, P., Semsey, S., Kereszt, A., Nagy, T., Papp, P., and Orosz, L. 1998. Integrative promoter cloning plasmid vectors for *Rhizobium meliloti*. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 159:7-13.

Engelberg-Kulka H., Glaser G. 1999. Addiction modules and programmed cell death and anti-death in bacterial cultures. Annu. Rev. Microbiol. 53:43–70.

Engelberg-Kulka, H. and Hazan, R. 2003. Cannibals defy starvation and avoid sporulation. Science 301:467-468.

Engelberg-Kulka H., Sat B., Reches M., Amitai S., and Hazan R. 2004. Bacterial programmed cell-death systems as targets for antibiotics. Trends Microbiol. 12:66-71.

Fahreus, G. 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J Gen Microbiol. 2:374-81.

Fozo EM, Hemm MR, Storz G. 2008. Small toxic proteins and the antisense RNAs that repress them. Microbiol. Mol. Biol. Rev. 72:579–589.

Gerdes, K., and Helin, K.. 1988. Translational control and differential RNA decay are key elements regulating postsegregational expression of the killer protein encoded by the *parB* locus of plasmid R1. J. Mol. Biol. 203:119-129.

Gerdes, K., Poulsen, L.K., Thisted, T., Nielsen, A.K., Martinussen, J. and Andreasen, P,H. 1990. The hok killer gene family in gram-negative bacteria. New Biol. 2, 11:946-956.

Gerdes, K. 2000. Toxin-antitoxin molecules may regulate synthesis of macromolecules during nutritional stress. J. Bacteriol. 182:561-572.

Gerdes, K., Christensen, S. K., and Lobner-Olesen, A. 2005. Prokaryotic toxin-antitoxin stress response loci. Nat. Rev. Microbiol. 3:371-382.

Gerdes, K. Wagner, E. G. H. 2007. RNA antitoxins. Curr. Opin. Microbiol. 10: 2 117-24.

Gibson, K. E., Kobayashi, H., and Walker, G. C.2008. Molecular determinants of a symbiotic chronic infection. Annu. Rev. Genet. 42:413-41.

Gordon, R. O., Reuhs, B. L., and Walker, G. C. 2002. Chronic intracellular infection of alfalfa nodules by *Sinorhizobium meliloti* requires correct lipopolysaccharide core. Proc. Natl. Acad. Sci. USA. 99: 3938-3943.

Guerinot, M. L., Meidl, E. J., and Plessner, O. 1990. Citrate as a siderophore in *Bradyrhizobium japonicum.* J. Bacteriol. 172:3298-3303.

Hacker, S., Sohlenkamp, C., Aktas, M., Geiger, O., and Narberhaus, F. 2008. Multiple phospholipid *N*-methyltransferases with distinct substrate specificities are encoded in *Bradyrhizobium japonicum*. J. Bacteriol. 190:571-580.

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

Hayes, F. 1998. A family of stability determinants in pathogenic bacteria. J. Bacteriol. 180:6415-6418.

Hayes, F. 2003. Toxins-antitoxins: plasmid maintenance, programmed cell death and cell cycle arrest. Science. 301:1496-1499.

Hazan, R., Sat, B., and Engelberg-Kulka, H. 2004. *Escherichia coli mazEF*-mediated cell death is triggered by various stressful conditions. J. Bacteriol. 186:3663-3669.

Horváth, I., Mansourian, A. R., Vigh, L., Thomas, P. G., Joó, F., and Quinn, P. J. 1986. Homogeneous catalytic hydrogenation of the polar lipids of pea chloroplasts in situ and the effects on lipid polymorphism. Chem.Phys.Lipids. 39:251-264.

Hutter, J. L., and Bechhofer, J. 1993. Calibration of atomic-force microscope tips. J. Rev. Sci. Instr. 64:1868-1873.

Jensen, R. B., and Gerdes, K. 1995. Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol. Microbiol. 17:205-210.

Jiang, Y., Pogliano, J., Helsinki, D.R. and Konieczny, I. 2002. ParE toxin encoded by the broad-host-range plasmid RK2 is an inhibitor of *Escherichia coli* gyrase. Mol. Microbiol. 44:971-979.

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., and Walker, G. C. 2007. How rhizobial symbionts invade plants: The *Sinorhizobium-Medicago* model. Nature reviews. 5:619-633.

