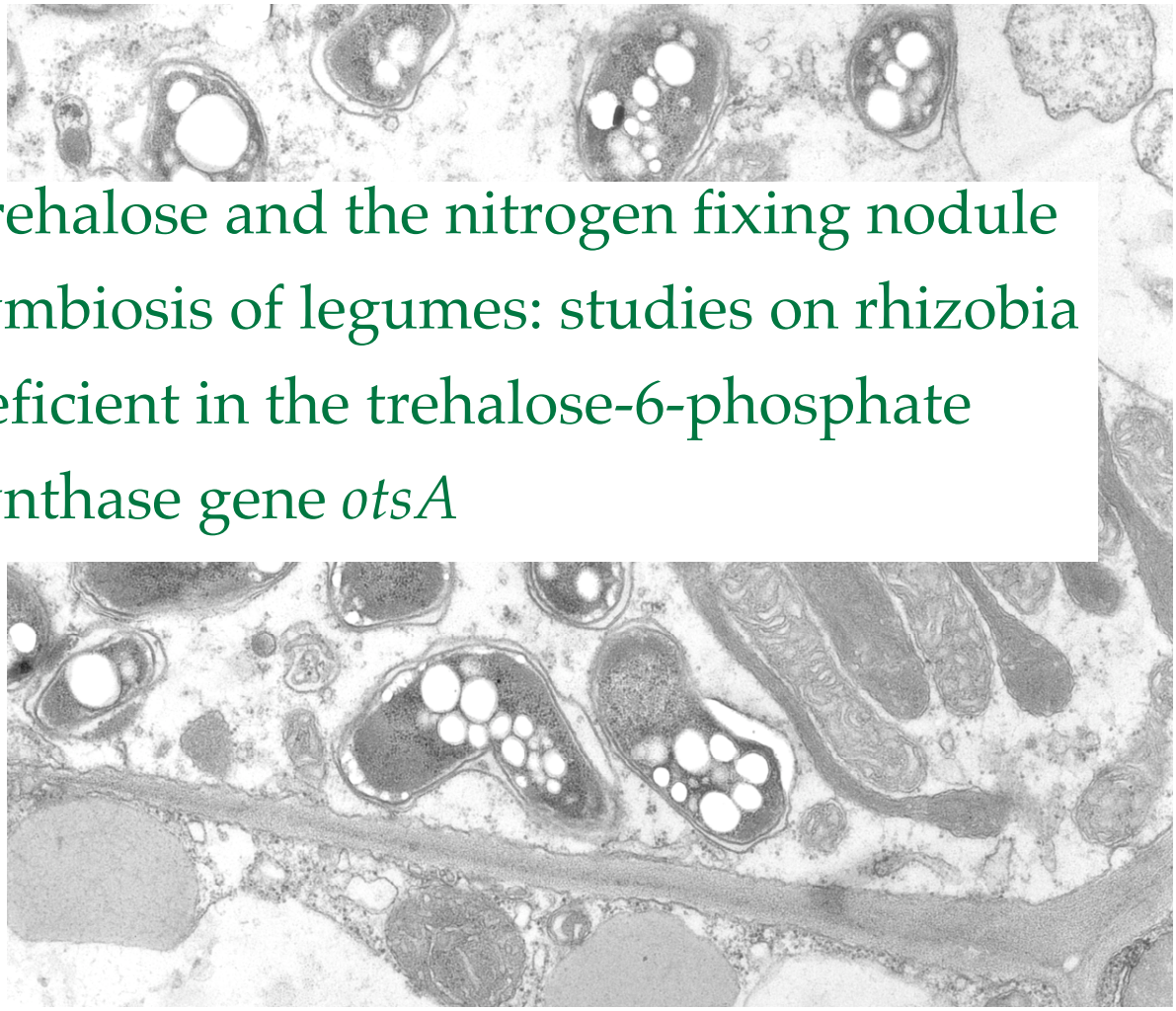

Trehalose and the nitrogen fixing nodule symbiosis of legumes: studies on rhizobia deficient in the trehalose-6-phosphate synthase gene *otsA*

Trehalose and the nitrogen fixing nodule symbiosis of legumes: studies on rhizobia deficient in the trehalose-6-phosphate synthase gene *otsA*

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Abstract

The non-reducing disaccharide trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is widespread in nature, but is normally not present in higher plants. With respect to plant-microbe interactions, it is interesting that trehalose is regularly found in plant roots interacting with antagonistic fungi, mycorrhizal fungi, and in nitrogen-fixing root nodules, probably as a microbial substance. The impact of trehalose on plant metabolism and its role in nitrogen fixing symbiosis is unclear. This work focuses on the nodule symbiosis. It represents a genetic approach to study the role of trehalose synthesis by the microsymbiont. One pathway for trehalose synthesis is the OtsA/B pathway. Trehalose is synthesized from UDP-glucose and glucose-6-phosphate in a two-step process by the action of trehalose-6-phosphate synthase (OtsA) and trehalose-6-phosphate phosphatase (OtsB). Homologues of the genes coding for these two enzymes in *Escherichia coli*, *otsA* and *otsB*, have been localized on the symbiotic plasmid of *Rhizobium* sp. NGR234 (pNGR234*a*). To study the significance of rhizobial trehalose synthesis in free living and symbiotic rhizobia, an Ω-cassette was inserted into the *otsA* homologue. Phenotypically, the deletion of the rhizobial *otsA*-homologue strongly reduced trehalose synthesis under microaerobic growth conditions. Thus, there are strong indications that the rhizobial trehalose synthesis induced under hypoxic conditions is directed by the symbiotic plasmid encoded *otsA*-homologue in conjunction with *otsB*. The functionality of *otsA* has therefore indirectly been demonstrated in *Rhizobium* sp. NGR234, which is the first time in α-proteobacteria in general. In addition, the induction of *otsA* and its homologues by low oxygen conditions has not been previously reported. The natural environment inside nodules is characterized by low oxygen. In contrast, trehalose synthesis under salt stress was not influenced by the mutation of *otsA*. This indicates that *Rhizobium* sp. NGR234 exhibits a second trehalose pathway. Activities of maltooligosyltrehalose synthase and maltooligosyl trehalohydrolase (MOS – pathway) had been demonstrated in *Rhizobium* sp. NGR234 in previous work. To study the role of rhizobial *otsA* in symbiosis, various host plants were infected with *Rhizobium* sp. NGRΔ*otsA*. In a number of hosts, average nodule size was reduced, nodule number was increased (up to 30 %) and nitrogen fixation was reduced compared to control plants infected with the wildtype strain NGR234. Analysis of the carbohydrate content of these nodules revealed significant increases in the levels of sucrose, hexoses and starch. Thus the deletion of the potential rhizobial *otsA*-homologue has a severe impact on rhizobium-legume symbiosis, and a signal function of trehalose in carbohydrate partitioning and root nodule development is proposed.

1 Introduction

The topic of trehalose and the nitrogen fixing symbiosis of legumes is approached from the perspective of nitrogen fixing symbiosis in the first part and from the perspective of trehalose and trehalose in rhizobia and symbiosis in the second part of the introduction. Special credit is given to the currently changing discussion on the definition of symbiosis, since the symbiosis of legumes represent together with the mycorrhizal symbiosis the most important symbiosis known.

1.1 Nitrogen fixation

1.1.1 Biological nitrogen fixation

Nitrogen is an essential nutrient for living organisms. Even though molecular nitrogen or dinitrogen (N_2) makes up to 78% of the atmosphere, it is often a limiting factor of growth since this stable gas is not directly metabolically available to eukaryotes. It is the domain of some prokaryotes to reduce atmospheric nitrogen to ammonia that afterwards can be assimilated into biological material. This process is called biological nitrogen fixation and is catalysed by an enzyme complex called nitrogenase. Microorganisms that are able to fix nitrogen are named diazotrophs. Biological nitrogen fixation is an essential element of the global nitrogen cycle, a major natural cycle. The contribution of nitrogen fixation to the global nitrogen cycle is approximately equivalent to the denitrification process that converts combined nitrogen back to atmospheric nitrogen and is driven by denitrifying bacteria. Fixation does not occur in excess because biological nitrogen fixation is inhibited by the presence of mineral nitrogen.

Due to the chemically inert nature of dinitrogen gas, the reduction of molecular nitrogen to ammonia is a reaction consuming much energy. A large group of nitrogen fixing bacteria lives in association with plants and they obtain the energy necessary for nitrogen fixation from compounds that they receive from their host plants. They are defined as associative or symbiotic diazotrophs. Associative diastrophic microorganisms occur in close proximity to plant roots

(that is, in the rhizosphere) and utilize plant exudates as source of energy. Symbiotic diazotrophic microorganisms obtain energy rich compounds directly from a plant partner in exchange to reduced nitrogen (for "symbiosis" see also 2.1.7). In contrast, a third group of diazotrophic microorganisms is free-living and independent from the association with other organisms. They require a chemical energy source for nitrogen fixation if nonphotosynthetic, whereas the photosynthetic diazotrophs utilize light energy. With a contribution of up to 30% of total biological fixed nitrogen per year the symbiotic association of bacteria of the family of *Rhizobiacea* and of plants of the family of the *Fabacea* are the most prominent ones. Nitrogen fixation in oceans contributes another 30% to the total biological nitrogen fixation. The remaining 40% are derived from bacteria living in an associative way with various other plant societies. Rice paddy fields, for example, contribute 3,5% to the total annual biological nitrogen fixation (calculations are on the basis of Strasburger, 1998).

1.1.2 Biological nitrogen fixation in comparison to industrial nitrogen fixation

Nitrogen is a main component of fertilizers since it is the plant nutrient that is most commonly deficient. With respect to agricultural yields throughout the world, nitrogen fertilization can be provided by biological or non-biological fixed nitrogen. In the first case, fertilization is achieved by intercropping with legumes that are capable of biological nitrogen fixation through their symbiotic diazotrophic partner, the rhizobia. Nitrogen from biological sources can also be introduced into the fields by applying compost and organic fertilizers (*organic* farming) to the fields. Since seventy years, however, nitrogen fixed in an industrial procedure has been introduced increasingly as a fertilizer into agriculture. The discoveries of the physical chemists Fritz Haber and Carl Bosch enabled industry to produce inorganic nitrogen fertilizer from N_2 from the air, and made the industrial production of ammonia economically feasible. In the so-called Haber-Bosch process ammonia is directly synthesized from nitrogen from the air and hydrogen under very high pressures (200 to 400 atmospheres), high temperatures (400°C to 650°C) and a catalyst made mostly from iron. Industrial fixation of N_2 to produce fertilizer for agriculture has increased from 3.5 million metric tons in 1950 to 80 million metric tons in 2001

(International Fertilizer Industry Association, www.fertilizer.org). In comparison, biological nitrogen fixation contributes an estimated 100-175 million metric tons of nitrogen annually to the global nitrogen cycle. Natural non-biological nitrogen fixation occurs by lightning and may fix 10 million metric tons of nitrogen a year (Haber *et al.*, 1994).

1.1.3 Environmental relevance of biological nitrogen fixation

The industrial production of ammonia and its application as a fertilizer has considerable environmental costs. The production of one ton of nitrogen fertilizer requires the burning of 1.7 tons of coal or its equivalent. Thereby carbon dioxide is released to the atmosphere and thus contributes indirectly to global warming. This problem might be solved in the long run by the application of alterative energy sources to this process, but there is a second source for the release of greenhouse gases during the use of synthetic nitrogen fertilizer in farming: Inorganic fertilizer that is supplied to the fields is only assimilated by the crops up to 50 percent and often the efficiency of utilization is much less. Before synthetic fertilizer is assimilated by the plant, it is usually converted to nitrate by microbial action. In parallel in wet soils denitrifying bacteria convert nitrate to nitrous oxide (N_2O) and atmospheric nitrogen in a ratio of 10:90. Nitrous oxide is a greenhouse gas that has an energy reflectivity that is 180-fold higher than CO_2. The increase of tropospheric nitrous oxide levels during the 1980s correlates with the increase in the use of fertilizer nitrogen. At present, the concentration of nitrous oxide in the atmosphere is out of balance and is increasing at 0.3 percent per year (Ledley *et al.*, 1999). Thus greenhouse gases arise during the production of synthetic fertilizer and during its application in farming. Food production may thereby contribute indirectly to global warming.

Another environmental problem is the loss of nitrate through leakage from the fields by surface-water movement. This nitrate is polluting streams and rivers, eventually affecting estuarine and marine ecosystems. Furthermore, nitrate may leach into groundwater. Drinking water pollution that leads to human health at risk is a permanent problem.

It is yet unclear if the key components of the global nitrogen cycle are being increasingly affected by the industrial conversion of atmospheric nitrogen and the accumulation of nitrous oxide. Since its invention seventy years ago the industrial fixation of nitrogen has reached quantities that are estimated to amount to up to 50% of the nitrogen that is fixed naturally by biological nitrogen fixation every year. With the current development of the growing population, and the resulting increased need in food production, the industrial production of inorganic fertilizer might reach 100% of the amount that is fixed naturally by biological nitrogen fixation in a few decades. It remains to be seen if this development has any impact on the global nitrogen cycle.

The natural process of biological nitrogen fixation has a critical role to bypass these problems and to achieve sustainable farming systems. An increased use of biological nitrogen fixation in farming would reduce the need for industrial produced nitrogen. This simultaneously would reduce negative effects of industrially produced nitrogen on global warming, ground- and surface-water contamination, and on the global nitrogen cycle. The energy that is required in biological nitrogen fixation is mostly derived from photosynthesis and therefore does not emit additional CO_2, in contrast to industrial nitrogen fixation. Biological fixed nitrogen is also directly and fully assimilated into biological material preventing the 50% loss of fixed nitrogen as in the case of cropping systems that are supplemented with industrial fixed nitrogen. Thus fertilization with biologically fixed nitrogen reduces the problem of the emission of the strong greenhouse gas nitrous oxide and the ground- and surface-water contamination with nitrate. Due to the better exploitation of biologically fixed nitrogen, 50% less nitrogen has to be fixed to meet the demand of nutrient supply for the crops. Thus the risk of potential interference of the additional fixed nitrogen with the global nitrogen cycle is reduced. In addition, an increased use of biological nitrogen fixation for fertilization might reduce the cost of nitrogen fertilization. The current annual worldwide expenditure for fertilizer nitrogen exceeds 20 billion US$. This amount is comparable to that for synthetic chemical pesticides (Haber *et al.*, 1994). If a replacement of industrial nitrogen fertilizer by biological fixed nitrogen could be achieved without substantial loss in productivity, the huge cost factors of food production, which are based at least in part on the costs of industrial nitrogen fertilizer, could be reduced.

Thus biological nitrogen fixation is not only a major process in the global nitrogen cycle but might also play an important role in the development of sustainable farming systems to meet the increasing food demand of an increasing world population. The research on biological nitrogen fixation goes back to the year 1838. However, even today further research is needed to increase productivity of farming systems that are fertilized with biological fixed nitrogen.

1.1.4 Discovery of biological nitrogen fixation

The discovery of biological nitrogen fixation is an impressive and somehow typical example in the history of science. When Boussingault in 1838 first reported that legume plants were able to increase their nitrogen content by fixation from the air, his results met with disbelief in the scientific community. Especially, doubts were raised when a group at the Rothamsted Experiment Station in Great Britain repeated his experiments under even more careful controlled conditions without finding any evidence for nitrogen fixation. Nowadays, we know that in their experiments the soil bacteria rhizobium, which infects the plants and by that is becoming its microsymbiont, were destroyed because they had grown legume plants in heat-treated soil (Burris, 2000). Without being aware of, they had killed the organism that actually is capable to fix nitrogen from the air.

50 years after Boussingault's experiments, Hellriegel and Wilfarth found an increase in nitrogen content of peas carrying root nodules but not if the plants were free of them (reviewed by Quispel, 1988). Inside these nodules an abundance of bacteria was discovered. This first observation initiated studies on the general physiology of the root nodule bacteria and the nodule development. At the same time, Sergei Winogradsky demonstrated that a free-living bacterium, the anaerobic *Clostridium pasteurianum* could fix atmospheric nitrogen. In 1901 Martinus Beijerinck reported that the aerobic *Azotobacter chroococcum* was also able to fix nitrogen as a free-living organism (reviewed by Burris, 2000 and Brock *et al.*, 1994). Step by step it was shown that biological nitrogen fixation is a domain of prokaryotes. When attempts were made to purify the responsible enzyme, Mortenson found that two protein components were needed (Mortenson, 1966). One of these proteins carries molybdenum and iron and the

other is an iron protein. It was established that N_2 is reduced at the MoFe site of the so-called dinitrogenase. The MoFe protein itself is reduced by the Fe protein, called dinitrogenase reductase. The term nitrogenase is generally accepted for the combination of the two proteins. The analysis of nitrogenase required the development of techniques now commonly used in anaerobic biochemistry and served as a pioneer study in the research on metal cofactor containing and multi-component enzymes (Triplett, 2000).

1.1.5 Biochemistry of nitrogen fixation

Nitrogen fixation is catalyzed by the complex metalloenzyme nitrogenase, composed of two component proteins (dinitrogenase and dinitrogenase reductase). Both component proteins are very oxygen-labile. Until now three different forms of nitrogenase have been reported. All three different nitrogenases share similar structural properties but differ in the heterometal present in the active site of the dinitrogenase unit. The most widespread nitrogenase contains iron-molybdenum cofactors at the active site and is encoded by the *nif* gene family. Vanadium-containing and *vnf*-encoded (vanadium-dependent nitrogen fixation) nitrogenase exhibits an iron-vanadium cofactor. A third so-called "alternative" nitrogenase contains an iron-only cofactor (FeFe-co) and is *anf*-encoded (alternative nitrogen fixation) (Rangaraj *et al*, 2000). The reaction catalyzed by nitrogenase involves the MgATP-dependent reduction of nitrogen gas to yield two molecules of ammonia. In the course of this reaction protons are also reduced. The proportion of electrons donated to protons and to nitrogen is variable, but the minimum ratio is 25% to protons and 75% to nitrogen, which results in one molecule hydrogen produced for each molecule of nitrogen reduced (Dixon and Wheeler, 1986). Thus the equation for the whole reaction can be depicted as follows:

$$N_2 + 8H^+ + 16ATP + 8\ e^- \rightarrow 2\ NH_3 + H_2 + 16ADP + 16\ Pi$$

The ratio of hydrogen to ammonia produced can vary and is increased when conditions are not optimal for enzyme reaction and when the enzyme is inhibited. Some diazotrophs possess a so-called uptake hydrogenase to regain energy lost by hydrogen evolution by the reduction of

hydrogen and the generation of ATP and H_2O. Uptake hydrogenases occur in cyanobacteria, *Azotobacter* and some *Frankia* and *Rhizobium* strains.

1.1.6 Occurrence of biological nitrogen fixation and its classification in free-living and symbiotic nitrogen fixation

The ability to express nitrogenase and to fix nitrogen is restricted to prokaryotes. However, among the two domains of prokaryotic life - the eubacteria and archaebacteria - it is widely distributed, reflecting its early evolution even prior to the occurrence of oxygenic photosynthesis (Spent and Raven, 1992). Although nitrogen fixation is quite abundant, the number of species that are today still capable of nitrogen fixation is relatively low: 2 genera and 87 species of archaea and 58 genera of bacteria, including 20 genera of cyanobacteria (Dixon and Wheeler, 1986). Most of these species are free-living diazotrophs, which occur independently of other organisms. Many of them are autotrophic (chemoautotrophic or photoautotrophic) and therefore able to supply the required energy for nitrogen fixation themselves, such as e.g. the cyanobacteria. However, a number of N_2-fixing prokaryotes are able to derive their energy from plant photosynthesis by forming associations with a host plant. These diazotrophs are usually heterotrophic species that can occur in multiple transitions from free-living to associative stages: depending on their environment some N_2-fixing prokaryotes can exist as free-living diazotrophs or in a plant associated from. In addition, associations of diazotrophs and plants can be very loose as in the case of azopirilla on the surface of rice roots but can also reach very intimate contact, like in the case of the rhizobia and legume symbiosis. These latter even develop a specialized symbiotic organ to breed their diazotrophic partner – the root nodules (see also 2.1.9 and figure 1). In all cases new physiological properties are created, achieved only by both partners, bacteria and plants. These cases can be described as symbiotic nitrogen fixation according to the following definition of symbiosis.

1.1.7 Symbiosis

Even before most of such associations between plant and bacteria were known, the term "symbiosis" was first defined by the plant pathologist Anton de Bary in 1887. In order to describe the common life of parasites and hosts, de Bary pointed out that there is every conceivable degree of contact between the parasite and host reaching from fast destruction of its victim to those that "further and support" their hosts (Harley and Smith, 1983*)*. Nowadays two definitions are used simultaneously: In a first classification symbiosis is used for mutually beneficial associations, in which both partners can gain a new physiologically capacity through their partner and in this way colonize new habitats or improve their growth (Douglas, 1984). The terms parasite and parasitism are used synonymously with pathogen and pathogenesis. A second school defines symbiosis as "every form of organised association of two different species", that are neutral to each other (commensalistic symbiosis), benefit from each other (mutualistic symbiosis) or one partner benefits from the other, but harms the other one (antagonistic symbiosis) (Werner, 1992). Modern textbooks, with the exception of only a few, give usually only one definition without mentioning the other (Wilkinson, 2001). However, in the last years scientists gradually re-adopted de Barys original view that there are no strict borders between the different forms of coexistence because the character of a particular symbiosis can dramatically be changed by developmental status and environmental factors (Wilkinson, 2001; Harley and Smith, 1983*)*. However, the nitrogen fixing symbiosis of rhizobia and legumes is a prominent model system for classic mutualistic symbiosis. Research on this system became so important not only because of economic and ecological reasons, but also because it represents a model for plant-microbe interactions and for the mechanism of biological nitrogen fixation.

1.1.8 The *Rhizobium*/legume symbiosis

The plant family of *Fabacea* is a large family of about 750 genera and 20 000 species. Most species and genera of which are able to form nitrogen-fixing symbiosis with rhizobia. With a contribution of 30% to the total annual biological nitrogen fixation, the association of legumes and rhizobia is the most prominent group yielding among nitrogen fixing plant symbiosis. In addition, it is the first nitrogen-fixing plant symbiosis that has been discovered, and by now it is serving as the most common model system for the research on biological nitrogen fixation in general. Only a tiny fraction of the total number of species in the family of *Fabacea* is exploited by man, but nevertheless these legumes are of great importance. Seeds of grain legumes (e. g. soybean, beans, peas, cowpeas) are high in proteins and therefore important nutritionally for humans. Forage legumes (e. g. clovers, lucerne, alfalfa, vetches) also supply high-protein diet to livestock. These legumes are in addition important to serve as a nitrogen fertilizer in organic farming. In addition fast growing tropical legume trees are cultivated for their wood and for organic fertilizer as a by-product.