Jørgensen, M. G., Pandey, D. P., Jaskolska, M., and Gerdes, K. 2009. HicA of *Escherichia coli* defines a novel family of translation-independent mRNA interferases in bacteria and archaea. J. Bacteriol. 191:1191-1199.

Kaneko T., Nakamura Y., Sato S., Minamisawa K., Uchiumi T., Sasamoto S., Watanabe A., Idesawa K., Iriguchi M., Kawashima K., Kohara M., Matsumoto M., Shimpo S., Tsuruoka H., Wada T., Yamada M., and Tabata S. 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Res. 9:189-197.

Kates, M. 1986. Techniques in lipidology, pp. 232-254. *In*: Burdon, R. H., van Knippenberg, P. H. (Eds.), Isolation and Identification of Lipids, 2nd ed., Elsevier, Amsterdam.

Kawai, F., Shoda, M., Harashima, R., Sadaie, Y., Hara, H., and Matsumoto, K. 2004. Cardiolipin domains in *Bacillus subtilis* marburg membranes. J. Bacteriol. 186:1475–1483.

Kedzierska, B., Lian L.-Y., and Hayes F. 2007. Toxin–antitoxin regulation: bimodal interaction of YefM–YoeB with paired DNA palindromes exerts transcriptional autorepression. Nucleic Acids Res. 35:325–339.

Keren, I., Shah, D., Spoering, A., Kaldalu, N., and Lewis, K. 2004. Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J. Bacteriol. 186:8172-8180.

Kiss, E., Reuhs, B. L., Kim, J. S., Kereszt, A., Petrovics, G., Putnoky, P., Dusha, I., Carlson, R. W., and Kondorosi, A. 1997. The *rkpGHI* and -*J* genes are involved in capsular polysaccharide production by *Rhizobium meliloti*. J. Bacteriol. 179: 2132–2140.

Kobayashi, I. 2001. Behaviour of restriction–modification systems as selfish mobile elements and their impact on genome evolution. Nucleic Acids Res. 29, 18:3742-3756.

Kolodkin-Gal I., Verdiger, R., Shlosberg-Fedida, A., and Engelberg-Kulka, H. 2009. A differential effect of *Escherichia coli* toxin-antitoxin systems on cell death in liquid media and biofilm formation. PLoS One. 4(8):e6785.

Korch, S. B., Contreras, H., and Clark-Curtiss, J. E. 2009. Three *Mycobacterium tuberculosis* Rel toxin-antitoxin modules inhibit mycobacterial growth and are expressed in infected human macrophages. J. Bacteriol. 191:1618-1630.

Krauss, J. H., Weckesser, J., and Mayer, H. 1988. Electrophoretic analysis of lipopolysaccharides of purple nonsulfur bacteria. Int. J. Syst. Bacteriol. 38:157-163.

Kristoffersen, P., Jensen, G. B., Gerdes, K., and Piskur, J. 2000. Bacterial toxin-antitoxin gene system as containment control in yeast cells. Applied and Environmental Microbiology. 66:5524-5526.

Lagares A., Caetano-Anollés, G., Niehaus, K., Lorenzen, J., Ljunggren, H.D., Pühler, A., and Favelukes, G. 1992. A *Rhizobium meliloti* lipopolysaccharide mutant altered in competitiveness for nodulation of alfalfa. J. Bacteriol. 174: 5941–5952.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., and Higgins, D.G. 2007. Clustal W and Clustal X version 2.0. Bioinformatics. 23: 2947-2948.

Lehnherr, H., Magnuson, R., Jafri, S., and Yarmolinsky, M. B. 1993. Plasmid addiction genes of bacteriophage P1: *doc*, which causes cell death on curing of prophage, and *phd*, which prevents host death when prophage is retained. J. Mol. Biol. 233:414–428.

Lodeiro, A. R., and Favelukes, G. 1999. Early interactions of *Bradyrhizobium japonicum* and soybean roots: specificity in the process of adsorption. Soil Biol. Biochem. 31:1405-1411.

Lewis, K. 2005. Persister cells and the riddle of biofilm survival. Biochemistry (Moskow) 70:267-274.

Magnuson, R. D. 2007. Hypothetical functions of toxin-antitoxin systems. J. Bacteriol. 189:6089-6092.