The symbiosis between legumes and the nitrogen-fixing rhizobia occurs within specialized organs. These organs are called nodules and are situated mainly on the root, and in a few cases on the stem. The only other nitrogen fixing symbiosis that has developed such organs occurs between a number of woody plant species and the diazotrophic actinomycete Frankia. In both cases the architecture of these nodules provides the specific physiological and anatomical requisites for the activity of nitrogenase (the key enzyme of this symbiosis) and the nutrient exchange between the symbiotic partners.

1.1.9 Root nodules – specialized plant organs that meet requirements for symbiotic nitrogen fixation

As mentioned above, the crucial enzymatic activity for biological nitrogen fixation is the nitrogenase complex. Since nitrogenase is oxygen sensitive, its activity is limited to microaerobic conditions, - although oxygen is needed for respiration in order to supply the necessary energy. Since N_2 fixation evolved prior to oxygenic photosynthesis, oxygen sensitivity of the nitrogenase was initially not a problem because of the general low oxygen concentration in the atmosphere (Sprent and Raven, 1992). Today, organisms that are still able to fix nitrogen have developed different strategies to ensure hypoxic milieu: some are themselves microaerophilic and occur only in these environments either in the soil, in aqueous systems or - like many diazotrophic grass endophytes - in the microaerobic zones above the meristematic area of the root tip. Some aerobic bacteria remove O_2 by high respiration rates to prevent inactivation of the nitrogenase. Cyanobacteria have evolved diverse mechanisms to protect the nitrogenase from oxygen, ranging from cellular differentiation in filamentous strains to temporal compartmentalization in unicellular strains (Haselkorn and Buikema, 1992). Finally, in legumes, specialized structures - the root nodules - ensure optimal conditions for nitrogenase activity and nutrient exchange (figure 1 and 2).

Nodules of legumes meet the specific physiological requirements for nitrogenase activity as follows. The activity of nitrogenase is limited to microaerobic conditions. These conditions are provided in symbiotic nodules by a cellular diffusion barrier in combination with a symbiosis specific molecule - leghemoglobin – that facilitates a targeted and well-dosed transport of O_2 to the endosymbiont. In addition, leghemoglobin can serve as an intercellular oxygen buffer, so that nodules can hold a large amount of oxygen at a very low concentration of free oxygen (Dixon and Wheeler, 1986). This trick circumvents the paradox that nitrogen fixation is oxygen sensitive but needs a lot of O_2 for the respiration to provide the required energy. The cellular diffusion barriers consist of an uninfected cortex, which surrounds a large central zone containing both infected and uninfected plant cells. In addition infected plant cells possess a thicker plant cell wall as a diffusion barrier (see cell wall (CW) of infected cell in comparison to the cell walls of uninfected cells in figure 1, E). Legume nodules are highly compartmentalized

through which nutrient exchange can be channelled (figure 1). Prior to nodule development and the root infection, recognition and targeting of rhizobia to appropriate sites of infections at the root surface occurs in the rhizosphere of the plant. This process includes the release of rhizobial stimuli (the so called nodulation- or Nod-factors), their recognition by the plant and the consequential activation of plant specific nodulation genes that lead to an release of plant stimuli (flavonoids) and in parallel to the development of nodule premodia. Interestingly, most genetic studies on the *rhizobium*/legume-symbiosis focus on these pre-symbiotic processes (i.e. Nod^-mutants, that are deficient in Nod-factor synthesis) and not on the symbiosis itself. This work represents a genetic approach that focus on the symbiosis itself. During the infection process and nodule development rhizobia attach to the root hairs of its host by lectin binding and are enclosed by root hair curling, which is induced by rhizobial nodulation factors. Thereafter, the bacteria are surrounded by cell wall material and divide in an initial infection thread. Rhizobia penetrate the cortex of roots via the infection thread and enter the inner cortical cells of the nodule premodium via endocytosis of the infection thread membrane. This leads to a structure termed symbiosome that consists of a host-derived membrane (the peribacteroid membrane, PBM), surrounding the microsymbiont that still has its own membrane (the bacteroid membrane (BM)). This creates an internal space that has been called symbiosome space in analogy to other cellular organelles like ribosome, proteasome, or spliceosome. The term symbiosome was proposed by Roth *et al.* (1988) to describe its organelle-like structure. The newly formed symbiosomes proliferate through the host cell by division. Differentiation of the rhizobia into nitrogen-fixing bacteroids follows. Mature infected cells contain many thousands of bacteroid-containing symbiosomes (Day, 2000). For the infection process see also figure 1. The organelle like structure of the symbiosomes sets boundaries to the plant cytoplasm by the plant-derived peribacteroid membrane and to the microsymbiont-derived bacteroids membrane. Thus nutrient exchange between the micro- and macrosymbiont has to overcome this barrier but in parallel can be channelled at this gate.

Figure 1: Development of a soybean root nodule. Read clockwise from attachment of rhizobia, to infection thread formation, infection of the root cells via endocytosis, formation of symbiosomes, their division and nodule formation (figure was adapted from Campell *et al.*, 1999)

1.1.10 Nutrient exchange in the nodule symbiosis

The main aspect of nitrogen fixing nodule symbiosis is the nutrient exchange between the two partners. The plant supplies energy to the diazotrophs, which in turn reduce atmospheric nitrogen to ammonia. This reduced nitrogen is transferred from the bacteria to the plant to meet the plant's nutritional nitrogen needs for the synthesis of proteins, enzymes, nucleic acids, chlorophyll, and so forth. Nutrients that are exchanged have to be transported from the plant organs that they are

derived from, as the leaves, via the phloem to the symbiotic organ, the nodules, and there to the infected plant cell cytoplasm and across the symbiosome membranes to the microsymbiont and vice versa. The nutrients that are exchanged include nitrogen and carbon compounds. Biologically fixed nitrogen is thought to be transported in the form of ammonia or amino acids via an ammonium channel or an aspartate/H^+ porter on the peribacteroid membrane respectively to the cytoplasm of the host cell (Tyerman *et al.*, 1995; Rudbeck *et al.*, 1999). The favourite transport form is still under debate (Day, 2000). Ammonia is then incorporated by glutamine synthase into glutamine, before it is further metabolised and thereafter exported from the plant cytoplasm and to the xylem. In legumes from temperate climates nitrogen is primarily transferred to the shoot in the form of amides. In tropical and subtropical legumes nitrogen is transported via ureides. These compounds are favourable in the terms of carbon economy because of their C: N ratio of 1.

The carbon, by which the nitrogen fixation in the bacteroids is fuelled, derives from the photosynthetic product sucrose that is exported from the leaves via the phloem to the sink, the nodules. Once unloaded in the nodule cortex, sucrose diffuses into the infected region of the nodule and into the cells. Since sucrose is not directly used by the bacteroids, it is cleaved in the plant cytoplasm by a nodule specific sucrose synthase first. Mutants of pea that are deficient in 90% of the sucrose synthase activity are incapable of effective nitrogen fixation, demonstrating the key role of this enzyme (Gordon *et al.*, 1999*)*. The produced UDP-glucoses and free hexoses, which, after phosphorylation by hexokinase, enter the glycolytic or oxidative pentose phosphate pathway, are metabolised to phosphoenolpyruvate (PEP). This is believed to be converted to malate by high levels of PEP carboxylase and malate dehydrogenase present in the plant tissue. There appears to be a consensus that malate or other C_4-dicarboxic acids like succinate are the major carbon compounds supplied to the bacteroids. Several $^{14}CO_2$ labelling studies have shown that malate is the dominant carbon source produced (Rosendahl *et al.*, 1990; Snapp and Vance, 1986; Vance *et al.*, 1983; Reibach and Streeter, 1983).

Prior to the utilization in the bacteroids, the C_4-dicarboxic acids must traverse both, the host-derived peribacteroid membrane and the bacteroid membrane. The peribacteroid membrane is relatively impermeable to sugars, but it does contain a dicarboxylate transporter capable of

rapidly transferring C_4-dicarboxic acids to the bacteroid (Streeter, 1987). Mutants of rhizobia that lack C_4-dicarboxylate transport are deficient in nitrogen fixation in association with their plant hosts (e.g. Finan *et al.*, 1983, Ronson *et al.*, 1981). Furthermore, isolated bacteroids are themselves capable of taking up C_4-dicarboxylates at rates that are significantly higher than that of sugars such as glucose (Salminen and Streeter, 1987). These studies together with the $^{14}CO_2$ labelling experiments provide the best data in favour of C_4-dicarboxic acids serving as carbon source for nitrogen fixation. Within the bacteroids the C_4-dicarboxic acids are further oxidized by entering the tricarboxylic acid cycle (TCA).

Despite the fact that it has been demonstrated that the transport of dicarboxylic acids is crucial for nitrogen fixation, it seems unlikely that dicarboxylic acids are required by bacteroids as a carbon and energy source for all other cellular processes. *Sinorhizobium meliloti dct* mutants, which fail to transport dicarboxylic acids, cannot utilize carbon sources such as succinate in the free-living state and are Fix^-. However, they are able to induce and invade nodules, and they proceed through several stages of bacteroid development, becoming blocked just prior to active nitrogen fixation (Vasse et al., 1990). The efficiency of the tricarboxylic acid cycle is reduced in the *dct* strain, and the defect in nitrogen fixation may be due to the failure to produce enough ATP to fuel the reaction of nitrogen fixation. Thus dicarboxylic acids are only crucial for sufficient energy supply for nitrogen fixation. After the discovery of corresponding transport systems it has recently been suggested that sucrose itself or hexoses could act as carbon and energy source during infection, bacteroid development and bacteroid maintenance (Willis and Walker, 1999; Lambert *et al.*, 2001).

Although the key steps of carbon metabolism are well characterized, many pathways, their function and the regulation of carbon allocation are still unclear. One example concerns the trehalose metabolism in nodules. Its metabolic and/or anabolic fate as well as its function is only little understood. In the following the present day knowledge about trehalose occurrence, metabolism and its general function as well as its function in nodules is summarized.

1.2 Trehalose

1.2.1 Occurrence of trehalose

A crucial aspect of nodule symbiosis is the exchange of plant energy sources against fixed nitrogen. It is interesting to consider the role of sugars and in particular trehalose in this respect. Trehalose is a non-reducing disaccharide consisting of two glucose units linked with an α,α linkage (alpha-D-glucopyranosyl-1, 1-alpha-D-glucopyranoside). It is regularly accumulated in symbiotic organs of many plants and it is thought to be derived from the microsymbiont. Some examples are mycorrhized roots, nitrogen fixing root nodules or clubroots of some *Brassicaceae,* which are infected with *Plasmodiophora brassicae* (Streeter, 1980; Schubert *et al*., 1992; Brodmann, 2002). In general trehalose is widespread, if not ubiquitous, among fungi and also found in many animals and bacteria, as well as in some lower vascular plants such as *Selaginella lepidophylla* (rose of Jericho). The presence of trehalose in angiosperms had been demonstrated in leaves of desiccation tolerant angiosperms such as *Myrothamnus flabellifolius* (Bianchi *et al*., 1993; Atar-Zwillenberg, 1998). Recently trehalose had also been detected in very low concentrations in tobacco- and potato- and in *Arabidopsis*-plants indicating a more general occurrence in angiosperms (Goddijn *et al*., 1998, Vogel *et al*., 2001; Müller *et al*., 2001). The ubiquitous presence of trehalose in the animal, fungal, bacterial and plant kingdom is indicating its ancient history. The functions of trehalose are manifold in the diverse organisms and also within one organism (see section 2.2.3). In addition trehalose has gained increasing importance in industrial applications (section 2.2.3). These commercial interests have intensified the search for different biosynthetic traces for trehalose synthesis. For the biosynthesis of trehalose three pathways have been described.

1.2.2 Biosynthesis of Trehalose

So far three pathways for trehalose synthesis have been described to occur in nature. The most common route of trehalose synthesis is the OtsA/B pathway that forms trehalose-6-phophate from UDP-glucose and glucose-6-phophate with subsequent dephosphorylation, yielding free trehalose. This pathway has mostly been described for its involvement in stress protection. Two additional, but less prominent, routes for trehalose synthesis have been discovered in the past decade that are mostly not connected to stress protection. One catalyses the conversion of oligo/polymaltodextrines/glycogen into trehalose in a two-step reaction by maltooligosyltrehalose synthase and maltooligosyltrehalose trehalohydrolase. This pathway is referred to as the MOTS-pathway. The last pathway – the TreS- pathway- comprises a single transglycolysation reaction producing trehalose from maltose by the action of trehalose synthase.

I. Trehalose synthesis via the OtsA/B pathway

Cabib and Leloir (1958) described that trehalose in yeast is synthesized from UDP-glucose and glucose-6-phosphate in a two-step process by the action of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase. In the first step, trehalose-6-phosphate synthase (TPS; UDP-glucose: D-glucose-6-P 1-glucosyltransferase) transfers the glycosyl residue from UDP-glucose (UDPG) to glucose-6-P (G6P) to yield trehalose-6-P (Tre6*P*). In the second step, Tre6*P* is split into trehalose and inorganic phosphate by a specific trehalose-6-phosphate phosphatase (TPP; trehalose-6-P-phosphohydrolase):

UDP-glucose + glucose-6-P ➔ UDP + □,□-trehalose-6-P ➔ UDP + P_i + □,□-trehalose

This pathway is analogous to the pathway of sucrose synthesis. In *Saccharomyces cerevisae* TPS and TPP form a multimeric trehalose synthase complex together with additional subunits, which regulate activation in response to stress (Reinders *et al*., 1997). The trehalose-6-phosphate synthase is encoded by *tps1*, the trehalose-6-phosphate phosphatase by *tps2* and the regulatory components of the trehalose-6-phosphate synthase/phosphatase complex by *tps3* and *tsl1*. In *E.*

coli - the second well studied model system for this pathway - trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase are encoded by *otsA* and *otsB* (osmotic trehalose synthesis). OtsA has homology to the full-length TPS1, the C-terminal part of TPS2 and an internal region of TPS3. OtsB has homology to the C-terminal part of TPS2, but no homology to other subunits (Kaasen *et al*., 1994). Homologues to *otsA*/*B* or *tps*/*tps* genes have been described in many organisms including 15 bacteria, 3 archaebacteria, 16 fungi, 4 animals, 12 plants and *Plasmodiophora brassicae*, an organism that cannot be assigned to a specific kingdom (http://www.ncbi.nlm.nih.gov/Entrez/). However, the functionality of these homologues has only been shown in several yeasts, some enterobacteriaceae, in *Mycobacterium* spp. (De Smet *et al*., 2000) and in two plants, namely *Arabidopsis thaliana* and *Selginella lepidophylla* (Van Dijck *et al*., 2002).

In rhizobia, Salminen and Streeter (1986) reported the synthesis of trehalose from UDP-glucose and glucose-6-P in *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* indicating trehalose synthesis via the OtsA/B pathway. However, the same group reports that efforts to obtain transposon insertion mutants unable to make trehalose had failed (Streeter and Bhagwat, 1999). Thus the final proof that rhizobia synthesise trehalose via the OtsA/B pathway still awaits an answer. Recently homologues of *otsA* and *otsB* have been located on the symbiotic plasmid of *Rhizobium* sp. NGR234 (pNGR234*a*) (Freiberg *et al*., 1997), and an Ω-cassette insertion mutagenisis of *otsA* homologue is subject of this work.

II. Trehalose synthesis via the MOTS - pathway

An alternative pathway for the generation of trehalose from glucose polymers has been identified in *Arthrobacter* sp. strain "Q36", which was isolated from soil. In contrast to the OtsA/B pathway this pathway is phosphate-independent. Trehalose is formed upon intermolecular transglycosylation: The terminal α 1-4 linked residue of a variety of maltooligosaccharides with chain length of four and above is converted to an α 1-1 linkage by maltooligosyltrehalose synthase (MOT synthase, encoded by *treY*). The terminal disaccharide is subsequently cleaved

off by a hydrolase (MOT trehalohydrolase, encoded by *treZ*), releasing a free trehalose molecule (Maruta *et al*., 1995a; Maruta *et al*., 1995b):

glucose -α(1-4) [glucose -α(1-4)]$_n$ glucose -α(1-**4**)glucose ➔

glucose -α(1-4) [glucose -α(1-4)]$_n$ glucose -α(1-**1**)glucose ➔

glucose -α(1-4) [glucose -α(1-4)]$_n$ + glucose -α(1-**1**)glucose (trehalose)

In vitro, MOT synthase and MOT trehalohydrolase are also able to transform amylose into trehalose (Maruta *et al*., 1995a; Kim *et al*., 2000). So far, this pathway had been discovered in several other organisms including the thermophilic archaeon *Sulfolobus acidocaldarius* (Nakada *et al*., 1995a; Nakada *et al*., 1995b), *Brevibacterium helvolum* (Maruta *et al*., 1995b; Kim *et al*., 2000) and *Mycobacterium* spp. (De Smet *et al*., 2000). In the initial report Maruta *et al.* (1995a) reported the MOTS-pathway also to be present in an organism classified as *Rhizobium* sp.. However, Streeter and Bhagwat reported in 1999 that neither the ability of the organism to form nodules nor the species of this organism was established. Streeter and Bhagwat have subsequently analyzed a variety of rhizobia for the ability to synthesize trehalose from maltooligosaccharides and reported that this activity is present in *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Rhizobium* sp. NGR234, *Sinorhizobium meliloti*, *Rhizobium tropici* A, *Rhizobium leguminosarum bv viciae, Rhizobium leguminosarum bv trifolii*, and *Azorhizobium caulinodans*. Synthesis of trehalose from maltooligosaccharides could not be detected in *Rhizobium tropici* B or *Rhizobium etli* (Streeter and Bhagwat, 1999).

III. Trehalose synthesis via the TreS pathway

The TreS pathway has been identified in *Pimelobacter* sp. R48, *Thermus* spp. (Nishimoto *et al*., 1995) and *Mycobacterium* spp. (De Smet *et al*., 2000). As in the MOTS-pathway, trehalose is formed upon intermolecular transglycosylation as well. Both TreY and TreS catalyze an αα-1,1-transglucosylation but they differ in the number of additional sugar residues attached to the action

site (De Smet *et al.*, 2000). In the TreS pathway the α(1-**4**) link in maltose is flipped to form an α(1-**1**) link in trehalose by the action of trehalose synthase (encoded by *treS*):

glucose -α(1-**4**)glucose (maltose) ➔ glucose -α(1-**1**)glucose (trehalose)

This pathway has not been reported to occur in rhizobia so far.

In summary, in rhizobia there are first indications for the existence of two pathways - the OtsA/B - and the MOTS – pathway. The conditions that cause trehalose accumulation in free-living rhizobia, baceroids and in nodules are summarized in the following section.

1.2.3 General functions of trehalose

In nature a variety of functions have been proposed for trehalose, which depend on the specific biological system analyzed. These functions include sole carbon and energy source especially in bacteria, reserve and transport carbohydrate in i.e. fungi, compatible solute in i.e. *Bacillus*, stress protectant in for example yeasts and bacteria, signal in carbon flux possibly in plants and module of cell-wall glycolipids of *Mycobacterium* spp.. In rhizobia trehalose exhibits multiple functions depending on its occurrence as a free-living soil bacterium or in symbiosis (see 2.2.4.). Trehalose has first been isolated 1832 by Wiggers as an unknown sugar from sclerotia of the fungal disease ergot of rye (cited by Panek, 1991). A quarter century later, the chemist Berthelot discovered the same sugar in the pupae of the beetle *Larinus*, commercially used as “trehala manna”, which inspired Berthelot to name the sugar “trehalose”. It has a widespread occurrence in nature and its commercial applications proliferate. The success of trehalose, compared to other sugars, in industrial applications can be explained by its particular structure. In addition of being non-reducing, it possesses several unique physical properties, which include high hydrophilicity, chemical stability and the absence of internal hydrogen bond formation. These features account for the principal ability of trehalose for protein stabilization. It is thought that protein stabilization is achieved by the association of trehalose molecules with the protein without interfering with the protein. Thereby trehalose is helping the protein to keep in shape and concentrate the remaining

water next to the protein (Schiraldi *et al.*, 2002). These properties are exploited in industrial applications of trehalose. Trehalose is commercially used as additive for enzyme preservation, during cell lyophilisation and mammalian cell cryo-preservation. Recently, trehalose has been accepted as safe food ingredient by the European regulation system, following approval by the US Food and Drug Administration (Schiraldi *et al.*, 2002). In food industry trehalose is of interest in food-preservation with low sweetness, since trehalose is only 43% as sweet as sucrose. It is also discussed to utilize trehalose for the stabilization of vaccines during storage at room temperature, which could have great implications in the health care of developing countries (Schiraldi *et al.*, 2002).

1.2.4 Trehalose in free-living rhizobia

In the free-living state of rhizobia trehalose acts as a carbon and energy source, as a stress protectant and is accumulated in the late-logarithmic and in the stationary phase, and under mircoaerophilic growth conditions.

I. Function of trehalose as a carbon and energy source in free-living rhizobia

In addition to their ability to inhabit root nodules, rhizobia occur as free-living saprophytic bacteria in the soil. To successfully survive as a saprophyte, they must compete with other soil microorganisms for limited carbon sources and cope with different abiotic stresses, such as osmotic stress. As previously stated, trehalose is widely distributed among bacteria, fungi and insects and often occurs in high concentrations. In plants trehalose generally only occurs in very low concentrations, but in symbiotic plant tissues, such as the nearly ubiquitous mycorrhizal roots or nitrogen fixing nodules, trehalose accumulation is high enough to serve as a carbon source for saprophytic bacteria upon decomposition of these tissues. The ability to grow on trehalose as a sole carbon source is common to rhizobia with the exception of bradyrhizobia (Glenn and Dilworth, 1981; Streeter, 1985). Recently two transport systems for trehalose uptake have been described in *Sinorhizobium meliloti* (Willis and Walker, 1999; Jensen *et al.*, 2002).