Magnuson, R., and Yarmolinsky, M. B. 1998. Corepression of the P1 addiction operon by Phd and Doc. J. Bacteriol. 180:6342-6351.

Makarova, K. S., Aravind, L., Galperin, M. Y., Grishin, N. V., Tatusov, R. L., Wolf, Y. I., and Koonin, E. V. 1999. Comparative genomics of the Archaea (Euryarchaeota): Evolution of conserved protein families, the stable core, and the variable shell. Genome Res. 9:608-628.

Maniatis, T., Fritsch, E. F., and Sambrook, J. E. 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.

Marianovsky, I., Aizenman, E., Engelberg-Kulka, H., and Glaser, G. 2001. The regulation of the *Escherichia coli mazEF* promoter involves an unusual alternating palindrome. J. Biol. Chem. 276:5975-5984.

Martínez-Morales, F., Schobert, M., López-Lara I. M., and Geiger O. 2003. Pathways for phosphatidylcholine biosynthesis in bacteria. Microbiology 149:3461-3471.

Masuda, Y., Miyakawa, K., Nishimura, Y., and Ohtsubo, E. 1993. *chpA* and *chpB*, *Escherichia coli* chromosomal homologs of the *pem* locus responsible for stable maintenance of plasmid R100. J. Bacteriol. 175:6850-6856.

Mathur, A. B.,. Collinsworth, A. M,. Reichert, W. M,. Kraus, W. E, and Truskey, G. A. 2001. Viscous and elastic properties of endothelial, cardiac muscle and skeletal muscle cells measured by atomic force microscopy. J. Biomech. 34:1545–1553.

Mattison, K., Wilbur, J. C., So, M., and Brennan, R. G. 2006. Structure of FitAB from *Neisseria gonorrhoeae* bound to DNA reveals a tetramer of toxin-antitoxin heterodimers containing PIN domains and ribbon-helix-helix motifs. J. Biol. Chem. 281:37942-37951.

Miallau L., Faller M., Chiang J., Arbing M., Guo F., Cascio D., and Eisenberg D. 2009. Structure and proposed activity of a member of the VapBC family of toxin-antitoxin systems. VapBC-5 from *Mycobacterium tuberculosis*. The Journal of biological chemistry. 284(1): 276-83.

Miclea, P.S., Péter, M., Végh, G., Cinege, G., Kiss, E., Váró, G., Horváth, I., and Dusha, I. 2010. Atypical transcriptional regulation and role of a new toxin-antitoxin-like module and its effect on the lipid composition of *Bradyrhizobium japonicum*. Mol. Plant-Microbe Interact. 23:638-50.

Mileykovskaya, E., and Dowhan, W. 2005. Role of membrane lipids in bacterial division-site selection. Curr. Opin. Microbiol. 8: 135–142.

Minamisawa, K., 1989. Comparison of extracellular polysaccharide composition, rhizobitoxine production, and hydrogenase phenotype among various strains of *Bradyrhizobium japonicum*. Plant Cell Physiol. 30:877-884.

Minder, A. C., de Rudder, K. E., Narberhaus, F., Fischer, H. M., Hennecke, H., and Geiger, O. 2001. Phosphatidylcholine levels in *Bradyrhizobium japonicum* membranes are critical for an efficient symbiosis with the soybean host plant. Mol. Microbiol. 39:1186-1198.

Mine, N., Guglielmini, J., Wilbaux, M., and Van Melderen, M. 2009. The decay of the chromosomally encoded ccd_{O157} toxin-antitoxin system in the *Escherichia coli* species. Genetics, 181:1557-1566.

Ogura, T., and Hiraga, S. 1983. Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc. Natl. Acad. Sci. U.S.A. 80: 4784–4788.

Oláh, B., Kiss, E., Györgypál, Z., Borzi, J., Cinege, G., Csanádi, G., Batut, J., Kondorosi, A., and Dusha, I. 2001. Mutation in the *ntrR* gene, a member of the *vap* gene family, increases the symbiotic efficiency of *Sinorhizobium meliloti*. Mol. Plant-Microbe Interact. 14:887-894.

Pandey, D. P., and Gerdes, K. 2005. Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. Nucleic Acids Res. 33:966-976.

Pecota, D.C., Kim C.S., Wu K., Gerdes, K., and Wood, T. 1997. Combining the *hok/sok*, *parDE*, and postsegregational killer loci to enhance plasmid stability. Applied and Environmental Microbiology. 63**:**1917–11924.