They are both binding-protein-dependent ATP-binding cassette transport systems encoded by two operons called *thuEFGK* and *aglEFGAK* ("trehalose transport and utilization" and "α-glucoside utilization") respectively. These transport systems normally consist of a high-affinity substrate-binding protein located in the periplasm, two hydrophobic membrane proteins, and two cytoplasmic ATP-hydrolyzing subunits. Both the *thuEFGK* and *aglEFGA* transport system are involved in the uptake of the α-glucoside disaccharides trehalose, maltose and sucrose. AglE is the first example of a bacterial periplasmatic sugar binding protein, which specifically binds to these three disaccharides. Transport and growth experiments using mutants impaired in either or both of these transport systems revealed that these systems form the major transport systems for trehalose, maltose and sucrose. The transporter encoded by the *thuEFGK* operon is primary targeted towards trehalose, since *thuE* (that codes for the periplasmatic binding protein) is only induced by trehalose and not by cellobiose, glucose, maltopentaose, maltose, mannitol, or sucrose. The *aglEFGA* operon, on the other hand, is induced primarily by sucrose and to a lesser extent by trehalose (Jensen *et al*., 2002). The a*glEFGA* operon also encodes an α-glycosidase (AglA) that is able to cleave trehalose, sucrose and maltose after the transport (Willis and Walker, 1999). Further trehalose degrading enzymes have been described in *Rhizobium* sp. USDA 4280 (Berthelot and Delmotte, 1999), *Rhizobium* sp. TAL 1000 (Ghittoni *et al*., 1997), *Bradyrhizobium* sp. *(Lupinus)* (Wolska-Mitaszko and Malek, 1999).

It is intriguing that the ability of trehalose utilization also seems to be necessary for competitive root colonization by different rhizobia: Mutants of the *thuEFGK* transport system did not show any phenotype regarding nodule development or nitrogen fixation, but when these mutants where co-inoculated with the wild type strain, the transport mutants were significantly impaired in the ability to compete with the wild type strain (Jensen *et al*., 2002). When the availability of carbon sources becomes critical, the presence of a high-affinity transport system may be necessary to utilize scarce carbon sources. It has been shown that trehalose is available as a carbon source in the root exudates of *Medicago sativa* - the host of *Sinorhizobium meliloti* (Phillips and Streit, 1997). In addition the *thuEFGK* transport system is induced when growing on the root surface of *Medicago sativa*, indicating the occurrence of trehalose in the root exudates of this legume as well (Jensen *et al*., 2002).

The knowledge about competition for nodule occupancy is of importance for economical and environmental reasons. Rhizobial strains with beneficial characteristics are often outcompeted by indigenous strains that already exist in the field. A better understanding about the underlying mechanisms may solve this problem.

II. Function of trehalose as a stress protectant in free-living rhizobia

Trehalose is not only a carbon source for free-living rhizobia, but also plays an important role during stress protection in the free-living state of many rhizobia. The studies on stress protection in rhizobia have recently been intensified, because of the rising problem of salt-polluted fields due to improper water management and irrigation especially in India and Pakistan. A common strategy of living cells exposed to hyperosmotic environments to re-establish turgor pressure and circumvent detrimental consequences of the water loss is the accumulation of very large amounts of a few small organic solutes termed “compatible solutes”. Very high cytosolic concentrations of these solutes counteract cell dehydration without interfering with the central metabolism of the cell (Yancey *et al.*, 1982). Bacterial compatible solutes are accumulated either by de novo biosynthesis (endogenous osmolytes, such as glutamate, proline, ectoine and glucosylglycerol) or by uptake from the environment (exogenous osmolytes, so called “osmoprotectants” such as gycine betain, ectoine or 3-dimethylsulfonio-propionate). In rhizobia trehalose - in parallel to a number of other compatible solutes - has been described as endogenous as well as exogenous osmolyte in the response to salt stress.

In *Sinorhizobium meliloti* and *Rhizobium leguminosarum* bv. trifolii, trehalose is accumulated as an endogenous osmolyte in response to a 0.4 M NaCl and 0.23 M NaCl shock, respectively (Breedveld *et al.*, 1993). In addition, in *Sinorhizobium meliloti*, a growth phase dependent sequential accumulation of different compatible solutes is described in response to hypersalinity. When the exogenous osmolyte glycine betaine is present first during the lag and early exponential phase, glycine betaine is accumulated, and simultaneously the accumulation of endogenous osmolytes are suppressed. During the second half of exponential phase a sudden shift occurs from the exogenous osmolyte glycine betaine that is catabolized to the endogenous

osmolytes glutamate and the dipeptide N-acetylglutaminylglutamine amide. Trehalose appears only in the stationary phase as third major endogenous osmolyte (Talibart *et al*., 1997).

Fast-growing peanut rhizobia increase cellular content of trehalose upon hypersalinity regardless of carbon source, while slow-growing peanut rhizobia only accumulate trehalose when cultured with mannitol as carbon source (Ghittoni and Bueno, 1996). Similar to *Sinorhizobium meliloti*, in peanut rhizobia, an exogenously supplied osmolyte led to a decrease in the level of endogenous accumulated trehalose. But in the case of peanut rhizobia this solute is proline, while glycine betaine has no influence on the trehalose accumulation upon hypersalinity (Ghittoni and Bueno, 1995). As in *Sinorhizobium meliloti* in some peanut rhizobia the growth phase had an influence on the degree of trehalose content of salt stressed cells (Ghittoni and Bueno, 1995).

In summary, the accumulation of trehalose as endogenous osmolyte in salt stress is tightly regulated depending on growth phase, carbon source, occurrence of exogenous osmolytes or rhizobial strain.

Trehalose has also been described as an unusual exogenous osmolyte in *Sinorhizobium meliloti* and *Rhizobium leguminosarum*. Together with a group of other disaccharides - like sucrose or maltose - trehalose acts as a “nonaccumulating” osmoprotectant in these rhizobia. In salt stress, growth stimulation is achieved without accumulation of the supplied nonaccumulating osmoprotectant. Instead these osmoprotectants are taken up from the medium, catabolized and simultaneously the de novo synthesis of some endogenous osmolytes like glutamate is stimulated. Interestingly the level of endogenous accumulated trehalose remains unaffected by the addition of exogenously supplied trehalose or any other nonaccumulating osmoprotectant (Gouffi *et al*., 1999).

The role of trehalose accumulation in response to other stresses, such as high osmotic pressure, is so far not conclusively solved. For example, in *Bradyrhizobium japonicum* USDA 110, the level of trehalose remained constant upon high osmotic pressure (Pfeffer *et al*., 1994), but in the peanut *Rhizobium* sp. ATCC51466, trehalose accumulated after the osmotic pressure in the medium was augmented (Ghittoni and Bueno, 1995).

So far it remains unclear which trehalose-synthesizing pathway or regulatory network is involved in trehalose accumulation in response to stress. One aim of the present work is to test whether the OtsA/B pathway is involved in the salt stress response of free-living rhizobia.

III. Function of trehalose in the late logarithmic and the stationary phase of free-living rhizobia

In addition to the ability of free-living rhizobia to grow on trehalose, and to accumulate trehalose in the response to salt stress, trehalose accumulation in rhizobia can also occur - independently of salt stress stimuli - in late logarithmic and stationary phase of aerobically grown cells, before it is rapidly metabolized in the death growth-phase. This trehalose synthesis is very variable among different strains: some strains produce negligible amounts, while others accumulate significant amounts that can reach 90 – 100 % of the mono- and disaccharides (Streeter, 1985). In *Bradyrhizobium japonicum* trehalose is always the main carbohydrate among mono- and disaccharides accumulated, independent of the strain, while different strains of *Sinorhizobium meliloti*, *Rhizobium trifolii*, *R. leguminosarum*, *R. phaseoli* (Streeter, 1985) and additionally *Bradyrhizobium lupini* and *Rhizobium* sp. NGR234 (Müller *et al.*, 1994) regularly accumulate trehalose in the stationary phase, but the concentrations of trehalose varies wildly (Streeter, 1985).

The function of this late logarithmic and stationary phase trehalose accumulation in rhizobia is not yet established. Interestingly, a similar pattern of trehalose accumulation in the late logarithmic and stationary phase followed by a quick brake-down during cell death has been observed in yeast and *Cellulomonas* sp. (Lillie and Pringle, 1980; Schimz and Overhoff, 1987). Taking these examples into account, it has been suggested that the function of trehalose differs from the role as a nutritional reserve, since it is not produced previous to nutrient starvation, but just at the onset of starvation. Furthermore, the reserve polysaccharide glycogen, which accumulates earlier, is transformed in an energetically costly reaction to trehalose, which in addition yields less energy upon mobilization than glycogen. Therefore trehalose and glycogen are not expected to be equivalent in their functions (Wiemken, 1990). It was proposed that the

enormous quantities of trehalose could affect the water activity in the cytosol and in this way contribute to the retardation of the metabolism in this phase (Wiemken, 1990). In addition to this possible function, the trehalose accumulated in a growth phase dependent way can take over the role of other osmolytes in rhizobia if subjected to a salt stress environment, as described above.

IV. Function of trehalose in free-living rhizobia in microaerobic conditions

When it was found that rhizobia cultured under 1% oxygen accumulated trehalose, a possible link to the symbiosis was postulated (Hoelzle and Streeter, 1990). It is well established that the environment in active nodules is characterized by reduced internal O_2 tension, enabling activity of the oxygen sensitive nitrogenase. In addition many symbiotic genes are only expressed in anoxic conditions, like the *nif* genes coding for the nitrogenase complex (Hoover, 2000).

Hoelzle and Streeter (1990) conducted these experiments on the basis of the hypothesis that the trehalose formation in nodules may be induced by microaerobic environment as well, after the finding of trehalose accumulation in nodules containing rhizobia known to accumulate essentially no trehalose when cultured aerobically. *Rhizobium leguminosarum* bv. phaseoli, *R. leguminosarum* bv. vivicae, *R. fredii* and *Sinorhizobium meliloti* accumulated no or only up to 4.8 µg of trehalose per mg of protein when cultured under 21% oxygen, but when they where shifted to 1% oxygen they all produced significantly more – up to 85.6 µg of trehalose per mg of protein. When *R. leguminosarum* bv. phaseoli was switched back from 1% to 21% oxygen, the accumulated trehalose was metabolized.

So far the biochemical mechanism for trehalose production under low O_2 tension is not clear. The accumulation could be due to increased synthesis, decreased breakdown or both (Hoelzle and Streeter, 1990). It is again part of this work to test whether the OtsA/B pathway is involved in trehalose accumulation in response to low oxygen of rhizobia.

In summary, in free-living rhizobia trehalose has been described to act as a carbon source for most rhizobia, to play an important role in the salt stress response of different rhizobia, to be

accumulated in the late logarithmic and stationary growth phase and upon low O_2 tension. This diverse list demonstrates nicely that there is not “the one and only” function of trehalose in free-living rhizobia. The picture becomes even more diverse when the symbiotic plant partner comes into play. It is important to differentiate and again combine primary ancient functions of trehalose in free-living rhizobia and possible secondary symbiotic functions.

1.2.5 Trehalose in bacteroids and nodules

I. Occurrence of trehalose in bacteroids and nodules

In 1980 Streeter first described the appearance of trehalose as a major carbohydrate in soybean root nodules at the onset of nitrogen fixation. Trace amounts of trehalose were also detected in other plant parts, but the bulk of trehalose was specific to the symbiotic organs (Streeter, 1980). At this time trehalose was thought to be uncommon in plants, so it was speculated that the trehalose measured in other parts of the plant was nodule-derived. In later studies, trehalose appeared to be a common carbohydrate in almost all nodules tested. Phillips *et al*. (1984) detected trehalose accumulation in nodules of soybean (*Glycine max*), and in addition, in the nodules of white clover (*Trifolium repens*), kudzu (*Pueraria thunbergiana*), silk tree (*Alibizia julibrissin*), and even in the nodules of the nonlegume European alder (*Alnus glutinosa*) and Russian olive (*Elaeagnus angustifolia*). Nodules from all species were collected from field grown plants. Streeter (1985) discovered trehalose accumulation in the field grown nodules of peanut (*Arachis hypogenus*), alfalfa (*Medicago sativa*), common bird's-foot-trefoil (*Lotus corniculatus*), and again in white clover (*Trifolium repens*). Where pure strains had been employed in the cases of soybean (*Glycine max*), common bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) the concentration of trehalose varied with the *Rhizobium* species (Streeter, 1985; Farias-Rodriguez, *et al*., 1998). This could be an explanation, why Müller *et al*. (1994) could not detect trehalose accumulation in some legume species inoculated with pure *Rhizobium* strains, that had been previously tested positive by Streeter (1985) and Phillips *et al*. (1984) in field grown plants (namely *Trifolium repens* and *Medicago sativa*). In total Müller *et al*. tested 12 legumes including *Lablab purpureus*, *Psophocarpus tetragonolobus*, *Phaseolus vulgaris*, *Lupinus albus*, *Pisum sativum* and

Vicia faba, where trehalose was detected. No trehalose was detected in *Leucaena leucocephala*, *Vigna unguiculata*, *Cicer arietinum, Trifolium repens* and *Medicago sativa*. In addition in *Glycine max* trehalose accumulation clearly varied with rhizobial strain and genotype. Hence it cannot be excluded that the legumes that were once negative for trehalose accumulation do not accumulate trehalose at all stages of their lifecycle of the symbiosis or in symbiosis with different rhizobial strains. In summary most of the legumes tested so far accumulated trehalose in their nodules.

When trehalose was discovered as a carbohydrate in root nodules, it was predicted that trehalose was synthesized by bacteroids since the presence of trehalose in uninfected higher plants was not known at that time. This prediction was supported by the finding that trehalose was not depleted in senescing soybean nodules but accumulated while the concentration of other compounds declined (Streeter, 1981, see also Müller *et al.*, 2001). Trehalose is the major carbohydrate in senescing nonfunctional nodules thus it is likely to be bacteroid-born (Streeter, 1981). In addition, the trehalose concentration in bacteroids varied greatly depending on the rhizobial strain (Streeter, 1985). More direct evidence was obtained by the finding that trehalose was synthesized in bacteroids isolated from soybean nodules (Streeter, 1985).

Trehalose is distributed among the different nodule compartments in a growth phase dependent manner. In young soybean plants the bulk of trehalose is located in the cytosol, and only a small proportion in the bacteroids. The older the plants get, the more this distribution is changing to the opposite. In 65 days old plants up to 90% of the total trehalose accumulates in the bacteroids (Streeter, 1985). The increasing retention of trehalose in bacteroids with increasing nodule age is indicating that its function is changing during nodule development.

Generally, it should be noted that different *Rhizobium* and legume model systems are used in the trehalose research and that results are not automatically transferable.

II. Trehalose metabolism in nodules

Enzymes involved in trehalose metabolism in nodules have mainly been examined in soybean root nodules. The key biosynthetic enzyme trehalose-6-phosphate synthase is active in the bacteroid soluble protein fraction and in the bacteroid fragment fraction, but not in the plant cytosol fraction of 5 – 6 week old soybean nodules. It is currently unclear, if *in vitro* trehalose-6-phosphate synthase is always associated with membranes and the activity in the soluble protein fraction is due to one part of the enzyme being solubilized during preparation. The activity of trehalose-6-phosphate synthase differs with rhizobial strains used to form the nodules. Bacteroids formed by *Bradyrhizobium japonicum* strain 61A76 has 1.8 times the activity found in *B. j.* strain USDA110 bacteroids (Salminen and Streeter, 1986). This correlates with the relatively high trehalose accumulation seen with 61A76 (Streeter, 1985). $Mg^{2+}$and KCl were reported to be activators of Tre6*P* synthase in *Dictiostilium discoideum*, but in the bacteroid Tre6*P* synthase KCl do not have a major role in the activity (at least under these conditions).

The second enzyme of the OtsA/B pathway is - in contrast to trehalose-6-phosphate synthase - not specific to bacteroids. Trehalose-6-phosphate phosphatase is active in both the bacteroids and the host cytosol in similar proportions, indicating partial export of trehalose-6-phosphate to the plant cytoplasm. Again the activity in bacteroids formed by *Bradyrhizobium japonicum* strain 61A76 is higher (Salminen and Streeter, 1986).

The most prominent degrading enzyme is trehalase. Salminen and Streeter (1986) discovered trehalase in soybean root nodules. They reported trehalase to be present in bacteroids and the host cytosol, with its main activity in the cytosol. Mellor (1988) detected the bulk of trehalase activity in the plant cytosol as well, some activity in the peribacteroid space, but no activity in the bacteroids. In contrast, Kinnback and Werner (1991) reported trehalase to be 8 – fold more active in the peribacteroid space than in the nodule cytoplasm. Thus the exact compartmentalization of trehalase activity remains unresolved. However, all groups agree in the fact that trehalase is nodule enhanced (see also Müller *et al.*, 1994). Further characterisation of this enzyme activity revealed that it has two pH optima: pH 3.8 and 6.6 (Salminen and Streeter, 1986); pH 4 and 7 (Mellor, 1988); or in the most purified case pH 3.7 and 6.3 (Müller *et al.*, 1992). Previously it

was suggested that the acidic pH lacks physiological relevance, except it would be associated with the vacuole, as it was shown in yeast (Salminen and Streeter, 1986). However, in the meantime an acidification of the peribacteroid space was reported. Both ATPases located on the peribacteroid membrane and the bacteroid respiratory-electron-transport-chain pump protons into the peribacteroid space. In the presence of permanent anions this potential is rapidly converted in a ΔpH. Thus the acid pH optimum of the trehalase might have physiological relevance in root nodules.

Trehalase is the only enzyme of trehalose metabolism in nodules, for which further legumes have been tested: Besides in soybean, trehalase activity has been reported in *Lablab purpureus*, *Psophocarpus tetragonolobus*, *Phaseolus vulgaris*, *Lupinus albus*, *Pisum sativum, Vicia faba*, *Leucaena leucocephala*, *Vigna unguiculata*, *Cicer arietinum, Trifolium repens* and *Medicago sativa* (Müller *et al*., 1994). Interestingly trehalose accumulation and trehalase activity were not correlated. However, trehalase activity was constantly higher in effective soybean nodules than ineffective nodules. Thus trehalase activation in nodules is correlated with nitrogen fixation rather than the availability of its substrate (Müller *et al*., 1994). This regulation does not seem to occur at the transcriptional level, since *GMTRE1* coding for the soybean trehalase is expressed at low but constant levels in many tissues (Aeschbacher *et al*., 1999). In addition, in sterile soybean roots, it was shown that a treatment with the phytohormone auxin strongly increased trehalase activity (Müller *et al*., 1995).

Two additional degrading proteins, phosphotrehalase, acting on trehalose-6- phosphate forming glucose and glucose-6-phosphate, and trehalose phosphorylase, forming glucose and β-glucose-1-phosphate, were also detected in the soluble fraction of bacteroids bacteroid soluble protein fraction and cytosol fraction. In addition trehalase is present in large excess over trehalose-6-phosphate synthase. Therefore a tight regulation of activity and a compartmentalisation of these enzymes are required for trehalose accumulation to occur (Salminen and Streeter, 1986).

III. Factors influencing trehalose levels in nodules

Trehalose accumulation in nodules can further be altered by diverse abiotic factors like salt stress, drought or nitrate. The legume-Rhizobium symbiosis and nodule formation on legumes are more sensitive to salt or osmotic stress than free-living rhizobia (Zahran, 1999). Therefore it has been tested if trehalose is involved in stress protection in the legume rhizobium symbiosis.

In 5 weeks old *Medicago sativa* plants infected with *Rhizobium meliloti*, maltose and trehalose concentrations were significantly enhanced upon 0.15 M sodium chloride stress for two weeks, especially in the roots (4.6 and 3.5- fold respectively) and bacteroids (3.0 and 4.4 fold). However, these sugars remained minor compounds: they accounted for less than 2.5% of the carbohydrate pool. Thus the concentration was too low to contribute efficiently in direct osmoregulation in alfalfa (Fougère *et al.*, 1991).

In soybean root nodules, an increase of the trehalose pools size upon water stress was reported, but this accumulation was dependent on the rhizobial strain that was used for infection (Müller *et al.*, 1996). In addition, an increase of sucrose and pinitol pools was measured in these experiments. In common bean (*Phaseolus vulgaris*), an increase in nodule trehalose content during drought stress differed among rhizobial strains as well and varied between a 2-fold and 48-fold increase. Those cultivars exhibiting high nodule trehalose levels or a high degree of trehalose stimulation also exhibited high leaf relative water contents and were also more drought resistant (Farias-Rodriguez *et al.*, 1998).

Generally the sugar concentration in bean nodules is markedly depressed when plants are supplied with nitrate (Streeter, 1986), but in soybean nodules, an increase in sugar concentration has been reported (Streeter, 1981). However, trehalose concentration is depressed in both types of nodules in the presence of nitrate (Streeter, 1986; Müller *et al.*, 1993). This is analogous to the observation that the nitrogenase activity is inhibited in the presence of nitrate (Müller *et al.*, 1993) and the finding that trehalose accumulation is also depressed in soybean nodules infected with nitrogen fixing$^-$ mutant strains (Müller *et al.*, 1994).

IV. Factors influenced by different levels of trehalose in the symbiosis

The impact of trehalose on nodule metabolism has further been examined by an artificial alteration of trehalose levels by the addition of trehalase inhibitor or external supply of trehalose. The addition of validamycin A, a potent trehalase inhibitor, to soybean and cowpea nodules caused an increase in the amount of trehalose and a decrease in the sucrose and starch pools, but nitrogen fixation was not affected (Müller *et al*., 1995). Experiments with sterile soybean roots grown with trehalose added to the growth medium, strongly induced activity of sucrose synthase and to a lesser extent alkaline invertase (Müller *et al*., 1997). In addition, pinitol accumulation was significantly reduced in these roots (Müller *et al*., 1995). Soybean nodules with naturally occurring high levels of trehalose had significantly higher levels of sucrose synthase and alkaline invertase and significantly lower levels of sucrose than nodules with low levels of trehalose (Müller *et al*., 1997). Mutants of trehalose synthesis genes might provide insight into relationship between produced by the microsymbiont and sucrose metabolism in plants. It is again part of this work to test whether trehalose produced by the OtsA/B pathway is involved in a correlation between trehalose and carbon allocation.

1.3 *Vigna unguiculata* and *Rhizobium* sp. NGR234 as a model system

Rhizobium sp. strain NGR234 is a fast-growing *Rhizobium* with the broadest host-range of known rhizobia. It has first been isolated from the nodules of *Lablab purpureus* in Papua New Guinea ("NGR"). Up to now, 112 genera of legumes as well as the non-legume *Parasponia andersonii* have been described as a host (Pueppke and Broughton, 1999). Most symbiotic genes are carried by pNGR234*a*, a 536-kb self-transmissible plasmid. In addition to the chromosome, NGR234 possesses a ≥ 2 Mb mega-plasmid, which encodes several other loci involved in nodulation and nitrogen fixation. The symbiotic plasmid pNGR234*a* of *Rhizobium* sp. NGR234 is the first symbiotic plasmid that has fully been sequenced, providing a powerful tool to study genes involved in the symbiosis (Freiberg *et al*., 1997). Homologues of *otsA* and *otsB* have been located on the symbiotic plasmid of *Rhizobium* sp. NGR234 (pNGR234*a*).

The most important model legume for the research with *Rhizobium* sp. NGR234 is cowpea. *Vigna unguiculata* (L.) WALP. or cowpea (German: "Kuherbse, Kuhbohne, Kunde- oder Augenbohne") originates from tropical Africa and is nowadays cultivated in the tropics and subtropics all over the world. In addition to the seeds, which contain 23% protein, young pods and leaves are used as vegetables (Franke, 1997).

For the model system *Vigna unguiculata* and *Rhizobium* sp. NGR234 see also figure 2.

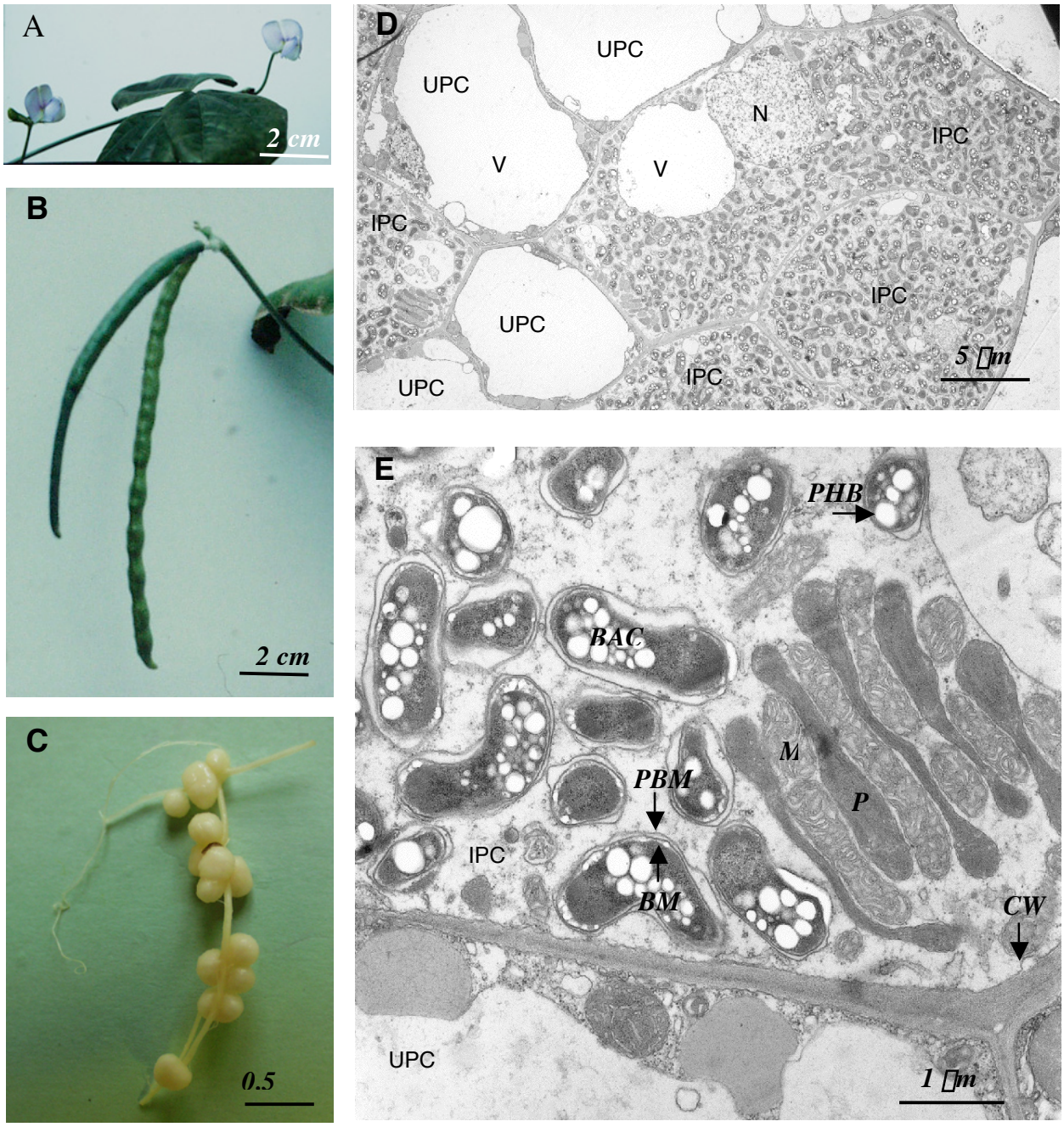


Figure 2: The *Rhizobium*–legume symbiosis: the characteristic flowers of a legume plant (A) and its pods (B) are demonstrated by 9 weeks old *Vigna unguiculata* plants. The symbiotic organ of the *Rhizobium*–legume symbiosis - the root nodules are shown in (C) and are derived from a five week old soybean root infected with *Bradyrhizobium japonicum*. Electron micrographic study of 7 week old *Vigna unguiculata* nodules inoculated with *Rhizobium* sp. NGR234 are shown in (D) and (E). Nucleus (N) and vacuole (V) of infected plant cells (IPC) and uninfected plant cells (UPC) are indicated in the

image (D). Sections of infected plant cells are shown in picture (E). The microsymbiont appears in its symbiotic form the bacteroids (BAC) that are filled with granules of □-hydroxybuturate (PHB). Bacteroids are confined by the plant derived peribacteroid membranes (PBM) and the microsymbiont derived bacteroid membranes (BM). The cell wall (CW) of the infected plant cell and a stack of plastides (P) and mitochondria (M) are indicated.

1.4 Aim of this work

Trehalose is widespread in nature and known to function as a carbon source, transport carbohydrate, stress protectant or signal in carbon metabolism in the very different organisms it has been described so far. It is common in symbiotic organs of ectomycorrizal short roots, roots colonized by arbuscular mycorrizal fungi and in nitrogen fixing nodules, which are comprising the most important plant-microbe symbiosis – both economically and ecologically – described in nature so far. In addition it is accumulated by all other N_2 – fixing bacteria that were tested up to now, like many cyanobacteria or *Frankia* sp. In rhizobia – the best studied model system for biological nitrogen fixation – possible functions of trehalose in the free-living state and symbiotic state remain unresolved, with exception of its role as a carbon source in free-living rhizobia.

So far the knowledge of trehalose in rhizobia adds up to the following observations:
In free-living rhizobia, trehalose acts as a carbon source and, the ability to use this carbon source is important in the competition for nodule occupancy with other rhizobial strains. Trehalose accumulates in the late logarithmic and the stationary phase of free-living rhizobia, in rhizobia under salt stress and when cultured in 1% oxygen. So far no clear function has been assigned to the trehalose accumulation in the late logarithmic and the stationary phase and in hypoxic conditions. In addition, the function of trehalose to act as a stress protectant in height salt is possible, but mutant studies are missing to finally proof this function.

In nodules trehalose is appearing at the onset of nitrogen fixation. Nodule trehalose is synthesised by bacteroids. A significant proportion of trehalose in nodules appears to be transferred from the bacteroids to the plant cytoplasm in young nodules, but this proportion is becoming smaller and

smaller with nodule age. Trehalose is present in nearly all nodules tested so far and its universal occurrence in nodules is likely. Trehalose concentration in nodules varies widely depending on the rhizobial strain used to form nodules. In field grown plant nodules, trehalose concentrations are not as diverse. Trehalose accumulation in nodules is further altered by salt stress, drought or nitrate. When the trehalose content in legumes is artificially altered by the addition of a trehalase inhibitor or external supply of trehalose, general sugar pools and activity of sucrose synthase and invertase are changed. In rhizobia there are first indications for the existence of two pathways - the OtsA/B pathway and the MOTS – pathway, but genetic evidence is missing. In addition it is unclear, which biosynthetic pathway is active at which timepoint in rhizobia and the symbiosis.

This diverse physiological picture is implying that there are also diverse functions of trehalose in rhizobia themselves and in the symbiosis. In addition it should be differentiated between primary prokaryotic functions of trehalose and secondary symbiotic functions. The following functions of trehalose are currently discussed: Trehalose could play a role in stress protection in free-living rhizobia and/or nodules, in functioning as carbon source or again stress protectant in aging nodules, to enhance rhizobial survival rates, or to act as a signal in carbon allocation in the symbiosis.

This study represents the first genetic approach to study trehalose metabolism in the nitrogen fixing symbiosis. So far, efforts to obtain transposon insertion mutants unable to make trehalose have failed (Streeter and Bhagwat, 1999). Here, a targeted approach is reported which leads to the disruption of trehalose synthesis via the OtsA/B pathway. Homologues of *otsA* and *otsB* have been located on the symbiotic plasmid of *Rhizobium* sp. NGR234 (pNGR234*a*) (Freiberg *et al.*, 1997), and an Ω-cassette insertion mutagenisis of *otsA* homologue is subject of this work. The involvement of *otsA* derived trehalose in free-living rhizobia and in the symbiosis are both examined, and an involvement of trehalose in root nodule development and carbon allocation is reported.

2 Materials and Methods

2.1 Molecular and microbiological techniques

2.1.1 Bacterial strains and plasmids used in this work

All bacterial strains and plasmids used in this work are listed in table 1 and table 2. *Escherichia coli* recombinants were grown on Luria-Bertani medium or Terrific broth (Sambrook *et al.,* 1998) shaking at 140 rpm at 37°C. Rhizobia were cultivated in Rhizobium Minimal Medium (RMM; Broughton *et al*., 1986) or 20E medium (Werner *et al*., 1975) both containing succinate (20 mM) as C-source shaking at 140 rpm at 27°C.

Table 1. Bacterial strains used in this work

Strains	Characteristics	Source
Rhizobium sp. NGR234	Broad host range, isolated from *Lablab purpureus*, Rif^R	Stanley *et al.* (1988)
Rhizobium sp. NGRΩ*otsA*	NGR234 derivative containing an insertion in *y4pC,* Rif^R, Sp^R	This work
Escherichia coli DH5	*recA*1, 80 *lacZ*M15	Hanahan (1983)
Escherichia coli 1046	*recA*1	Cami and Kourilsky (1978)

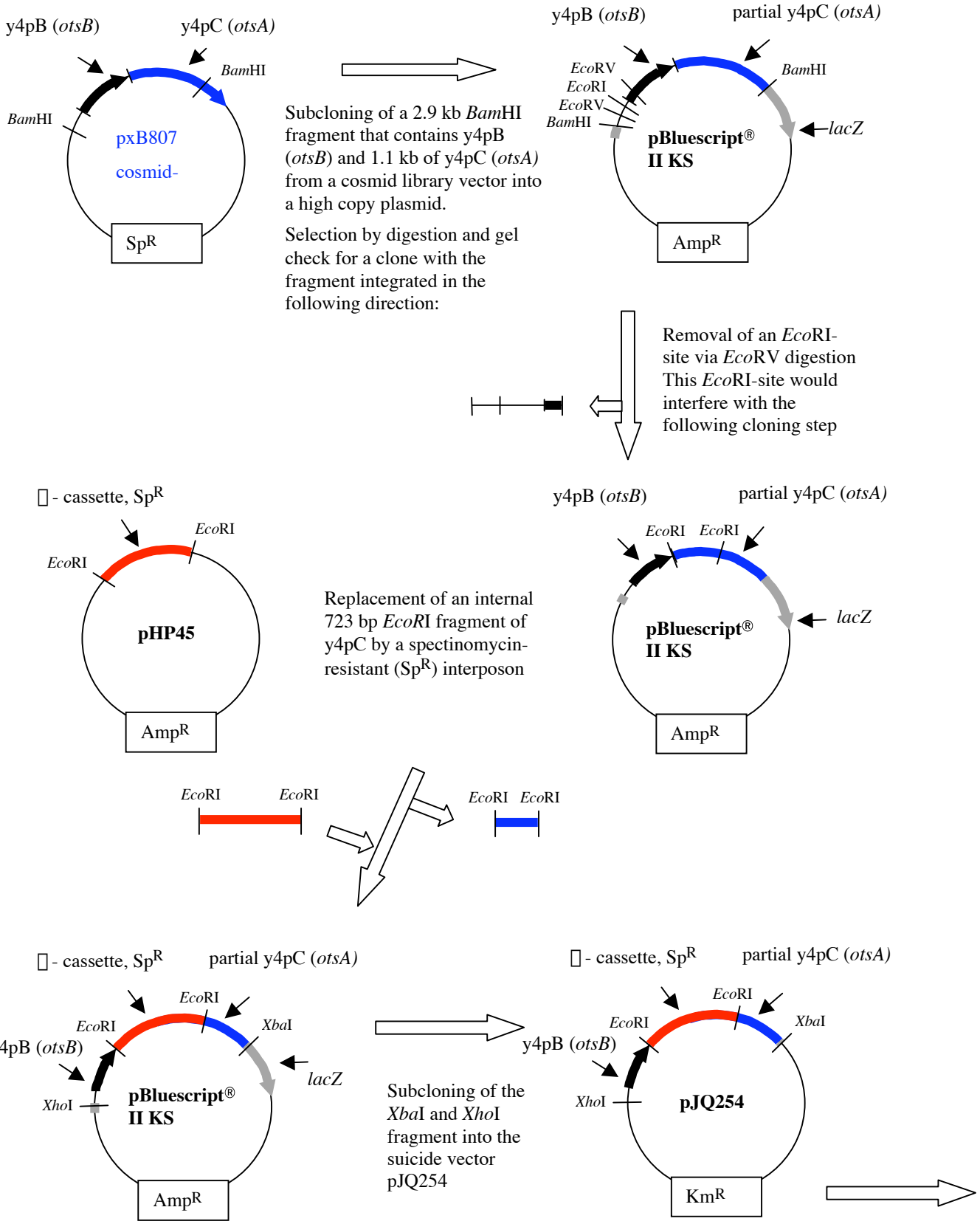
Table 2. Plasmids used in this work

Plasmids	Characteristics	Source
pBluescript KS+	ColEI based phagemid, lac□Z^{+}, Amp^{R}	Stratagene
pHP45	□-cassette, Amp^{R}	Prentki and Krisch (1984)
pJQ254	Versatile suicide vector, Gm^{R}	Quandt and Hynes (1993)
pRK2013	Tra^{+} helper plasmid, Km^{R}	Figurski and Helinski (1979)
pXB807	Lorist2 cosmid of pNGR234*a*	Perret *et al*. (1991)
pGMT	TA-cloning vector, Amp^{R}	Promega
pGEX-1	Glutathione-S-transferase fusion vector, Amp^{R}	Pharmacia

2.1.2 Mutagenesis of the OtsA-like ORF on pNGR234*a* of *Rhizobium* sp. NGR234

Rhizobium sp. NGRΩotsA was constructed as follows (see also figure 1). In the first part the *otsA*-like ORF (*y4pC*) of the symbiotic plasmid pNGR234*a* of *Rhizobium* sp. NGR234 was subcloned and disrupted by an insertion of an Ω-cassette interposon. In the second part this construct was transferred from *Escherichia coli* to *Rhizobium* sp. NGR234 where recombination with the endogenous *otsA*-like ORF could occur. In the first part the *otsA*-like ORF (*y4pC*) of the symbiotic plasmid pNGR234*a* of *Rhizobium* sp. NGR234 was obtained from a cosmid library (Perret *et al*. 1991). A 2.9 kb *Bam*HI fragment of the cosmid vector, pXB807 that is containing *y4pC* was cloned into pBluescript KS+ (Stratagene). A 1 kb fragment of this construct was excised using *Eco*RV to remove an interfering *Eco*RI site. Thereafter an internal 723 bp *Eco*RI fragment of *y4pC* was replaced by an interposon carrying spectinomycin-resistance (Sp^R), called "Ω-cassette", that is derived from pHP45 (Prentki and Kirsch, 1984) and that was excised using *Eco*RI. The resulting insert comprised the Sp^R interposon, the remaining part of *y4pC* and an additional 700 bp fragment upstream of *y4pC*. This insert was excised using *Xba*I and *Xho*I and subcloned into the suicide vector pJQ254 (Quandt and Hynes, 1993) resulting in pJQ254Ω*otsA*. In the second part this vector was transferred into *Rhizobium* sp. NGR234 by triparental mating using the helper plasmid pRK2013 (Figurski and Helinski 1979). For this 150 µl (LB, OD 2) of the *Escherichia coli* helper strain carrying pRK2013, 150 µl (LB, OD 2) of the *Escherichia coli* donor strain carrying the suicide vector pJQ254Ω*otsA* and 500 µl (RMM, OD 2,5) of *Rhizobium* sp. NGR234 were gently mixed (in order not to brake sex pili) and centrifuged at 8000 rpm for five minutes and resuspended in 20 µl RMM. This mixture was carefully dropped onto a RMM agar plate and was allowed to dry before it was incubated over night at 30°C to allow conjugation to occur. The bacterial mixture was then diluted in RMM and subcultured on RMM plates supplemented with rifampicin and spectomycine to select for transconjugants. In the next step marker exchange was forced by selection on RMM plates containing 5% (w/v) sucrose that is toxic to transconjugants that still contain the suicide vector pJQ254Ω*otsA*. Mutants were confirmed by probing Southern blots of restricted genomic DNA according to standard methods (Sambrook *et al*., 1989).

Rhizobium sp. NGR□ *otsA*: □-cassette insertion



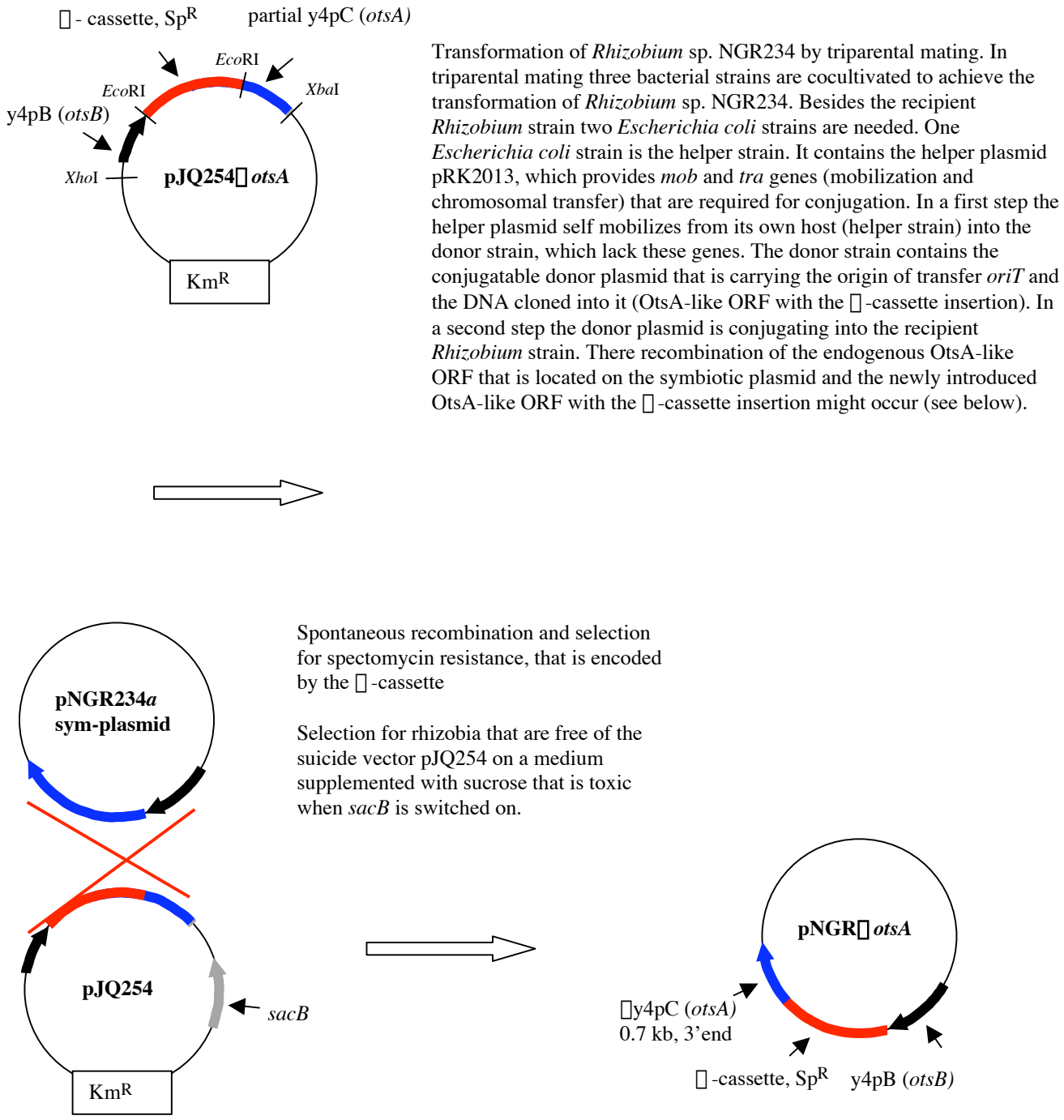


Figure 1. *Rhizobium* sp. NGR*otsA*: Ω-cassette insertion, triparental mating and recombination

2.1.3 Production of recombinant glutathione-S-transferase tagged rhizobial OtsA

Rhizobial OtsA was tagged with glutathione-S-transferase (GST) and expressed in *Escherichia coli* as follows. OtsA-like ORF (*y4pC*) of the symbiotic plasmid pNGR234*a* of *Rhizobium* sp. NGR234 was amplified from the cosmid vector pXB807 that is containing *y4pC* (Perret *et al.* 1991) by PCR using the following primers: otsAfor (5'- TCT AGA CCC GGG ATG AGT CGA CTC GTC ATT GTT TCC AAT CGC GT-3') and otsArev (5'-GAG CTC GCG GCC GCC CGG GTG GCA CGA TGA TGA TCT TTG ATT G-3'). The PCR product was cloned into the vector pGMT (Promega). In a second step pGMT-*otsA* was cut with the restriction enzyme *Xma*JI, and *otsA* was subcloned into the expression vector pGEX-1 (Pharmacia), (figure 2). Screening for recombinants with the insert in sense orientation was performed via restriction digest with *Bam*HI. The correctness of the obtained construct was verified by sequence analysis.