Pedersen, K., Christensen, S. K., and Gerdes, K. 2002. Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. Mol. Microbiol. 45:501-510.

Pedersen, K., Zavialov, A. V., Pavlov, M. Y., Elf, J., Gerdes, K., and Ehrenberg, M. 2003. The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. Cell. 112:131-140.

Pessi, G., Ahrens, C. H., Rehrauer, H., Lindemann, A., Hauser, F., Fischer, H. M., and Hennecke, H. 2007. Genome-wide transcript analysis of *Bradyrhizobium japonicum* bacteroids in soybean root nodules. Mol. Plant-Microbe Interact. 20:1353-1363.

Puskás, L. G., Nagy, Z. B., Kelemen, J. Z., Rüberg, S., Bodogai, M., Becker, A., and Dusha, I. 2004. Wide-range transcriptional modulating effect of *ntrR* under microaerobiosis in *Sinorhizobium meliloti*. Mol. Gen. Genomics 272:275-289.

Regensburger, B., and Hennecke, H. 1983. RNA polymerase from *Rhizobium japonicum*. Arch. Microbiol. 135:103–109.

Rietveld, A.G., Killian, J. A., Dowhan, W., and de Kruijff, B. 1993. Polymorphic regulation of membrane phospholipid composition in *Escherichia coli*. J Biol Chem. 15:12427-33.

Robson, J., McKenzie, J.L., Cursons, R., Cook, G.M. and Arcus, V.L. 2009. The *vapBC* operon from *Mycobacterium smegmatis* is an autoregulated toxin-antitoxin module which controls growth via inhibition of translation. Journal of Molecular Biology. 390:353-367.

Ruiz-Echevarria, M. J., Giménez-Gallego, G., Sabariegos-Jareño, R., and Diaz-Orejaz, R. 1995. Kid, a small protein of the parD stability system of plasmid R1, is an inhibitor of DNA replication acting at the initiation of DNA synthesis. J. Mol. Biol. 247:568-577.

Sat, B., Hazan, R., Fisher, T., Khaner, H., Glaser, G., and Engelberg-Kulka, H. 2001. Programmed cell death in *Escherichia coli*: Some antibiotics can trigger *mazEF* lethality. J. Bacteriol. 183:2041-2045.

Sat, B., Reches, M., and Engelberg-Kulka, H. 2003. The *Escherichia coli mazEF* suicide module mediates thymineless death. J. Bacteriol. 185:1803-1807.

Schmidt, O., Schuenemann, V. J., Hand, N. J., Silhavy, T. J., Martin, J., Lupas, A. N., and Djuranovic, S.. 2007. *prlF* and *yhaV* encode a new toxin-antitoxin system in *Escherichia coli*. J. Mol. Biol. 372:894-905.

Schultze, M., Kondorosi, E., Ratet, P., Buiré, M., and Kondorosi, A. 1994. Cell and molecular biology of Rhizobium-plant interactions. Int. Rev. Cytol. 156:1-76.

Smith, J. A., and Magnuson, R. D. 2004. Modular organization of the Phd repressor/antitoxin protein. J. Bacteriol. 186:2692-2698

Spoering, A. L., and Lewis, K. 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. J Bacteriol 183, 6746–6751.

Stacey, G., So, J. S., Roth, L. E., Lakshmi, S. K. B., and Carlson, R. W. 1991. A lipopolysaccharide mutant of *Bradyrhizobium japonicum* that uncouples plant from bacterial differentiation. Mol. Plant-Microbe Interact. 4:332–340.

Studholme, D.J., and Dixon, R. 2003. Domain architectures of sigma^{54}-dependent transcriptional activators. J Bacteriol. 185:1757-67.

Tsilibaris, V., Maenhaut-Michel, G., Mine, N., and Van Melderen, L. 2007. What is the benefit of *Escherichia coli* of having multiple toxin-antitoxin systems in its genome? J. Bacteriol. 189:6101-6108.

Vigh, L., Escribá, P. V, Sonnleitner, A., Sonnleitner, M., Piotto, S., Maresca, B., Horváth I., and John L. Harwood. 2005.The significance of lipid composition for membrane activity: New concepts and ways of assessing function. Progr. Lipid. Res. 44:303-344.

Vincent, J. M. 1970. A manual for the practical study of root nodule bacteria. International Biological Program handbook 15. Blackwell Scientific Publications, Oxford.

Vinckier, A., and Semenza, G. 1998. Measuring elasticity of biological materials by atomic force microscopy. Febs Letters. 430: 12-16.

Wall, D., and Kaiser, D. 1999. Type IV pili and cell motility. Mol. Microbiol 32:1-10.

Wilderman, P.J., Vasil, A.I., Martin, W.E., Murphy, R.C., and Vasil, M.L. 2002. *Pseudomonas* aeruginosa synthesizes phosphatidylcholine by use of the phosphatidylcholine synthase pathway. J. Bacteriol 184: 4792–4799.

Wilhelm, I., Farkas, A. E., Nagyőszi, P., Váró, G., Bálint, Z., Végh, G. A., Couraud, P-O., Romero, I. A., Weksler, B., and Krizbai, I. A. 2007. Regulation of cerebral endothelial cell morphology by extracellular calcium. Phys. Med. Biol. 52: 6261-6274.

Wolfgang, M., van Putten J. P. M., Hayes, S. F., Dorward, D., and Koomey, M. 2000. Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. EMBO J. 19:6408-6418.

Yang, B., and Larson, T. J. 1996. Action at a distance for negative control of transcription of the *glpD* gene encoding *sn*-glycerol 3-phosphate dehydrogenase of *Escherichia coli* K-12. J. Bacteriol. 178: 7090-7098.

Yang, B., and Larson, T. J. 1998. Multiple promoters are responsible for transcription of the *glpEGR* operon of *Escherichia coli* K-12. Biochim. Biophys. Acta 1396:114–126.

Yarmolinsky, M. B. 1995. Programmed cell death in bacterial populations. Science. 267:836-837.

Ye, S. Z., and Larson, T. J. 1988. Structures of the promoter and operator of the *glpD* gene encoding aerobic *sn*-glycerol-3-phosphate dehydrogenase of *Escherichia coli* K-12. J. Bacteriol. 170:4209-4215.

Zhang, Y., Zhang, J., Hoeflich, K. P., Ikura, M., Qing, G., and Inouye, M. 2003a. MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in *Escherichia coli.* Mol. Cell 12:913-923.

Zhang, J., Zhang, Y., and Inouye, M. 2003b. Characterization of the interactions within the *mazEF* addiction module of *Escherichia coli*. J. Biol. Chem. 278:32300-32306.

List of publications

Bodogai M., Ferenczi Sz., Bashtovyy D., **Miclea P**., Papp P., Dusha I. 2006. The *ntrPR* operon of *Sinorhizobium meliloti* is organized and functions as a toxin-antitoxin module. Mol. Plant-Microbe Interact. 19(7): 811-822. **IF: 3.936**

Miclea S. P., and Dusha I. 2007. Toxin-antitoxin modules affect the stress response and metabolism in Rhizobia, Acta Biologica Szegediensis. 51 (2): 137-160.

Bodogai M., Ferenczi Sz., **Miclea S.P.**, Papp P., Dusha I. 2008. Toxin-antitoxin modules and symbiosis. ED: Dakora FD, Chimphango SBM, Valentine AJ, Elmerich C, Newton WE. Sustainable agriculture. SPRINGER, 2008. pp. 237-238.

Miclea S. P., Dusha I. Mutant *Bradyrhizobium* bacteria for the improvement of nitrogen fixation at various leguminous plants, especially at soybean. Submited and registred to Hungarian Patent Office (Magyar Szabadalmi Hivatal) on 15.04.2008 under the number P0700337.

Miclea, S. P., Péter, M., Végh, G., Cinege, G., Kiss, E., Váró, G., Horváth, I., and Dusha, I. 2010. Atypical transcriptional regulation and role of a new toxin-antitoxin-like module and its effect on the lipid composition of *Bradyrhizobium japonicum*. Mol. Plant-Microbe Interact. 23:638-50. **IF: 4.136**

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Abstract

The recent availability of a high number of prokaryotic genome sequences promoted the identification of a new type of genetic modules in numerous bacterial plasmids and chromosomes. These are the so-called toxin-antitoxin (TA) modules.

The first TA modules were identified on plasmids acting as post-segregational killing systems, preventing the proliferation of plasmid-free progeny. More recently, many homologues of these plasmid-located TA loci were identified on the chromosome of various bacteria and they become of main interest. They are considered to be associated with the modulation of the global level of translation under various stress conditions.