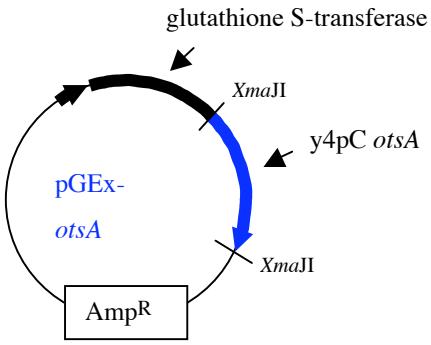


Figure 2. Expression vector pGEX-*otsA* with the glutathione-S-transferase – trehalose-6-phosphate synthase fusion protein

Recombinant *Escherichia coli* clones were grown in Luria-Bertani medium and the induction of expression of the fusion protein was tested. For that purpose different concentrations (0 □M, 50

µM, 100 µM, 200 µM) of isopropyl-beta-D-thiogalactopyranoside (IPTG) were added to 20ml cultures during the exponential growth phase 2.5 hours before harvest. To test the IPTG induction protein extracts from induced cells were analyzed on SDS polyacrylamide gels. Cells derived from 5ml culture were washed in 100mM Tris-buffer. 250µl thereof were boiled for 1 minute in SDS-PAGE loading buffer (62.5 mM Tris; 20% glycerol (v/v); 2.5% SDS (w/v); 5% b-mercaptoethanol (v/v); and 0.1% bromophenol blue) and 20µl were loaded onto the 10% SDS polyacrylamide gel (Laemmli, 1970). The gels ran at 180 V for about one hour by using the following SDS-PAGE gel running buffer (25 mM Tris; 250 mM glycine (pH 8.3); 0.1% SDS). Proteins were stained with Coomassie (0.25% (w/v) Coomassie Brilliant Blue R250, and 10% glacial acetic acid in methanol:H_2O (1:1 v/v)).

Activity tests were performed with raw cell-extracts and with isolated proteins. For the raw bacterial lysate 20 ml culture that were induced by 50 µM IPTG (see above) were centrifuged and resuspended in 500 µM lysis buffer (50 mM MES (pH 6.0); 100 mM NaCl; 5mM EDTA; 0,05% NP-40; 1mM DTT; 1mM PMSF). After 1 minute sonication on ice, 10 µl of this raw extract was used for activity tests. Activity tests were performed in 50 µl assay-buffer final volume (50 mM MES (pH 6.0); 10 mM UDP-glucose; 10 mM glucose-6-phophate; 15 mM MgCl; 1µl 50% (w/v) validoxidamine (trehalase inhibitor)). The assays were incubated for 1, 3, and 5 hours or over night at 30°C. Thereafter the assays were stopped by boiling for 1 minute. After centrifugation (10 000 g; 10 min), supernatants were analyzed by anion-exchange HPLC using pulse-field amperometric detection (Dionex, Sunnyvale, Cal., USA). Since trehalose is better detectable by HPLC than trehalose-6-phophate, probes were analyzed a second time by HPLC after phosphatase treatment (10 µl (0.4 U) acid phosphatase dissolved in 0.2 M acetate (pH 5.2) incubated at room temperature for one hour). In additional experiments, the extraction buffer was altered to achieve better solubility of the protein. Salt concentrations of 150 mM NaCl and 200 mM NaCl and detergent concentrations of 0.07% NP-40 and 0,1% NP-40 were tested. Isolated protein was obtained by purification of 1 ml sonicate obtained from an induced 50 ml culture using a glutathione sepharose column (Pharmacia) according to the manufacturer's instructions, with a slight adaptation: 50 mM Tris-HCl (pH 8.0) in the elution buffer had been replaced by 50 mM Na-phosphate buffer (pH 8.0), because Tris-HCl could interfere during HPLC analysis. Activity tests on different fractions of eluate were performed as described above for the raw

bacterial lysate. The protease inhibitor “complete Protease Inhibitor Cocktail Tablet“ (Roche) originally used in the lysis buffer proved to be inappropriate, since it contains trehalose.

2.1.4 *in vitro* experiments

For studies with free-living bacteria, bacteria were cultivated in 50 ml 20E medium in a 300 ml Erlenmeyer flask. For high salt conditions 0.2 M NaCl was supplied to the 20E medium. Low oxygen pressure was applied by sealing the Erlenmeyer flask with a rubber seal, followed by flushing with N_2 under sterile conditions. To achieve 1% oxygen pressure air was added under sterile conditions. Bacteria were harvested after 24 h by centrifugation (5000 g, 10 min at 4°C), cell pellets were washed with 20 mM KCl, resuspended in 1 ml double distilled water and boiled for 3 minutes. Extracts were centrifuged (10 000 g; 10 min) and supernatants were analyzed by anion-exchange HPLC using pulse-field amperometric detection (Dionex, Sunnyvale, Cal., USA).

Soluble proteins were assayed with the dye-binding assay (Bradford 1976) modified for microtitre plates (Felix and Meins, 1985).

2.2 Analysis of trehalose-6-phophate synthase sequences

2.2.1 Sequence alignments

A sequence alignment of eight deduced protein sequences of trehalose-6-phophate synthases and potential trehalose-6-phophate synthases was performed as follows. Deduced amino acid sequences of *otsA* and *otsA*-homologous were obtained by a net search in the database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nih.gov/). Sequence alignments were performed by ClustalX (1.81) (Higgins and Sharp, 1988). Thereafter conserved areas and amino acids that are involved in substrate binding in trehalose-6-phophate synthase of *Escherichia coli* (Gibson *et al.*, 2002) were detected and marked manually in the alignment.

2.2.2 Phylogenetic analysis

Phylogenetic analyses were performed on deduced amino acid sequences of *otsA* and *otsA*-homologs that were obtained by a net search in the database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nih.gov/) and that were aligned by ClustalX (1.81) (Higgins and Sharp, 1988). A tree was obtained by heuristic search under the parsimony criterion using PAUP, Version 4.0b10 (2002), Sinauer Associates, Sunderland, Massachusetts (Swofford, 1993). The data matrix comprised 470 parsimony-informative characters. Bootstrap confidence levels were calculated on the basis of 1000 bootstrap replicates.

2.2.3 Southern analysis

For southern blot analysis *Pst*I digested genomic DNA from *Rhizobium* sp. NGR234 and *Rhizobium* sp. NGR *otsA* according to standard methods (Sambrook *et al.*, 1989). As a probe a radioactively labeled 1.9 kb *Eco*RV – *Bam*HI probe that includes 1.2 kb of the rhizobial *otsA*-homologue was used This probe was produced as follows. The symbiotic megaplasmid

pNGR234a of *Rhizobium* sp. NGR234 was isolated by the cesium-chloride method (Ish-Horowicz and Burke, 1981). After restriction digest with *Eco*RV – *Bam*HI, the 1.9 kb fragment was gel purified and radiolabeled with [□- ^{32}P]dCTP using a random primer labeling kit (Stratagene) to specific activities of 1-10□x□10^{8}cpm/μg. The blot was hybridized with radiolabeled probe overnight at 65□C.

2.3 Plant assays

2.3.1 Establishment of the symbiosis

Legume plants used in this work are listed in table 3.

Table 3. Legume plants used in this work. All seeds were kindly provided by W.J. Broughton, Laboratoire de Biologie Moléculaire des Plantes Supèrieures (LSMPS), Université de Génève, Switzerland.

Line	Nodule Type
Vigna unguiculata	Determinate nodules
*Leucaena leucocephala**	Indeterminate nodules
Macroptilium atropurpureum	Determinate nodules
Psophocarpus tetetragonolobus	Determinate nodules
Pachyrhizus tuberosus	Determinate nodules

*tree

For nodulation experiments seeds of various legumes (see table 2) were treated with 30% sulfuric acid for 10 minutes, washed with tap water and then surface sterilized with 31% (v/v) H_2O_2 for 20 minutes and washed with sterile tap water for several times. These seeds were pregerminated on

1.5% (w/v) water agar plates in the dark at 27°C for 3-6 days. The seedlings were afterwards transferred to sterilized Leonard jars (Staehelin *et al.*, 1992), filled with washed Perlite/Vermiculite (1:1) in the upper part, covered with black glass beads to prevent algae contaminations in the nutrient solution (Werner *et al.*, 1975) in the lower compartment. For infection, 3 ml of a 3 days old, washed culture of *Rhizobium* sp. NGR234 or *Rhizobium* sp. NGR otsA were applied to the plants. The infected plants were grown for 4 weeks up to 6 month in a phytotron (14 h day at 240 mol m^{-2} s^{-1} and 26°C, 10 h night at 20°C, 60% relative humidity).

2.3.2 Nodule carbohydrate analysis

Carbohydrates were analyzed as described previously (Schubert *et al.*, 1992; Müller *et al.*, 1994). Nodules (about 10 mg dw) were chilled in ice-cold methanol (0.5 ml; 80% (v/v)) and lyophilized immediately after harvesting. Lyophilized samples of harvested nodules were weighed and ground in methanol (0.5 ml; 80% (v/v)) containing 1% insoluble polyvinyl-pyrrolidone (Polyclar AT, SERVA, Heidelberg, Germany) and mannoheptulose (50 g/sample) as internal standard. The homogenized samples were incubated at 60°C during 10 minutes, followed by centrifugation (13,000 x g, 10 minutes) and collection of the supernatant. This procedure was repeated three times. The supernatants were collected and vacuum-dried. The pellets were resuspended in 600 l distilled water. Charged components were removed with a mixed-bed ion-exchanger (Serolit micro blue and red 2 : 1 (v/v), SERVA, Heidelberg, Germany). After adding 50 l of the wet ion-exchange mixture, the samples were vortexed and centrifuged (13,000 x g, 15 min). The supernatants were lyophilized, redissolved in methanol (50% (v/v)) and transferred to gas chromatography (GC) vials. The solvents were removed by vacuum drying. Water was completely removed by adding twice pure methanol, followed by vacuum drying. The vials were tightly closed with a teflon-lined silicon septum (Varian, Sunnyvale, Cal., USA), and 50 l pyridine containing 625 g hydroxylamine and 50 g phenyl- -glucopyranoside (derivatization standard) were added to each sample by injection through the septum. The vials were incubated for 30 min at 80°C. For derivatisation, 50 l N-methyl-N-trimethylsilyl-heptafluoro-butyramide

containing 1 % (v/v) trimethyl-chorsilane were added. The vials were again incubated for 30 min at 80°C.

Gas chromatography of the derivatisized carbohydrates war performed on a capillary gas chromatograph (Carlo Erba Mega 3500; Zürich; Switzerland). The injections (4 µl) were done using an auto-injector and auto-sampler (Models AOC 14 and AOC 1400, Shimazu, Kyoto, Japan). The silylated carbohydrates were separated using linear temperature gradient from 70°C to 300°C over 40 min. Carbohydrates were detected and quantified using a flame ionization detector by comparison of peak areas with those of standard carbohydrates and internal standards.

Starch content was analyzed enzymatically as described by Müller *et al*. (1995). After the removal of soluble carbohydrates, starch remaining in the residue of the lyophilized samples was solubilized by adding 0.2 ml NaOH (0.5 M) followed by heating at 60°C for 1 h. Afterwards 0.2 ml HCl (0.5 M) was added to neutralize the mixture. Starch was quantified by adding 1 U of glucoamylase (from *Rhizopus*, Boehringer Mannheim, Germany) in 0.4 ml Na-acetate (0.2 M, pH 4.5). After overnight incubation at 37°C the glucose was quantified with the glucose-oxidase-peroxidase method using a test-kit according to the manufacturer's instructions (Boehringer Mannheim, Germany).

2.3.3 Nitrogenase activity

To estimate nitrogenase activity, the acetylene reduction activity (ARA) of nodulated roots was determined as described earlier (Turner and Gibson, 1980). Nodulated roots were put into an Erlenmeyer flask of known volume (600 ml) with 10% (v/v) acetylene in the atmosphere. Gas samples (1 ml) were taken at intervals (up to 30 min) and the ethylene content was assayed by gas chromatography (Shimazu, Kyoto, Japan). The ARA was expressed on the basis of nodule fresh weight, determined by harvesting and weighing all nodules at the end of the experiment.

2.3.4 Isolation of rhizobia from infected nodules

After harvest nodules were surface sterilized with H_2O_2 (purum, 31% (w/v), Fluka) for 20 min and washed several time with sterile tap water. Nodules were crushed and dilution series in rhizobium minimal medium (RMM) lacking the C-source were performed before plating on 20E plates. The 20E plates contained the relevant antibiotics and additional 200 mM mannitol to osmotically stabilize the isolated bacteroids. Plates were incubated for 4 to 5 days at 27°C before counting.

2.3.5 Transmission electron microscopy

Vigna unguiculata nodules inoculated with *Rhizobium* sp. NGR234 or *Rhizobium* sp. NGR *otsA* respectively were harvested seven weeks after infection and were embedded directly thereafter. For this, "small" and normal sized nodules were chosen for comparison. Embedding and transmission electron microscopy was performed in collaboration with Ursula Sauder of the Interdivisional Electron Microscopy (IEM) facility at the Biozentrum of the University of Basel. For transmission electron microscopy, the nodules were immersion fixed for at least 4 hours in a buffered glutaraldehyde and paraformaldehyde solution (Karnofski fixative: 0.1 M Na-cacodylate (pH 7.2); 3% (v/v) glutaraldehyde; 4% paraformaldehyde) at 4°C. Thereafter nodules were cut in pieces and washed in 10 mM PBS (pH 7.4). This was followed by one hour post fixation in buffered 1% osmium tetroxide at 4°C and subsequent washing in water. Nodules were then dewatered and stained in 50% EtOH for 15 minutes, 70% EtOH for 15 minutes, 70% EtOH + 2% uranyl acetate for 2 hours, 90% EtOH for 15 minutes and 98% EtOH for 15 minutes. After dehydration nodules were washed in 99% acetone and embedded in 1:1 acetone:Epon (Electron Microscopy Sciences, Euromedex, Strasbourg, Franc) for 1 hour. This was followed by two treatments with pure Epon for two hours each. After being sectioned and stained with 6% uranyl acetate for 1 hour and lead citrate (Millonig, 1961), the specimens were examined by transmission electron microscopy.

3. Results

Rhizobia are known to synthesize trehalose under two different conditions, namely at high osmolarity (Breedveld et al. 1993) and under low oxygen pressure (Hoelzle & Streeter 1990). Trehalose also accumulates in root nodules of many legumes, most likely as a product of the rhizobial microsymbiont (Streeter, 1980; Streeter 1981; Streeter 1985; Müller *et al*. 1994; Farias-Rodriguez, *et al*., 1998).

Here we present a genetic approach to study trehalose-synthesizing pathways in rhizobia. Furthermore the impact of microbial trehalose synthesis on the symbiosis is investigated. The classic pathway for trehalose synthesis involves the condensation of UDP-glucose and glucose-6-phophate to afford trehalose-6-phophate. This reaction is catalyzed by the glycosyltransferase trehalose-6-phophate synthase. In a second step trehalose-6-phophate is dephosphorylated by trehalose-6-phophate phosphatase yielding free trehalose. In eukaryotic systems like in yeasts or in *Arabidopsis thaliana* this pathway is commonly called TPS/TPP pathway (TPS= "trehalose-6-phophate synthase"; TPP= "trehalose-6-phophate phosphatase"), whereas in prokaryotic systems it is in general named the OtsA/B pathway (OTS= "osmotic trehalose synthesis"). Thus, in rhizobia, we will refer to this pathway as the OtsA/B pathway.

3.1 OtsA/B pathway in rhizobia

3.1.1 Occurrence of the OtsA/B pathway

Fifty-two independent OtsA-like sequences are known; they come from a wide taxonomic range of organisms (http://www.ncbi.nlm.nih.gov/Entrez/). Yet, in contrast to the abundance of sequence data, studies on the functionality of these sequences remain scarce. Functionality of the genes that code for trehalose-6-phophate synthase has been shown by i.e., enzyme assays followed by N-terminal amino acid sequencing of the purified protein as in *Mycobacterium*

smegmatis (De Smet *et al*., 2000), or by restoring a yeast tps1 mutant by expressing the heterologous cDNA in the yeast tps1 mutant (Blázquez *et al*., 1998). To date, functionality has been demonstrated for four genes that code for trehalose-6-phophate synthase in prokaryotes: in one α-proteobacterium (*Escherichia coli*) and in three gram+ bacteria (two *Mycobacteria* spp. and *Corynebacterium glutamicum*). In addition in eukaryotes functionality of *tps* genes have been shown in several yeasts and in two plants, namely in *Arabidopsis thaliana* and *Selginella lepidophylla*. Thus in the phylum of proteobacteria the functionality of *otsA* has been established in one α-proteobacterium, but not yet in β-proteobacteria, the class that comprises the order of rhizobiales. In rhizobia, OtsA-like open reading frames (ORFs) have been published for *Rhizobium* sp. NGR234 (accession No.: NP444016), *Sinorhizobium meliloti* (accession No.: NP435371), *Mesorhizobium loti* (accession No.: AP003017) and *Bradyrhizobium japonicum* USDA110 (accession No.: AP005936). The homologues of *otsA* and *otsB* in *Rhizobium* sp. NGR234 are located on the symbiotic plasmid pNGR234*a* (Freiberg *et al*., 1997). These two predicted ORFs are named y4pC and y4pB and are clustered. They represent the only sequence data of the complete OtsA/B pathway in fast growing rhizobia that are available so far. Thus y4pC was chosen for directed mutagenesis to study its function

Y4pC and y4pB of *Rhizobium* sp. NGR234 share 42 % identity with OtsA and OtsB of *Escherichia coli* (accession No.: X69160) on the protein level, but only 29% identity with TPS1 (accession No.: CAA48510) and 27% identity with TPP (accession No.: P31688) of *Saccharomyces cerevisae* (Table 1 and Table 2). As compared to the remaining published OtsA and OtsB-like ORF in rhizobia, y4pC (OtsA-like ORF) of *Rhizobium* sp. NGR234 and the OtsA-like ORF that is located on pSymA of *Sinorhizobium meliloti* (accession No.: NP_435371) possesses with 85% the highest identity. But *Sinorhizobium meliloti* appears to lack an OtsB-like ORF on the entire pSymA megaplasmid (Barnett *et al*., 2001). The second highest identity of y4pC and y4pB and other rhizobial ORFs is exhibited in comparison with the OtsA- and OtsB-like ORF of *Mesorhizobium loti* with 45% and 44% identity respectively. It should be noted that the size of the *Mesorhizobium loti* OtsA-like ORF possesses only 57% of the size of the average size of OtsA or OtsA-like ORFs. The forth known rhizobial OtsA and OtsB-like ORFs are published for the slow rhizobium *Bradyrhizobium japonicum* (accession No.: AP005936). They

share 43% and 40% identity with y4pC and y4pB of *Rhizobium* sp. NGR234 respectively (Table 1 and Table 2).

Table 1. Percentage identity and similarity of y4pC, an OtsA-like ORF located on pNGR234*a* in *Rhizobium* sp. NGR234, in comparison to OtsA of *Escherichia coli*, TPS1 of *Saccharomyces cerevisae* and OtsA-like ORF of other rhizobia which are published to date. The comparison was base on the 464 amino acids that Y4pC consists of. Its accession number is P55612.

Selected OtsA like ORF	Accession number	Percentage identity/similarity
OtsA of *Escherichia coli*	P31677	42/60
TPS1 of *Saccharomyces cerevisae*	CAA48510	29/52
OtsA-like ORF of *Bradyrhizobium japonicum* sp.	AP005936	43/55
OtsA-like ORF on pSymA of *Sinorhizobium meliloti*	NP_435371	85/88
OtsA-like ORF of *Mesorhizobium loti*	AP003017	45/58

Table 2. Percentage identity and similarity of y4pB, an OtsB-like ORF located on pNGR234*a* in *Rhizobium* sp. NGR234, in comparison to OtsB of *Escherichia coli*, TPP of *Saccharomyces cerevisae* and OtsB-like ORF of other rhizobia which are published to date. The comparison was base on the 265 amino acids Y4pB consists of. Its accession number is P55611.

Selected OtsA like ORF	Accession number	Percentage identity/similarity
OtsB of *Escherichia coli*	E90654	42/58
TPP of *Saccharomyces cerevisae*	P31688	27/40
OtsB-like ORF of *Bradyrhizobium japonicum*	AP005936	40/53
Sinohizobium meliloti appears to lack OtsB	NP_435371	-
OtsB-like ORF of *Mesorhizobium loti*	AP003017	44/54

3.1.2 Differences in highly conserved areas of OtsA-like ORFs of rhizobia in comparison to functional OtsA and TPS of different phyla

The homology of the OtsA-like ORF in *Rhizobium* sp. NGR234 is strikingly low when compared to the known sequence of trehalose-6-phophate synthases of *Saccharomyces cerevisae*: They share only 27% identity and 52% similarity. A more detailed analysis of consensus sequences of OtsA-like ORF in rhizobia and selected sequences for trehalose-6-phophate synthases was performed. A sequence alignment of eight protein sequences of trehalose-6-phophate synthases or putative trehalose-6-phophate synthases is shown in figure 1. The alignment includes all known and so far uninvestigated rhizobial sequences and compares them to representative examples of protein sequences of trehalose-6-phophate synthases, with proven functionality. The rhizobial sequences include OtsA-like ORF of *Rhizobium* sp. NGR234 (accession No.: NP444016), *Sinorhizobium meliloti* (accession No.: NP435371), *Mesorhizobium loti* (accession No.: AP003017) and *Bradyrhizobium japonicum* USDA110, (accession No.: AP005936). They are compared with trehalose-6-phophate synthase sequences of *Saccharomyces cerevisae* (accession No.: CAA48510), *Arabidopsis thaliana* (accession No.: NP_173142.1), *Mycobacterium tuberculosis* CDC1551 (accession No.: NP_338139.1) and *Escherichia coli* (accession No.: X69160).

Based on the analysis of the three-dimensional structure of the *Escherichia coli* OtsA (in complex with both UDP and glucose-6-phophate) and sequence alignments, Gibson *et al*., 2002 published regions that are “invariant” in trehalose-6-phophate synthase. In figure 1 these regions are marked in yellow. They represent 20% of the average total sequence length. Surprisingly in the OtsA-like ORF of rhizobia we detected an average of 14% of amino acids within these regions that were variant. They are marked in red. In *Arabidopsis thaliana*, *Mycobacterium tuberculosis* and *Escherichia coli* all amino acids within these regions are invariant, and in *Saccharomyces cerevisae* only two of ninety-two amino acids that comprise these regions are variant. Most of these highly conserved regions are involved in substrate binding. In *Escherichia coli* clusters of invariance centered around Arg9, Trp40, Tyr76, Trp85 and Arg300 (written in green below the alignment) are involved in the binding of the glucose-6-phophate. Clusters of invariance centered around Gly21, Gly22, Asp130, His154, Arg262, Asp361 and Glu369 (written in orange below

the alignment) are involved in the binding of the UDP. Furthermore there are patches of conservation whose function is unclear. Clusters around Leu181, Pro242 and Arg319 (written in blue below the alignment) lie in exposed surface regions. Such sites may be involved in the binding of a regulatory factor (Gibson *et al*., 2002).

The OtsA-like ORF (y4pC) of *Rhizobium* sp. NGR234 varies in 11 amino acids within the highly conserved regions that comprise a total of 92 amino acids. In addition one amino acid is missing at the end of the ORF. Amino acids that are directly involved in the binding of glucose-6-phophate in OtsA of *Escherichia coli* are not affected by these alterations. But in the clusters of "invariance" around Arg9 and Arg300, which are both directly, involved in the binding of glucose-6-phophate in OtsA of *Escherichia coli*, the y4pC locus of *Rhizobium* sp. NGR234 exhibits changes in one amino acid per region. Amino acids that are directly involved in the binding of UDP in *Escherichia coli* are not changed in the OtsA-like ORF of *Rhizobium* sp. NGR234 as well. But again the "invariant" regions around these amino acids are affected: one amino acid in the cluster around Gly21 and Gly22 is altered. In addition two amino acids in the "invariant" region around His154 and one in the region around Asp361 differ to the one in the active center of *Escherichia coli*. All clusters of "invariance" that lie in the exposed surface regions in *Escherichia coli* differ in the OtsA-like ORF of *Rhizobium* sp. NGR234: Leucine at the position 181 in *Escherichia coli* is replaced by valine and a proline at the position 242 is represented by alanine on the y4pC locus of *Rhizobium* sp. NGR234. In addition in the "invariant" area around Arg319 one amino acid is changed in *Rhizobium* sp. NGR234. The last two different amino acids in the OtsA-like ORF of *Rhizobium* sp. NGR234 in comparison to OtsA of *Escherichia coli* lie within "invariant" areas with unknown functions.

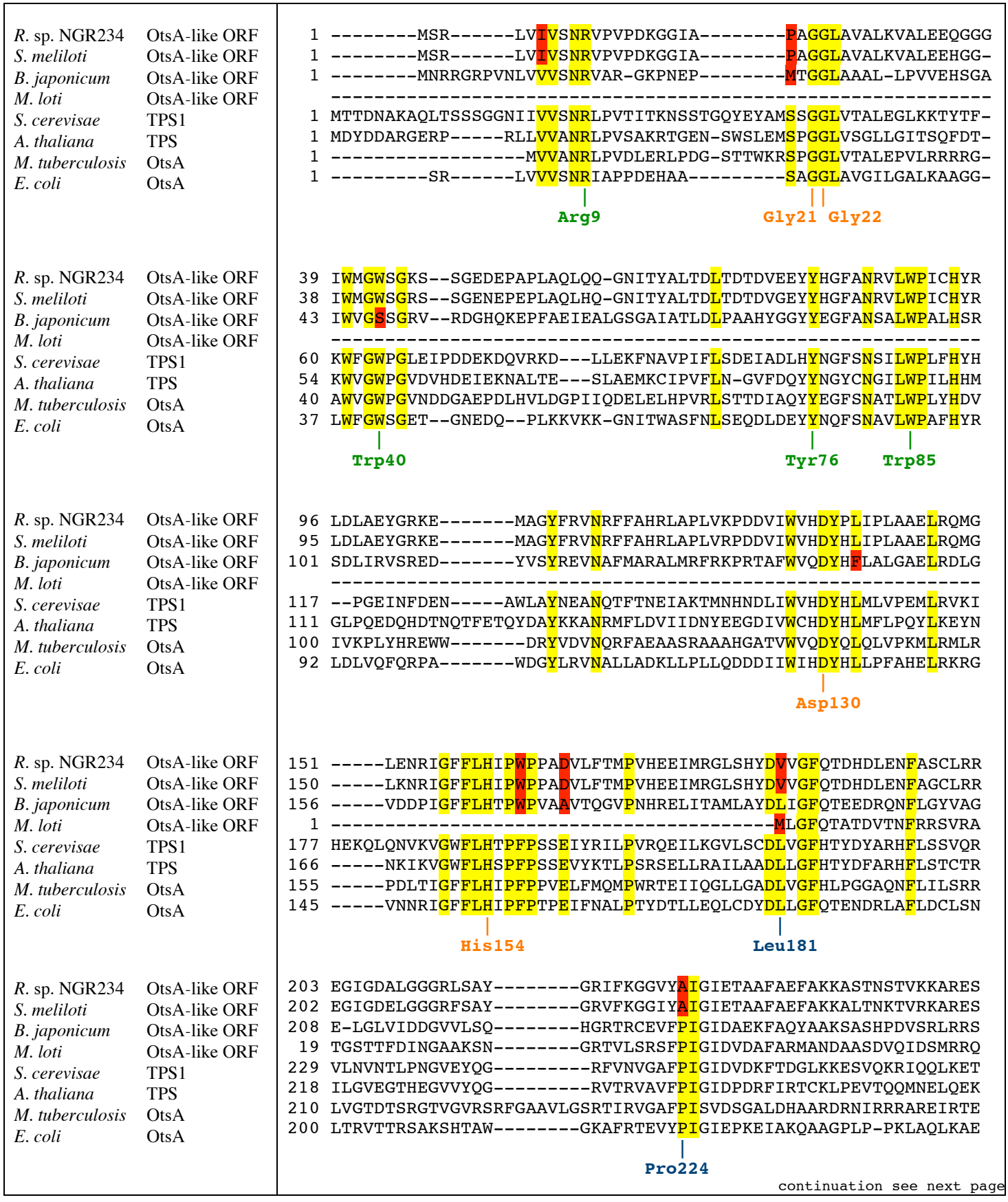
Sinorhizobium meliloti and *Rhizobium* sp. NGR234 are nearly 100% identical within the highly conserved regions that comprise a total of 92 amino acids. Only the last amino acid that is missing in NGR234 exists in *Sinorhizobium meliloti* - as it does in all the other organisms studied. With respect to the overall sequence *Sinorhizobium meliloti* and *Rhizobium* sp. NGR234 are 85% identical (see table 1) but within the highly conserved regions they are entirely identical (see figure 1). Thus the differences within the active centers and other highly conserved regions of the OtsA-like ORFs in *Sinorhizobium meliloti* and *Rhizobium* sp. NGR234 are the same in

comparison to OtsA of *Escherichia coli*: Two amino acids are changed in regions of "invariance" involved in the binding of glucose-6-phophate in OtsA of *Escherichia coli* and four amino acids differ in regions of "invariance" involved in the binding of UDP. Three amino acids are altered in the conserved areas that lie in the exposed surface regions of OtsA in *Escherichia coli* and two (or three in the case of *Sinorhizobium meliloti*) lie in regions of "invariance" with unknown function.

The OtsA-like ORF of *Bradyrhizobium japonicum* differs in the highly conserved regions in a similar manner from OtsA in *Escherichia coli* than *Sinorhizobium meliloti* and *Rhizobium* sp. NGR234. From a total of 92 amino acids in the regions of invariance 14 are altered in *Bradyrhizobium japonicum* in comparison to *Escherichia coli*. Half of them are situated at the same position as the variations in the OtsA-like ORFs of *Sinorhizobium meliloti* and *Rhizobium* sp. NGR234. Despite the fact that these alterations are at the same position, only two of them are changed into an identical amino acid. In addition Trp40 that is directly involved in the binding of glucose-6-phophate in OtsA of *Escherichia coli* is changed to a serine in the OtsA-like ORF of *Bradyrhizobium japonicum*. Furthermore in the clusters of "invariance" around Arg300 that is involved in the binding glucose-6-phophate one amino acid is altered. Amino acids that are directly involved in the binding of UDP are not altered but eight amino acids differ in the highly conserved regions around them. Two amino acids are changed in the conserved areas that lie in the exposed surface regions of OtsA in *Escherichia coli*. Again *Bradyrhizobium japonicum* does not exhibit the same pattern of changes in these regions when compared to *Sinorhizobium meliloti* and *Rhizobium* sp. NGR234 that shown an identical pattern of changes. The remaining two amino acids that are changes in *Bradyrhizobium japonicum* when compared to OtsA in *Escherichia coli* lie in regions of "invariance" with unknown functions.

The OtsA-like ORF of *Mesorhizobium loti* exhibits the most drastic differences in comparison to the sequences of OtsA with proven functionality. Kaneko *et al*. (2000) published an OtsA-like ORF with the accession No.: AP003017 (gil14028198) as "trehalose-6-phophate synthase". This published OtsA-like ORF of *Mesorhizobium loti* starts only at the position 181 of the corresponding OtsA of *Escherichia coli*. Thus it is missing three of four amino acids that are directly involved in the binding of glucose-6-phophate. In addition all highly conserved regions

around these three amino acids are completely missing. Also the OtsA-like ORF of *Mesorhizobium loti* is changed in one amino acid in the conserved area around the only existing amino acid that is involved in the binding of glucose-6-phophate: Arg300. Four out of eight amino acids involved in the binding of UDP are missing as well, just like the conserved areas surrounding them. But in the remaining four conserved areas around the amino acids involved in the binding of UDP the OtsA-like ORF of *Mesorhizobium loti* exhibits only one amino acid that is changed in comparison to OtsA of *Escherichia coli*. Three amino acids are altered in the conserved regions that lie in the exposed surface regions of OtsA in *Escherichia coli* and three that lie in regions of "invariance" with unknown functions. Thus the OtsA-like ORF of *Mesorhizobium loti* is missing 33% of the active center and an additional 4% are changed in comparison with the conserved regions of OtsA in *Escherichia coli*. It possess therefore hardly functionality as OtsA and it will not be included in further studies on trehalose-6-phohate synthase in rhizobia in this work.


R. sp. NGR234 OtsA-like ORF
S. meliloti OtsA-like ORF
B. japonicum OtsA-like ORF
M. loti OtsA-like ORF
S. cerevisae TPS1
A. thaliana TPS
M. tuberculosis OtsA
E. coli OtsA
Arg9
Gly21 Gly22
Trp40
Tyr76
Trp85
Asp130
His154
Leu181
Pro224
continuation see next page

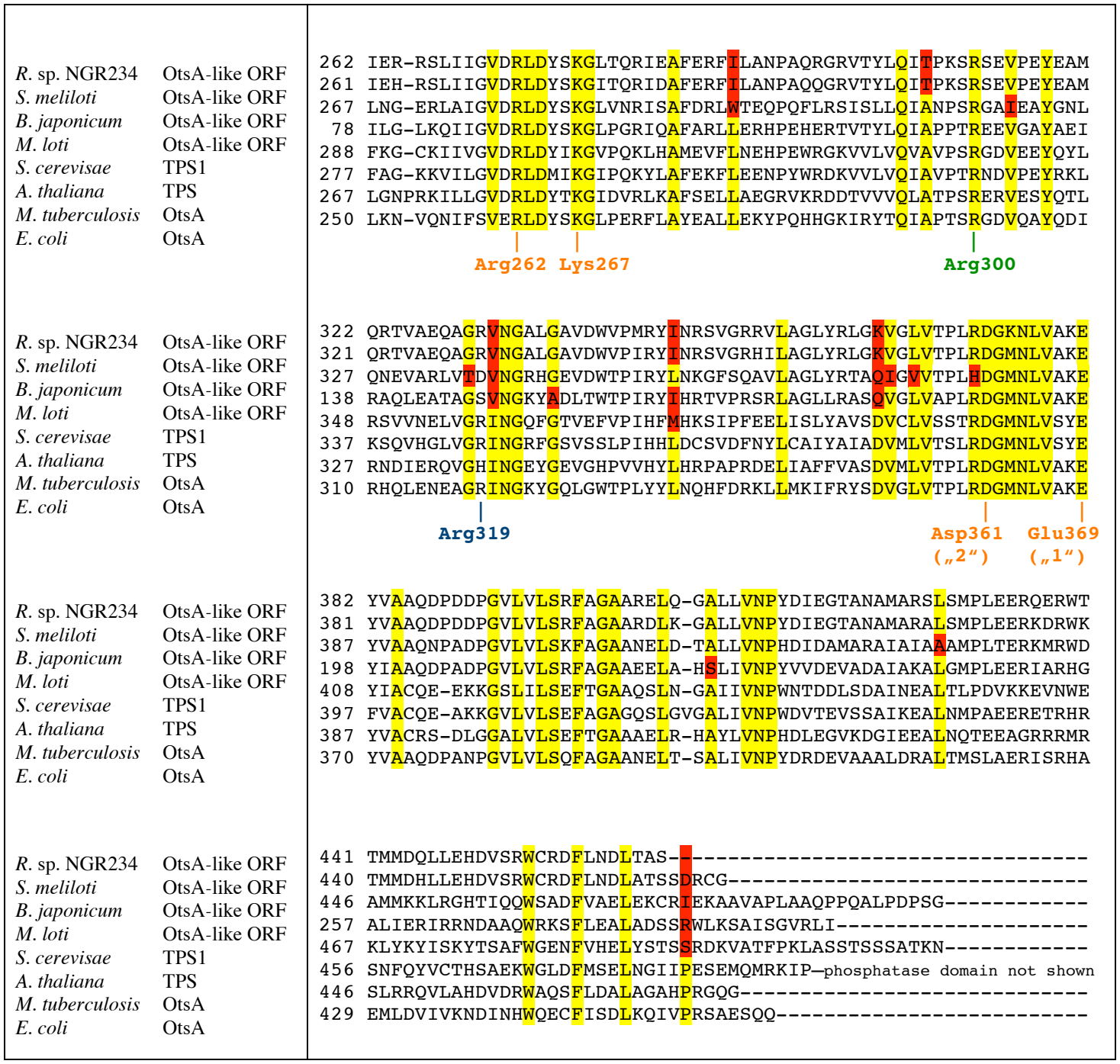


Figure 1. Sequence alignment of eight primary sequences of trehalose-6-phophate synthases and potential trehalose-6-phophate synthases. They include all published OtsA-like ORFs in rhizobia (*Rhizobium* sp. NGR234, accession No.: NP444016; *Sinorhizobium meliloti*, accession No.:

NP435371; *Mesorhizobium loti*, accession No.: AP003017 and *Bradyrhizobium japonicum* USDA110, accession No.: AP005936) In addition four other representative trehalose-6-phophate synthase sequences are shown (*Saccharomyces cerevisae*, accession No.: CAA48510; *Arabidopsis thaliana*, accession No.: NP_173142.1; *Mycobacterium tuberculosis* CDC1551 accession No.: NP_338139.1; *Escherichia coli*, accession No.: X69160). Regions that were published as "invariant" by Gibson *et al.*, 2002 are marked in yellow. Amino acids that are variant within these regions are marked in red. Arg9, Trp40, Tyr76, Trp85 and Arg400 are involved in the binding of glucose-6-phophate in OtsA of *Escherichia coli* and are written in green below the alignment. Gly21, Gly22, Asp130, His154, Arg262, Asp361and Glu369 are involved in the binding of UDP in OtsA of *Escherichia coli* and are written in orange below the alignment. Amino Acids corresponding to Wable and Grishin's "glycogen phophorylase glycosyltransferase" motif position 1 and 2 are indicated. Clusters around Leu181, Pro242 and Arg319 (written in blue below the alignment) lie in the exposed surface regions of OtsA in *Escherichia coli* (Gibson *et al.*, 2002).

3.1.3 Molecular evolutionary analysis of OtsA, TPS and OtsA-like sequences

To examine the evolutionary roots of the variations in different trehalose-6-phophate synthases and potential trehalose-6-phophate synthases, a phylogenetic analysis was performed (figure 2). This analysis was carried out on the basis of 22 protein-sequences that are derived from a wide range of taxa. During the selection of appropriate sequences it was put emphasis on the fact that all three superkingdoms (eukaryota, bacteria, archaebacteria) should be represented in the resulting phylogenetic tree to generate a general picture of the relations of the different trehalose-6-phophate synthases and potential trehalose-6-phophate synthases. Furthermore within the superkingdom of bacteria all phyli that possess OtsA, TPS or OtsA-like ORFs are represented in our phylogenetic analysis. The twenty-two protein-sequences that are included in the phylogenetic tree comprise seven protein-sequences with proven functionality as OtsA or TPS, and fifteen OtsA-like ORFs that include the three remaining OtsA-like ORFs of different rhizobia. Protein-sequences with proven functionality as OtsA or TPS are marked with a red star. The OtsA-like ORFs of rhizobia are written in blue (figure 2). One of the superkingdoms was chosen as outgroup to root the tree. The overall picture did not change if this outgroup was represented by the superkingdom of eukarya or archaebacteria. Here a tree is shown with TPS-sequences that are derived from eukarya as outgroup. The phylogenetic tree was obtained by heuristic search under the parsimony criterion. It is based on protein sequences that were obtained by a net search in the database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nih.gov/). The data matrix comprised 470 parsimony-informative characters. Bootstrap confidence levels are shown above each node. The number of bootstrap replicates is 1000. Most of the bootstrap values reach the maximum value of 100, whereas only two lineages are characterized by bootstrap values of 55 and 57 that are close to the minimum value of 50.

In the resulting tree all OtsA-like ORFs of rhizobia are located on the same lineage together with the OtsA-like ORF of *Rhodopseudomonas palustris*. Thus all OtsA-like ORFs of α-proteobacteria are clustered. In addition they are located on the same branch as OtsA-like ORFs and OtsA of three other proteobacteria: of the two γ-proteobacteria *Salmonella typhimurium* and *Escherichia coli* and of a β-proteobacterium that is diazotrophic: *Burkholderia fungorum*. For one

protein on this lineage – for OtsA of *Escherichia coli* the functionality has been shown. Thus on the basis of the overall protein sequence the OtsA-like ORFs of rhizobia and OtsA of *Escherichia coli* are closely related. They show a pattern that is reflecting their phylogenetic trace, since they all belong to the phylum of proteobacteria. Two other proteins, for which the functionality as OtsA has been shown in prokaryotes, lie on a deeply branching lineage in comparison to the lineage of rhizobia. They are derived from two gram positive actinobacteria: OtsA of *Mycobacterium tuberculosis* and *Corynebacterium efficiens*. They appear on the same branch and are clustered on a lineage with OtsA-like ORFs of two □-proteobacteria, one cyanobacterium, and two additional gram-positive actinobacteria. The last four sequences of trehalose-6-phophate synthases with proven function are clustered as well and are derived from eukaryotic organisms - two plants and two fungi. They are displayed as outgroup in this tree.

When overall sequences of OtsA, TPS or OtsA-like ORFs are considered they exhibit a relation that is reflecting their phylogenetic relationship (figure 2). But when homologies in the active centers and highly conserved areas on the surface areas of the proteins with proven function are compared they revealed to be 100% identical, even though they are derived from a wide taxonomic range (figure 1). Thus the evolutionary pressure to keep these areas unchanged is very high.

In contrast the OtsA-like ORFs of rhizobia differ in this pattern, when compared to protein sequences of trehalose-6-phophate synthases with proven function: With respect to the overall protein sequence they display a relation to the other sequences of trehalose-6-phophate synthases that is reflecting their phylogenetic relationship to the organism that they are derived from as well (figure 2). But in the areas that are 100% identical in the proteins with proven functions the OtsA-like ORFs of rhizobia differ. OtsA-like ORFs of *Rhizobium* sp. NGR234 and *Sinorhizobium meliloti* reveal to be only 87% identical to the highly conserved areas of trehalose-6-phophate synthases with proven function. In addition the OtsA-like ORF of *Bradyrhizobium japonicum* is even only 84% identical (figure 1). Thus the evolutionary pressure to keep these areas unchanged seems not as high in the OtsA-like ORFs of rhizobia as in the proteins with proven function as trehalose-6-phophate synthase.