Typically, TA modules are organized into operons in which the first gene encodes an antitoxin protein and the second gene encodes the toxin. These proteins form a complex, inhibiting in this way the damaging effect of the toxin. Both the antitoxin and the toxin-antitoxin complex are capable of binding to their own promoter and repress the transcription of the module. Under specific conditions, the unstable antitoxin protein is degraded faster by cellular proteases than the stable toxin protein. Cellular targets are then degraded by the free toxin protein.

TA loci have been grouped into seven families based on protein domain structure and module organization. The most abundant family present in Gram-positive and Gram-negative bacteria as well as in Archaea, the *vapBC*, is composed of a toxin carrying a PIN domain, and an antitoxin containing a DNA binding motif.

Previous work demonstrated that the first identified TA system in Rhizobial species, namely the *vapBC*-type *ntrPR* operon of *S. meliloti*, is involved in the adjustment of bacterial metabolism under symbiotic conditions. Toxin-antitoxin systems of symbiotic bacteria may be important regulators of metabolic rates required for bacterial survival under stressful conditions and/or for the transition from free-living to symbiotic life style.

Objectives

Our aim was to identify and structurally and functionally characterize *vapBC*-type TA modules in the symbiotic nitrogen-fixing bacterium *Bradyrhizobium japonicum*, the microsymbiont of soybean (*Glycine max*).

Results

Based on similar organization, size and sequence homology to the *vapBC*-type TA family, one TA-like module (designated as *bat/bto* operon) was identified in *Bradyrhizobium japonicum*. The *bat/bto* locus consists of an upstream 285 bp gene (*bat*) that encodes a putative antitoxin containing a Phd/YefM like domain and a downstream 426 bp gene (*bto*) that encodes a putative toxin presenting a PIN protein domain. The translational start codon for *bto* overlaps with the last base of the translational stop codon of *bat*, strongly indicating a translational coupling.

Further sequence analysis revealed that the *bat/bto* operon may be composed of two evolutionarily independent modules coupled by a transition region. The *bat/bto* operon seems to be the product of a recombination event between the *vapBC* (SpoVT/AbrB domains) and *phd/doc* families of TA systems, presenting a mixture of Phd and SpoVT-type antitoxin and a PIN domain-type toxin.

The negative autoregulation is characteristic for most of the TA systems. In contrast, the expression level determined from a *bat/bto* promoter-*lacZ* fusion was higher in the wild-type background than in the mutant strain, suggesting a positive role for the toxin-antitoxin complex. The putative promoter region of the *bat/bto* operon in *B. japonicum* overlaps the putative promoter region of the neighbouring *glpD* gene. We identified two putative *glpD*-repressor binding regions in this intergenic sequence, and one of them partially overlapped with a direct repeat sequence which may represent potential binding site for the toxin-antitoxin complex. We supposed that the regulation of the *bat/bto* system may be influenced by a competition of the toxin-antitoxin complex and *glpD*-repressor for the overlapping binding sites. Indeed, we demonstrated that the expression of the *bat/bto* module was positively influenced by the inhibition of the *glpD*-repressor binding.

The expression of Bto toxin in *E. coli* cells did not produce any damaging effect, while the ectopic expression of either the Bat antitoxin or the complete module resulted in a remarkable loss of cell viability. This suggests that the Bto protein may have lost its toxic function characteristic for the TA modules.

Deletion of the *bat/bto* operon resulted in the alteration of various metabolic pathways in the mutant bacteria. The generation time of the mutant was considerably decreased in media containing complex sources of carbon and nitrogen, but the mutant cells were unable to grow in minimal medium. Different imaging techniques (AFM, LSM) revealed the altered (shorter and wider) shape of mutant cells. Force measurements with the help of AFM indicated a softer cell surface for the mutant, suggesting considerable changes of the mutant membrane.

The lipopolysaccharide production of the mutant was four-fold lower than that of the wild type cells, and resulted in the synthesis of mainly incomplete molecules. By determining the fatty acid and phospholipid components, remarkable differences were observed in the membrane composition of the wild type and mutant strains, which may explain the observed phenotypic properties of the mutant bacteria. In the wild type strain, 80% of the total fatty acids were represented by *cis*-vaccenic acid. In contrast, a variety of fatty acids were present in the mutant membranes. The analysis of phospholipid content revealed the absence of phosphatidylcholine, and the increased amount of cardiolipin and phosphatidylethanolamine in mutant membranes. The higher amount of cardiolipin domains may greatly contribute to the increased division rate. Changes in the division rate and cell shape may also be provoked by the alterations in the cell wall elongation and in the membrane composition.

Today, the generally accepted idea about the function of these chromosomally located systems is that they act as bacterial metabolic stress managers, being associated with the modulation of the global level of translation under conditions of nutrient limitation, or under various stress conditions. In contrast to this hypothesis, *bat/bto* operon of *B. japonicum* seems to be involved in the maintenance of normal physiological state of the cell, modulating the metabolic rates to a level which will assure a longer survival and a better adaptation to environmental conditions.

Összefoglalás

Az utóbbi években ismertté vált nagyszámú prokarióta genomszekvencia elősegítette számos új típusú genetikai modul azonosítását bakteriális plazmidokon és kromoszómákon. Ezek az un. toxin-antitoxin (TA) modulok.

Az első TA modulokat plazmidokon azonosították mint un. „post-segregational killing" rendszereket, melyek megakadályozzák a plazmid nélküli utódsejtek proliferációját. Később ezeknek a plazmidon lokalizált TA moduloknak számos homológját mutatták ki különböző baktériumok kromoszómáján is, és e rendszerek az érdeklődés előterébe kerültek. Az eredmények arra utaltak, hogy a TA rendszerek a globális transzlációs szintnek a különböző stressz körülmények között bekövetkező modulálásában szerepelnek.

A TA modulok tipikusan olyan operonokba szerveződnek, melyekben az első gén az antitoxin fehérjét, a második gén pedig a toxin fehérjét kódolja. A fehérjék komplexet alkotnak, eképpen akadályozva meg a toxin káros hatását. Mind az antitoxin, mind a komplex képes az operon saját promóteréhez kötődni és represszálni a modul transzkripcióját. Specifikus körülmények hatására az instabil antitoxin a proteázok hatására gyorsabban lebomlik, mint a stabil toxin. Az így szabaddá váló toxin a sejtben lévő targeteken fejti ki hatását.

A TA lókuszokat a kódolt fehérjék domén szerkezete és a modulok szerveződése alapján hét családba sorolták. A legnépesebb család a Gram pozitív-, Gram negatív- és archebaktériumokban is előforduló *vapBC* család, melynek tagjai egy PIN domént tartalmazó toxinból és egy DNS kötő motívumot tartalmazó antitoxinból állnak.

Korábbi kísérletekben kimutatták, hogy a *Rhizobium* fajokban először azonosított TA rendszer, a *Sinorhizobium meliloti*ban található *vapBC*-típusú *ntrPR* operon részt vesz a baktérium metabolizmusának szimbiótikus körülmények közötti átállításában. A szimbiótikus baktériumok TA rendszerei fontos szabályozói lehetnek az olyan metabolikus folyamatoknak, melyek biztosítják a baktérium túlélését stresszkörülmények között, illetve a szabadon élő állapotból a szimbiótikus életformává való átalakulás során.

Célkitűzés

Célunk volt, hogy azonosítsunk, valamint szerkezetileg és funkcionálisan jellemezzünk egy *vapBC*-típusú TA modult a szimbiótikus nitrogénkötő *Bradyrhizobium japonicum* baktériumban, mely a szója (*Glycine max*) mikroszimbiontája.

Eredmények

A hasonló szerveződés, valamint a *vapBC*-típusú TA családdal való szekvencia homológia és hasonló méret alapján egy TA modult azonosítottunk *Bradyrhizobium japonicum*ban, melyet *bat/bto* operonnak neveztünk el. A *bat/bto* lókuszt két gén alkotja: a 285 bp méretű *bat*, mely a Phd/YefM domént tartalmazó feltételezett antitoxint kódolja, és a 426 bp méretű *bto*, mely a PIN doménnel homológ feltételezett toxint kódolja. A *bto* transzlációs start kodonja átfed a *bat* transzlációs stop kodonjának utolsó nukleotidjával, jelezve a szoros transzlációs kapcsoltságot.