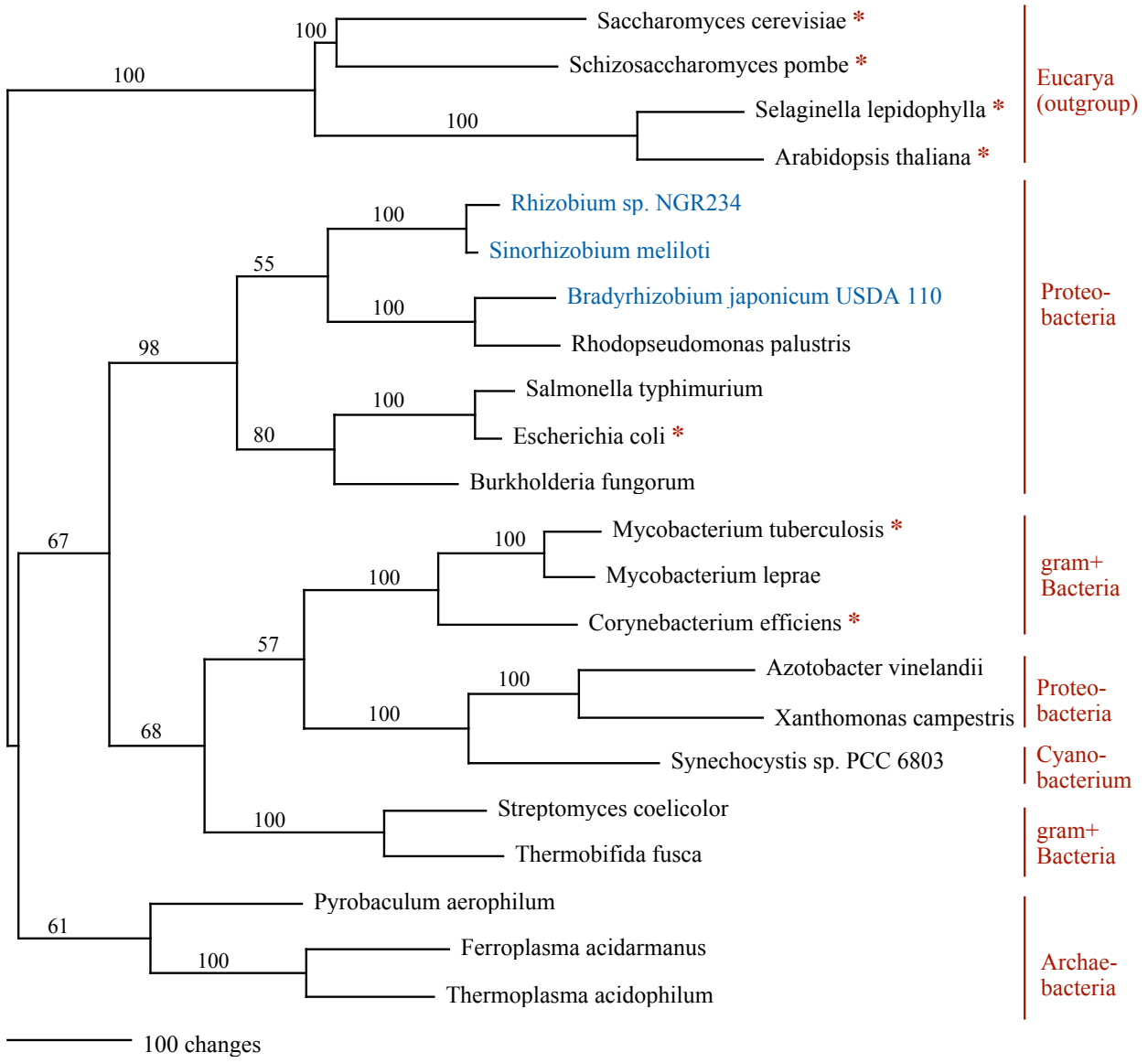


Figure 2: Phylogenetic relationship between OtsA and OtsA-like protein sequences. The tree was obtained by heuristic search under the parsimony criterion using the deduced amino acid sequences of OtsA and OtsA-homologous regions of a brought variety of taxa. The data matrix comprised 470 parsimony-informative characters. Bootstrap confidence levels are shown above each node. The number of bootstrap replicates is 1000. Organisms for which the functionality of

OtsA or TPS has been demonstrated are marked with a red star. All rhizobia are written in blue. The accession number and sources for the aliening sequences are as follows:
Saccharomyces cerevisae (gi|4629|emb|CAA48510.1), *Schizosaccharomyces pombe* (gi|8894854|emb|CAB95998.1), *Selaginella lepidophylla* (gi|6741654|emb|CAB69549.1), *Arabidopsis thaliana* (gi|15219969|ref|NP_173142.1), *Rhizobium* sp. NGR234 (gi|16519896|ref|NP_444016.1), *Sinorhizobium meliloti* (gi|16262578|ref|NP_435371.1), *Bradyrhizobium japonicum* USDA 110 (gi|27348570|dbj|BAC45587.1), *Rhodopseudomonas palustris* (gi|22961825|ref|ZP_00009431.1|COG0380), *Salmonella typhimurium* (gi|17865667:1-473), *Escherichia coli* (gi|603543|emb|CAA48913.1), *Burkholderia fungorum* (gi|22987002|ref|ZP_00032088.1|COG0380), *Mycobacterium tuberculosis* CDC1551 (gi|15843102|ref|NP_338139.1), *Mycobacterium leprae* (gi|15828204), *Corynebacterium efficiens* YS-314 (gi|23494351|dbj|BAC19318.1), *Azotobacter vinelandii* (gi|23103785|ref|ZP_00090259.1|COG0380), *Xanthomonas campestris* pv. *campestris* str. ATCC 33913 (gi|21114248|gb|AAM42306.1), *Synechocystis* sp. PCC 6803 (gi|16330944|ref|NP_441672.1), *Streptomyces coelicolor* A3(2) (gi|21222683), *Thermobifida fusca* (gi|23019438|ref|ZP_00059148.1|COG0380), *Pyrobaculum aerophilum* (gi|18312516|ref|NP_559183.1), *Ferroplasma acidarmanus* (gi|22406833|ref|ZP_00001675.1| COG0380), *Thermoplasma acidophilum* (gi|16082218|ref|NP_394666.1)**.**

3.1.4 Ω-cassette insertion mutagenesis resulting in *Rhizobium* sp. NGRΩotsA

To test the function of OtsA-like ORF in rhizobia, the only fast growing rhizobium was chosen for which the sequence data for the complete OtsA/B pathway is available. Homologues to *otsA* and *otsB* of *Escherichia coli* have been found during complete sequencing of the symbiotic plasmid pNGR234*a* of *Rhizobium* sp. NGR234 (Freiberg *et al.*, 1997). These two loci are named *y4pC* (encoding OtsA-like ORF) and *y4pB* (encoding OtsB-like ORF) and are clustered (figure 3).

We have mutated *y4pC* of *Rhizobium* sp. NGR234 by insertion of an Ω-cassette. The genomic organization of the *y4pC* and *y4pB* locus of pNGR234*a* and the Ω-cassette insertion mutagenesis resulting in *Rhizobium sp*. NGRΩotsA is shown in figure 3. *Y4pB* (OtsB-like ORF) is located upstream of *y4pC*, the homologue of *otsA*. The Ω-cassette is 2 kb long and carries a spectomycine resistance gene, followed by several transcriptional and translational stop codons. It replaces the first 723 bp of y4pC.

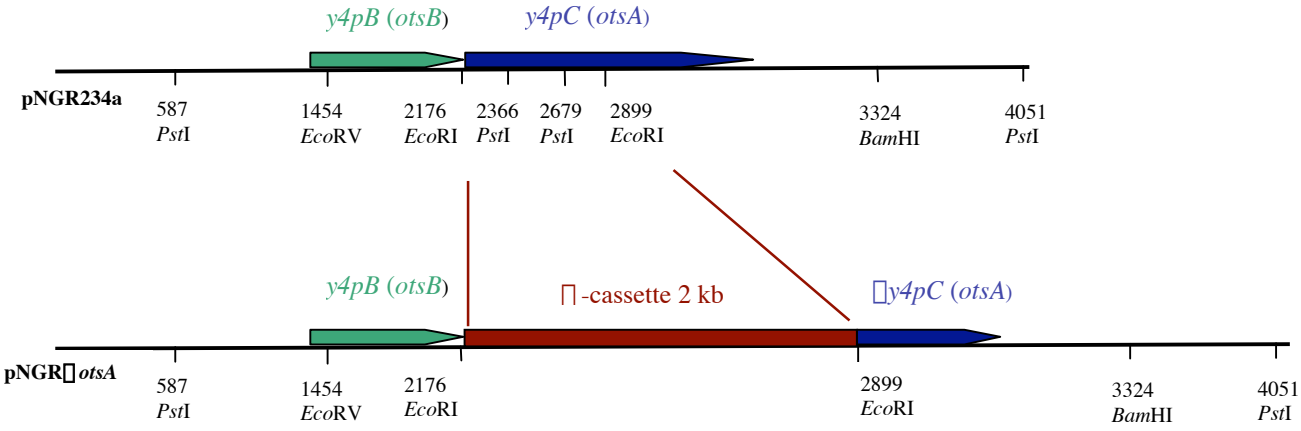


Figure 3: Genomic organization of the *y4pC*/*y4pB* locus of pNGR234*a* and pNGRΩ*otsA*. Predicted ORFs are represented as arrows oriented according to the sense of transcription. Position of the Omega interposon within *y4pC* is shown in red. The relevant restriction sites are marked according to their position.

For the analysis of these mutants a southern blot analysis was performed with DNA of the mutant and the wild type strain (see figure 4). The DNA was digested with *Pst*I and hybridized with a probe that carried 1.2 kb of the wild type *y4pC*. In the wild type the expected 0.3 kb and 1.4 kb *Pst*I fragments were detected (lane 3, figure 4). In the mutant stain two *Pst*I restrictions sites have been removed with the effect that the 0.3 kb fragment does not occur, as well as the 1.4 kb band. Instead only one 4.7 kb band is expected and detected (lane 1 and 2, figure 4). It is derived from a 3.4 kb *Pst*I fragment that is shortened by the 0.7 kb *Eco*RI fragment that had been removed and replaced and prolonged by the 2 kb Ω-cassette (3.4 kb – 0.7 kb + 2 kb = 4.7 kb). See also figure 3. For further studies the clone in lane 1 was chosen.

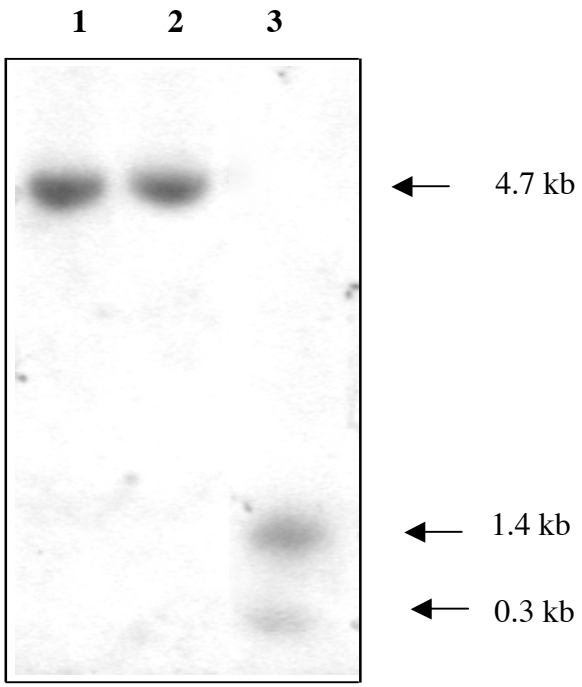


Figure 4: Southern blot of *Rhizobium* sp. NGR234 and *Rhizobium* sp. NGRΩ*otsA* genomic DNA. The DNA was digested with *Pst*I and analyzed by Southern blotting using a radioactive labeled 1.9 kb *Eco*RV – *Bam*HI probe that includes 1.2 kb of the rhizobial *otsA*-homologue. Lane 1 and 2: NGRΩ*otsA* (Ω-cassette insertion); lane 3: NGR234 (wild type). Molecular weights are indicated on the right.

3.2. Trehalose biosynthesis in free-living rhizobia

3.2.1 Trehalose biosynthesis in rhizobia growing under low oxygen is directed by the symbiotic plasmid encoded *otsA*

In *Rhizobium* sp. NGR234 an Ω-cassette insertion mutagenesis of *otsA* was performed revealing *Rhizobium* sp. NGRΩ*otsA*. To test the phenotype of this mutant and thereby the functionality of the *otsA*-like ORF in rhizobia, appropriate induction conditions of trehalose accumulation had to be worked out. In fast growing rhizobia trehalose accumulation has been described in *Sinorhizobium meliloti* and *Rhizobium leguminosarum* in response to salt stress (Breedveld *et al.,* 1993) and in *Rhizobium leguminosarum* bv. phaseoli, *Rhizobium leguminosarum* bv. vivicae, *Rhizobium fredii* and *Sinorhizobium meliloti* in microaerobic culture conditions (1% oxygen) (Hoelzle and Streeter, 1990).

Based on these findings trehalose accumulation was tested in *Rhizobium* sp. NGR234 and *Rhizobium* sp. NGRΩ*otsA* upon atmospheric, microaerobic or high salt (0.2 M NaCl) liquid culture conditions (table 3). In contrast to bradyrhizobia, all fast growing rhizobia previously tested do not accumulate trehalose under atmospheric exponential growth conditions. Here these findings were verified in *Rhizobium* sp. NGR234 (table 3), since no trehalose accumulation was detected under unstressed culture conditions. These culture conditions represent an appropriate negative control for trehalose accumulation in *Rhizobium* sp. NGR234. The remaining two potential induction conditions for trehalose synthesis were both tested positive in the wild type of *Rhizobium* sp. NGR234 for the first time: Trehalose accumulation was detected in *Rhizobium* sp. NGR234 cultured under salt stress (0.2 M NaCl) and under low oxygen (1%).

To test the phenotype of the *y4pC* (encoding OtsA-like ORF) deletion mutant, *Rhizobium* sp. NGRΩ*otsA* was subjected to non–inducing culture conditions (unstressed and well aerated) or inducing (high salt and low oxygen) growth conditions for trehalose accumulation respectively. The deletion of the rhizobial *otsA*-like ORF impaired trehalose synthesis in *Rhizobium* sp. NGRΩ*otsA* under microaerobic growth conditions. Thus most probably, trehalose synthesis

induced in hypoxic milieu is directed by the symbiotic plasmid encoded *otsA*-like ORF (*y4pC*) in *Rhizobium* sp. NGR234. So far, the functionality of *otsA* homologues genes had not been shown in any *Rhizobium* species and activity of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase had only been shown in slow growing bradyrhizobia. Although homologues to *otsA/B* genes have been described in 52 organisms (http://www.ncbi.nlm.nih.gov/Entrez/), the functionality of these homologues has only been shown in several yeasts, one enterobacterium (-proteobacteria), in three gram-positive bacteria (De Smet *et al*., 2000) and in two plants (Van Dijck *et al*., 2002).

The trehalose accumulation under salt stress was not affected by the mutation of *otsA* in *Rhizobium* sp. NGR234. Earlier studies have demonstrated that *Rhizobium* sp. NGR234 exhibit trehalose synthesizing activity by maltooligosyltrehalose synthase and maltooligosyltrehalose trehalohydrolase (MOTS – pathway) (Streeter and Bhagwat, 1999). Here it is shown that the trehalose accumulation is impaired in low oxygen conditions but not in salt stress by the mutation of *otsA*. Therefore it is suggested that *Rhizobium* sp. NGR234 contains at least two independent pathways for trehalose biosynthesis: the OtsA/B – pathway that is induced by hypoxia and the MOTS – pathway, that is likely to be induced by salt stress.

Table 3. Trehalose accumulation in *Rhizobium* sp. NGR234 and *Rhizobium* sp. NGRΩ*otsA* grown in liquid culture under the stated growth conditions. The carbon source was 20 mM succinate and the bacteria were harvested after 24h. Mean values and SE correspond to four independent experiments

Strain	Growth conditions	Trehalose (mg/g protein)
NGR234	Unstressed conditions	<0.01
	High salt (0.2 M NaCl)	7.5 ± 2.1
	Low oxygen (1%)	**6.0** ± 1.7
NGRΩ*otsA*	Unstressed conditions	<0.01
	High salt (0.2 M NaCl)	8.7 ± 2.8
	Low oxygen (1%)	**<0.01***

*, p< 0.05

3.2.2 The ability to utilize diverse carbon sources is not affected by the deletion of *otsA*

To further analyze the general phenotype of *Rhizobium* sp. NGRΩ*otsA*, it was tested, if the ability to grow on different carbon sources is affected. The deletion of *otsA* did not affect the ability to use trehalose, sucrose or succinate as a carbon source. It was the first time to demonstrate that *Rhizobium* sp. NGR234 is able to grow on trehalose as sole carbon source.

Table 4. *Rhizobium* sp. NGR234 and *Rhizobium* sp. NGRΩ*otsA* grown in liquid RMM-medium at 27°C with the in the table stated carbon sources added.

Strain	Carbon source	OD_{600} values (after 38h)
NGR234	no carbon source, control	0.09
NGRΩ*otsA*	no carbon source, control	0.09
NGR234	succinate	0.25
NGRΩ*otsA*	succinate	0.25
no inoculum	succinate	0.00
NGR234	sucrose	2.43
NGRΩ*otsA*	sucrose	2.37
no inoculum	sucrose	0.00
NGR234	trehalose	2.32
NGRΩ*otsA*	trehalose	2.32
no inoculum	trehalose	0.00

3.2.3 Recombinant glutathione-S-transferase tagged rhizobial OtsA is insoluble and inactive

To measure activity and further analyze the special characteristics of rhizobial OtsA, a molecular approach was chosen. Rhizobial *otsA* was tagged with glutathione-S-transferase (GST) and expressed in *Escherichia coli.* The correctness of the construct was verified by sequencing analysis. In a second step the induction of expression of the fusion protein was tested with

different concentrations of isopropyl-beta-D-thiogalactopyranoside (IPTG). Induction was already detectable at an IPTG concentration of 50 µM (figure 5). The expected size of the fusion protein is 75 kD.

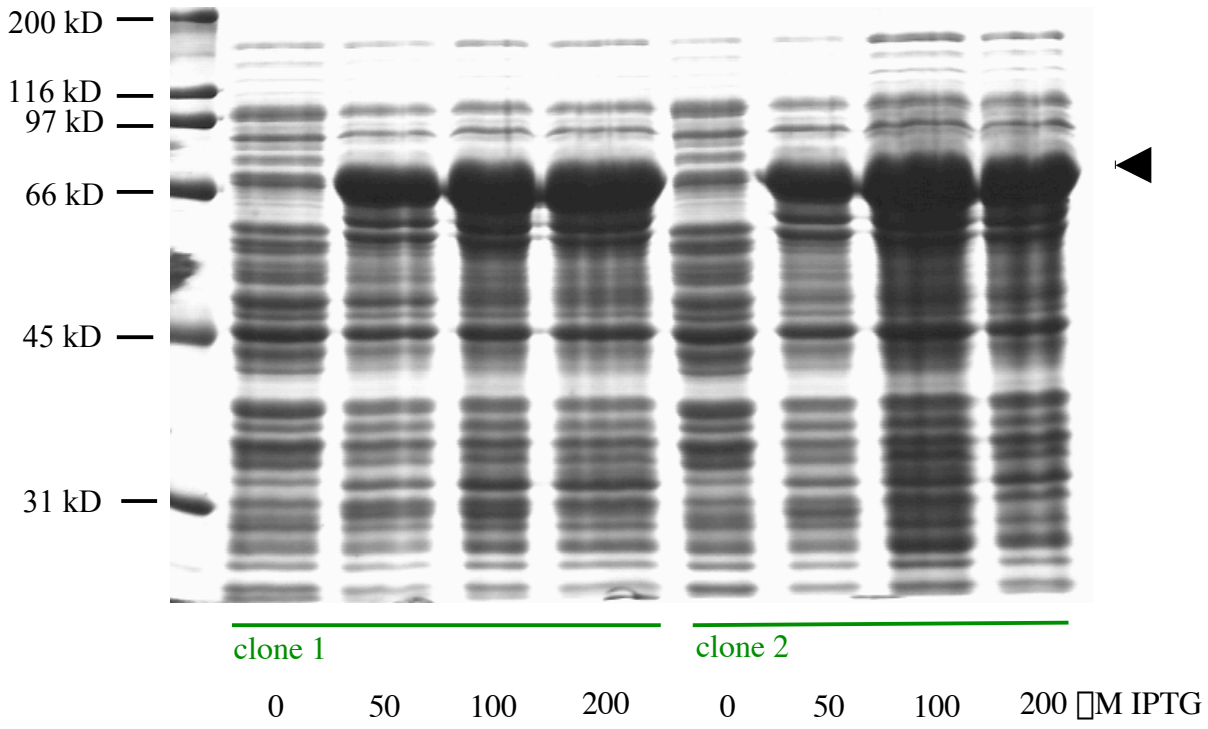


Figure 5: Coomassie stained denaturing polyacrylamide gel electrophoresis of total protein extracts of two *Escherichia coli* clones that possess the recombinant GST-tagged rhizobial OtsA, that were induced with differed concentrations of isopropyl-beta-D-thiogalactopyranoside (IPTG) for 2.5 hours. The arrow is pointing to the induced band.

The recombinant rhizobial OtsA was used for enzyme assays. Activity tests with raw cell-extracts and with isolated protein that had been isolated on a glutathione sepharose column did not reveal any activity. Therefore the solubility of the recombinant protein was tested. The protein extract was resuspended in native conditions, centrifuged and supernatant and pellet were analyzed separately on a denaturing polyacrylamide gel (figure 6). This experiment revealed that the bulk of the recombinant rhizobial OtsA is located in the pellet and therefore insoluble. Attempts to increase the solubility of the protein and thereafter measure activity of the recombinant rhizobial

OtsA failed. These assays included experiments with different salt concentrations and different concentrations of detergent in the extraction buffer. In addition in one experiment the temperature to grow the *Escherichia coli* cultures had been lowered from 37°C to 27°C. In summary the recombinant GST-tagged rhizobial OtsA is insoluble and inactive. Thus tagging rhizobial OtsA with GST followed by expression in *Escherichia coli* is not suitable to measure its activity.

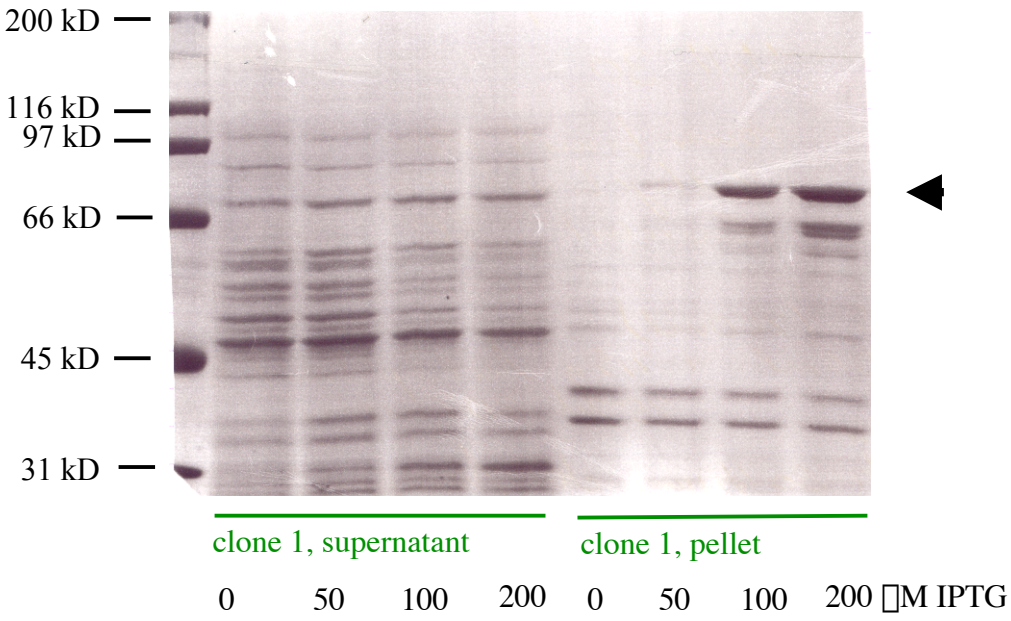


Figure 6: Coomassie stained denaturing polyacrylamide gel electrophoresis different fractions of total protein extracts of two *Escherichia coli* clones that possess the recombinant GST-tagged rhizobial OtsA. They were induced with differed concentrations of isopropyl-beta-D-thiogalactopyranoside (IPTG) for 2.5 hours. The arrow is pointing to the induced band.