További szekvencia analízis azt bizonyította, hogy a *bat/bto* operon két, evolúciósan független modulból épülhet fel, melyeket egy tranzíciós régió kapcsol össze. Úgy tűnik, a *bat/bto* operon egy olyan rekombináció eredménye, mely a SpoVT/AbrB doménnel homológ *vapBC* és a *phd/doc* típusú TA rendszerek között jött létre, és egy PhD illetve SpoVT típusú antitoxin, valamint egy PIN domén típusú toxin keverékét eredményezte.

A negatív autoreguláció a legtöbb TA rendszer jellemző tulajdonsága. A *bat/bto* modul expressziós szintje azonban, melyet egy *bat/bto* promóter-*lacZ* fúzió segítségével határoztunk meg, magasabb volt a vad típusú sejtekben, mint a mutáns törzsben, jelezve, hogy a toxin-antitoxin komplexnek pozitív szerepe lehet a folyamatban. A *B. japonicum bat/bto* operonjának feltételezett promóter régiója átfed aszomszédos *glpD* gén promóter régiójával. Ebben a gének közötti régióban két feltételezett *glpD* represszor kötőhelyet azonosítottunk, melyek közül az egyik részben átfedi a toxin-antitoxin komplex lehetséges kötőhelyét, egy direkt ismétlődő szekvenciát. Feltételeztük, hogy a *bat/bto* rendszer szabályozását befolyásolhatja a toxin-antitoxin komplex és a *glpD* represszor versengése az átfedő kötőhelyekért. Kimutattuk, hogy valóban, a *bat/bto* modul kifejeződését pozitívan befolyásolja a *glpD* represszor kötődésének gátlása.

A Bto toxin kifejeződése nem volt káros hatással az *Escherichia coli* sejtekre, azonban akár a Bat antitoxin, akár a teljes modul ektopikus kifejeződése a sejtek életképességének jelentős csökkenésével járt. Ez arra utalt, hogy a Bto protein elvesztette a TA modulokra jellemző tipikus funkcióját.

A *bat/bto* operon delációja különböző metabolikus utak megváltozását okozta a mutáns baktériumban. A mutáns generációs ideje jelentősen csökkent komplex szén és nitrogénforrást tartalmazó táptalajokban történő növesztéskor, de minimál táptalajban a mutáns sejtek egyáltalán nem szaporodtak. Különböző képalkotó technikákkal (AFM, LSM) kimutattuk a mutáns sejtek megváltozott (rövidebb és szélesebb) alakját. Atomerő mikroszkóppal végzett mérések lágyabb sejtfelszínt jeleztek a mutánsban, melyek a mutáns sejtmembrán jelentős változására utaltak.

A mutáns sejtek a vad típusú sejtekhez képest négyszer kevesebb lipopoliszacharidot termeltek, főleg inkomplett molekulák formájában. A zsírsav és a foszfolipid komponensek meghatározásával figyelemreméltó különbségeket tapasztaltunk a vad típusú és a mutáns törzsek membrán összetételében, melyek magyarázatot adhatnak a mutáns baktériumoknál tapasztalt fenotípus változásokra. A vad típusú sejtekben az összes zsírsav 80%-a cisz-vakszénsav sav, ezzel ellentétben, a mutáns membránokban számos más zsírsav is jelen volt. A foszfolipid tartalom analízise kimutatta, hogy a foszfatidilkolin hiányzik, míg a kardiolipin és a foszfatidiletanolamin megnövekedett mennyiségben van jelen a mutáns membránokban. A kardiolipin domének nagyobb mennyisége nagyban hozzájárulhat az osztódási ráta megnövekedéséhez. Az osztódási rátának és a sejt alakjának megváltozását ugyancsak kiválthatta az átalakult sejtfal megnyúlás és membrán összetétel.

A kromoszómán lokalizált TA rendszerek funkciójáról általánosan elfogadott nézet, hogy a baktérium metabolizmusának stressz menedzsereiként működhetnek, mivel tápanyaghiány vagy különböző stressz körülmények esetén a transzláció általános szintjének modulálásában játszanak szerepet. Úgy tűnik azonban, hogy ezzel szemben a *B. japonicum bat/bto* rendszere a sejt normális fiziológiai állapotának fenntartásában lehet jelentős azáltal, hogy a metabolikus ráták modulálásával biztosíthatja a hosszabb túlélést, és a környezeti feltételekhez való jobb alkalmazkodást.