3. 3. Symbiotic properties of *Rhizobium* sp. NGRΩ*otsA*

In the previous part of this work, we have demonstrated that rhizobia exhibit a functional OtsA/B pathway to synthesize trehalose via trehalose-6-phophate. In table 3 we show that the trehalose

accumulation is impaired in low oxygen growth conditions when *otsA* of *Rhizobium* sp. NGR234 is mutated. Since the accumulation of trehalose in salt stress was not affected by the mutation of *otsA,* and earlier studies have demonstrated that *Rhizobium* sp. NGR234 exhibit protein activity of a second pathway the MOTS – pathway (Streeter and Bhagwat, 1999), it is suggested that *Rhizobium* sp. NGR234 contains at least two independent pathways for trehalose biosynthesis: the OtsA/B – pathway and the MOTS – pathway. The finding's that in rhizobia trehalose synthesis is triggered by hypoxia, lead to the hypothesis that the same type of trehalose synthesis might be involved in the nitrogen fixing symbiosis of rhizobia and legumes. It is well established that the environment in active nodules is characterized by reduced internal O_2 tension, enabling activity of the oxygen sensitive nitrogenase. In addition many symbiotic genes are only expressed in anoxic conditions, like the *nif* genes coding for the nitrogenase complex (Hoover, 2000). Here we test the impact of the deletion of rhizobial *otsA* on the symbiosis.

3.3.1 Deletion of rhizobial *otsA* affects nodulation parameters

To test the symbiotic properties of *Rhizobium* sp. NGR *otsA* in comparison to the wild type strain various host plants, namely *Vigna unguiculata*, *Macroptilium atropurpureum* and *Psophocarpus tetetragonolobus* were infected with *Rhizobium* sp. NGR234 and its isogenic *otsA* deletion mutant. The mutant was able to colonize all hosts, but in most cases the nodulation behavior showed a clear phenotype. In a number of hosts, average nodule size was reduced, nodule number was increased and nitrogen fixation was reduced compared to control plants infected with the wild type strain *Rhizobium* sp. NGR234. Analysis of the carbohydrate content of these nodules revealed significant increases in the levels of sucrose, hexoses and starch (table 5, section 4.3.3-4.3.6). In *Vigna* and *Psophocarpu*s the deletion of *otsA* affected nodulation parameters: a strong increase in nodule number (up to a duplication) in combination with reduced average nodule size was detected, while the total nodule mass did not change as compared to control plants infected with the wild-type strain. In the case of *Psophocarpu*s this change in nodule development occurred only after three month but not in the young 6 weeks old symbiosis. In *Vigna* a change in nodule development was already detected four weeks after infection and the differences between wild-type and the *otsA* deletion mutant increased with time. This indicates a

role of the symbiotic plasmid encoded *otsA* in the negative regulation of nodule development in a late stage of symbiosis. At the time-point that was tested in *Macroptilium* no significant change in nodule development was detected.

Table 5. Nodule number and fresh weight (fw) in nodules of various host plants. Plants were infected with *Rhizobium* sp. NGR234 and its isogenic *otsA* deletion mutant (*Rhizobium* sp. NGR□*otsA)* and harvested on different time points that are stated in the table. Mean values and SE correspond to five independent plants.

Host plant, age of symbiosis	**Rhizobial Strain**	**Total number of nodules per plant**	**Total fw (g) of nodules per plant**	**Average fw (mg) per nodule**
Vigna unguiculata	NGR234	39 ±7	0.4 ±0.1	9.8 ±0.7
4 weeks	NGR□*otsA*	52 ±7*	0.4 ±0.1	7.4 ±0.7*
	NGR234	245 ±15	2.6 ±0.4	11.6 ±0.9
7 weeks	NGR□*otsA*	335 ±12*	3.1 ±0.2	8.5 ±0.7*
Macroptilium atropurpureum	NGR234	161 ±50	1.1 ±0.3	7.4 ±1.7
7 weeks	NGR□*otsA*	129 ±26	1.0 ±0.3	8.8 ±3.7
Psophocarpus tetetragonolobus	NGR234	26 ±7	2.1 ±0.5	86 ±7
6 weeks	NGR□*otsA*	29 ±3	1.4 ±0.4	47 ±7*
	NGR234	58 ±10	5.0 ±0.2	92 ±16
3 months	NGR□*otsA*	110 ±3**	4.9 ±0.8	44 ± 6*

*, $p < 0.05$; **, $p < 0.01$

3.3.2 Electron micrographic studies on “small” and “normal” nodules revealed no differences on the structural level

To analyze whether reduced sized nodules formed by *Rhizobium* sp. NGR *otsA* exhibit any structural abnormalities electron micrographic study were performed (figure 7, figure 8). Seven weeks old *Vigna unguiculata* nodules inoculated with *Rhizobium* sp. NGR234 or *Rhizobium* sp. NGR *otsA* respectively were embedded directly after harvest and later cut for the electron

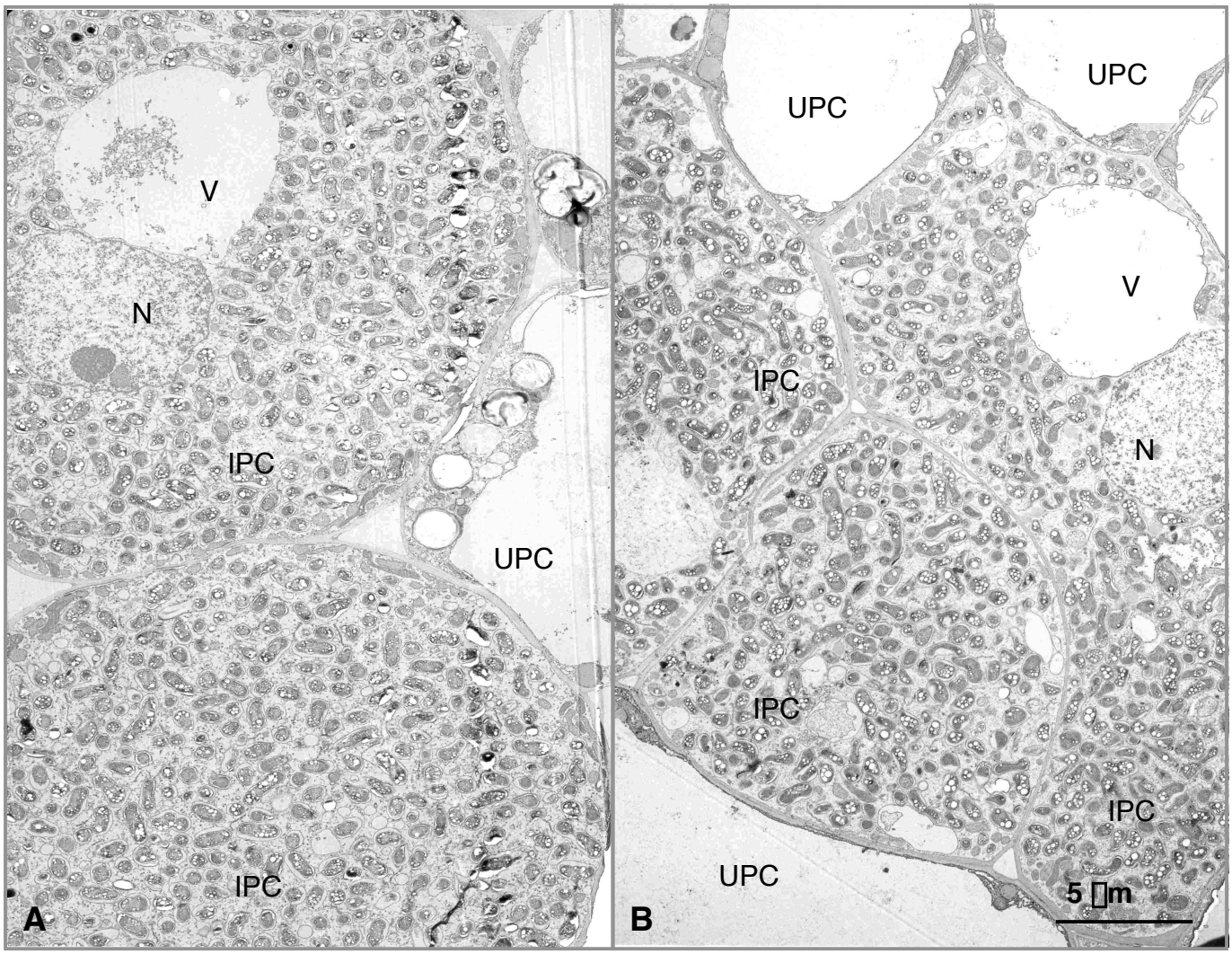


Figure 7: Electron micrographic study of 7 weeks old *Vigna unguiculata* nodules inoculated with *Rhizobium* sp. NGR234 (A) and *Rhizobium* sp. NGR *otsA* (B). Nucleus (N) and vacuole (V) of infected plant cells (IPC) and uninfected plant cells (UPC) are indicated in the image. Significant

cytological differences were not observed between normal sized nodules formed by wild-type rhizobium and the reduced sized nodules formed by *otsA* deficient mutant strain. The status of both infected plant cell (IPC) and uninfected plant cell (UPC) was independent from the genotype of the microsymbiont. On the subcellular level symbiotic structures are fully developed in both nodule types (figure 8). Concentrations of granules of □-hydroxybuturate (PHB) are similar in both nodule types. Moreover, the structure of bacteroids (B) that are surrounded by peribacteroid membranes (PBM) and the symbiosome membranes (S) is not affected by the deletion of rhizobial *otsA*. Thus the structure of the nodules is not affected by the reduced nodule size caused by the deletion of *otsA*.

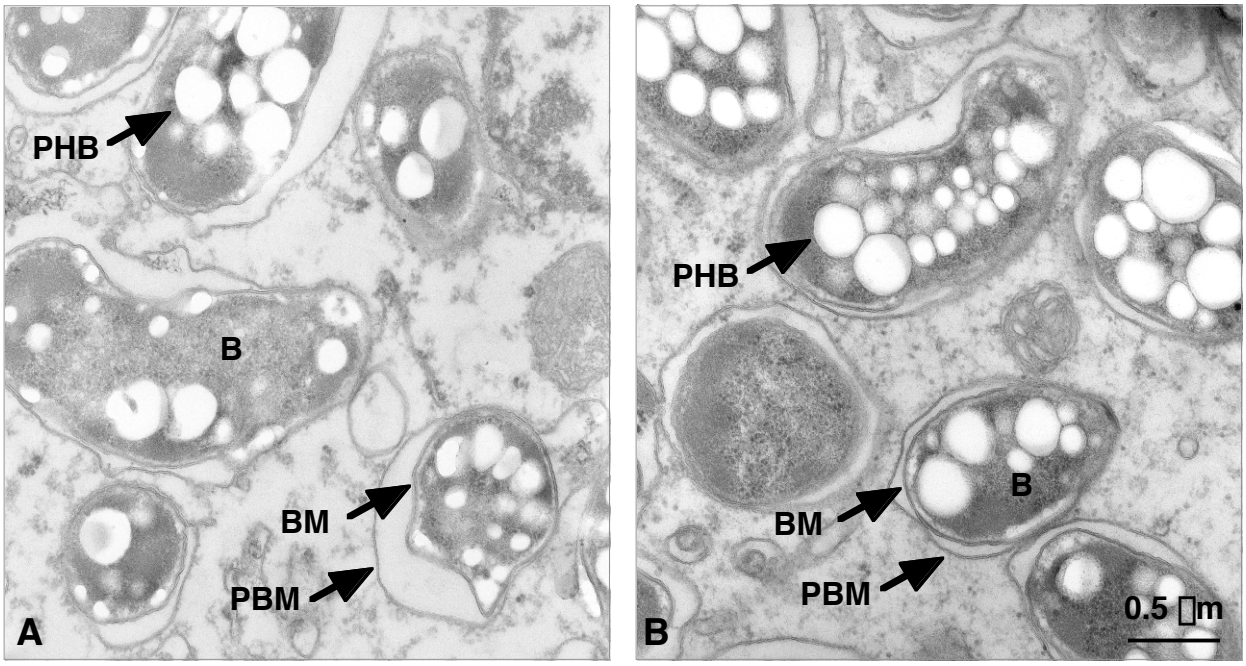


Figure 8: Electron micrographic study of 7 weeks old *Vigna unguiculata* nodules inoculated with NGR234 (A) and NGR□ *otsA* (B). Sections of infected plant cells. Granules of □-hydroxybuturate (PHB) are accumulated in bacteroids (B), which are surrounded by the plant derived peribacteroid membranes (PBM) and the microsymbiont derived bacteroid membranes (BM).

3.3.3 *Pachyrhizus tuberosus* becomes nodulating when infected with *Rhizobium* sp. NGRΩ*otsA*

To further study the capacity of rhizobial *otsA* to negatively regulate root nodule development, an additional plant was examined for its nodulation capacity. *Pachyrhizus tuberosus* is a poor nodulating host of *Rhizobium* sp. NGR234. Here *Pachyrhizus tuberosus* seedlings were inoculated with *Rhizobium* sp. NGR234 and its isogenic *otsA* deletion mutant (*Rhizobium* sp. NGR *otsA*). Five weeks after infection all plants that were inoculated with the wild type strain of *Rhizobium* sp. NGR234 did not show any nodule. In contrast plants that were inoculated with the *otsA* deficient rhizobium strain were nodulated (table 7, figure 9). These plants even possessed low nitrogenase activity (Acetyleenreduction Assay, ARA). Thus the ability of *otsA* to influence root nodule development in a negative way was demonstrated again (compare also table 3).

Table 7. Nodulation capacity of *Rhizobium* sp. NGR234 and its isogenic *otsA* deletion mutant (*Rhizobium* sp. NGR *otsA)* on *Pachyrhizus tuberosus*. Mean values and SD correspond to five independent plants.

Host plant, age of symbiosis	Rhizobial Strain	Total number of nodules per plant	ARA (nkat/g fw nodule)
Pachyrhizus tuberosus			
5 weeks	NGR234	0 ±0	0 ±0
	NGR *otsA*	2.6 ±0.6*	0.02±0.01

*, p< 0,05

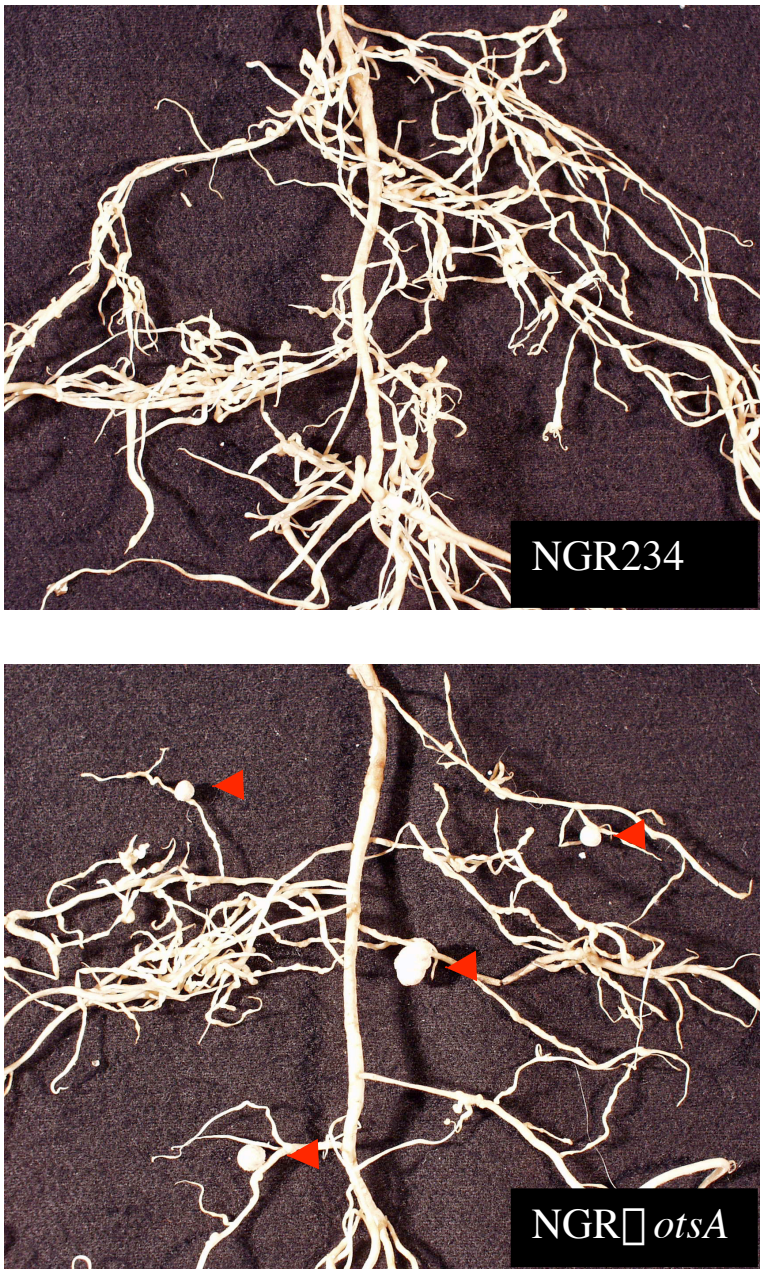


Figure 9. Roots system of *Pachyrhizus tuberosus* five weeks after infection with *Rhizobium* sp. NGR234 or *Rhizobium* sp. NGR *otsA* respectively. Nodules are indicated with red arrows.

3.3.4 Deletion of rhizobial *otsA* affects nitrogenase activity

The deletion of rhizobial *otsA* did not only affect the root nodule development but did also alter nitrogenase activity in some hosts. *Vigna unguiculata*, *Macroptilium atropurpureum* and *Psophocarpus tetetragonolobus* have been infected with *Rhizobium* sp. NGR234 and *Rhizobium* sp. NGR *otsA* respectively. Nitrogenase activity (Acetylene Reduction Assay, ARA) at the

Table 8. Nitrogenase activity (ARA) in nodules of various host plants. Plants were infected with *Rhizobium* sp. NGR234 and its isogenic *otsA* deletion mutant. Mean values and SE correspond to fife independent plants.

Host plant, age of symbiosis	*Rhizobial Strain*	**ARA (nkat/g fw nodule)**
Vigna unguiculata	NGR234	1.4 ±0.7
4 weeks	NGR *otsA*	1.0 ±0.5
	NGR234	3.1 ±0.3
7 weeks	NGR *otsA*	3.1 ±0.2
Macroptilium atropurpureum	NGR234	2.1 ±0.1
7 weeks	NGR *otsA*	0.6 ±0.2*
Psophocarpus tetetragonolobus	NGR234	0.2 ±0.1
6 weeks	NGR *otsA*	0.9 ±0.4*
	NGR234	0.50 ±0.11
3 months	NGR *otsA*	0.06 ±0.01**

*, p< 0.05; **, p <0.01

timepoint of harvest was affected by the deletion of rhizobial *otsA* when *Macroptilium* and *Psophocarpus* served as a host but not in combination with *Vigna* (table 8). Over the time of the experiments no difference in the green leaf color was observed when the plants were infected with *Rhizobium* sp. NGR234 or its isogenic *otsA* deletion mutant. Thus the host plants did not suffer a severe nitrogen starvation even when nitrogenase activity at the timepoint of the harvest was changed.

3.3.5 Deletion of otsA affects sucrose, hexose and starch contents in nodules of various legumes

Previous experiments have shown that an artificial alteration of trehalose levels by the addition of trehalase inhibitor can influence sucrose and starch pools in soybean and cowpea nodules (Müller *et al.*, 1995). To tests the impact of *otsA* on the level of sucrose, hexoses and starch *Vigna unguiculata* (V.), *Macroptilium atropurpureum* (M.), *Psophocarpus tetetragonolobus* (P.) and *Leucaena leucocephala* (L.) were infected with *Rhizobium* sp. NGR234 and NGR *otsA* (figure 10 and figure 11). Analysis of carbohydrates in nodules infected by NGR *otsA* reveals significant increases of sucrose, hexoses and starch. The starch pools increased in all hosts and the increase was significant in *Macroptilium*, *Vigna* and *Psophocarpus*. In *Vigna* the starch pool even reached about three times the size of the starch pool that was measured in the nodules inoculated with the wild type rhizobium (figure 10). In nodules inoculated NGR *otsA* the pool of sucrose and hexoses increased in all four hosts. This increase was significant in *Vigna* nodules 7 week after inoculation, in *Psophocarpus* nodules 3 month after infection but not in 6 weeks old nodules and in the tree legume *Leucaena* in 6 month old nodules but not in the 3 month old symbiosis. In 7 weeks old *Macroptilium* nodules only the sucrose pool and not the pool of hexoses increased significantly when compared with wild type nodules. Just like it was the case in the nodule development the later the stage of symbiosis the stronger the effect of the deletion of the rhizobial *otsA* on carbon partitioning. Trehalose itself was below the detection limit in these growth phases.

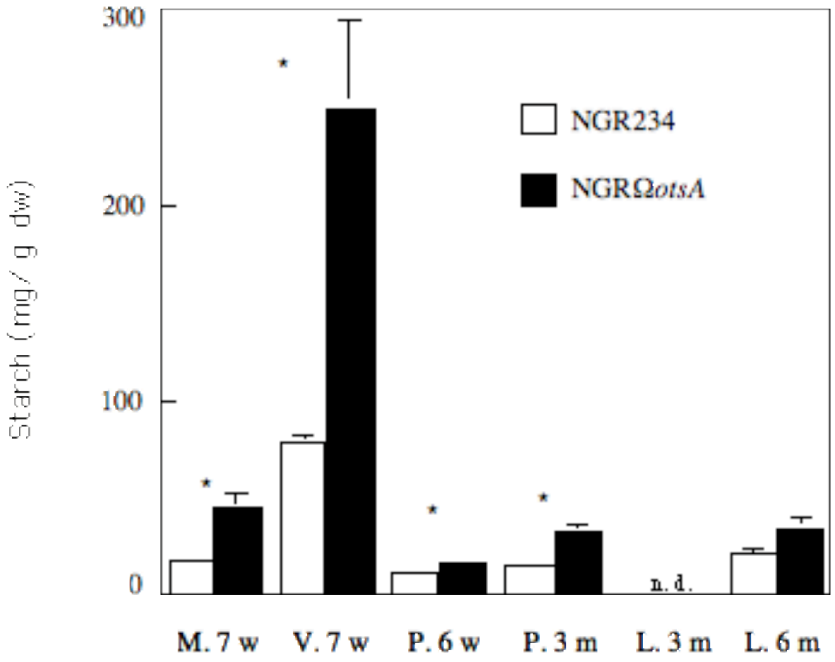


Figure 10: Starch contents in nodules of various legumes colonized by *Rhizobium* sp. NGR234 and its isogenic *otsA* deletion mutant (*Rhizobium* sp. NGR *otsA*). Mean values and SE correspond to five independent plants. Capital letters correspond to the host plant: *Macroptilium* (M.), *Vigna* (V.), *Psophocarpus* (P.), *Leucaena* (L.); small letters correspond to the age of symbiosis: week (w); month (m); *, p< 0,05

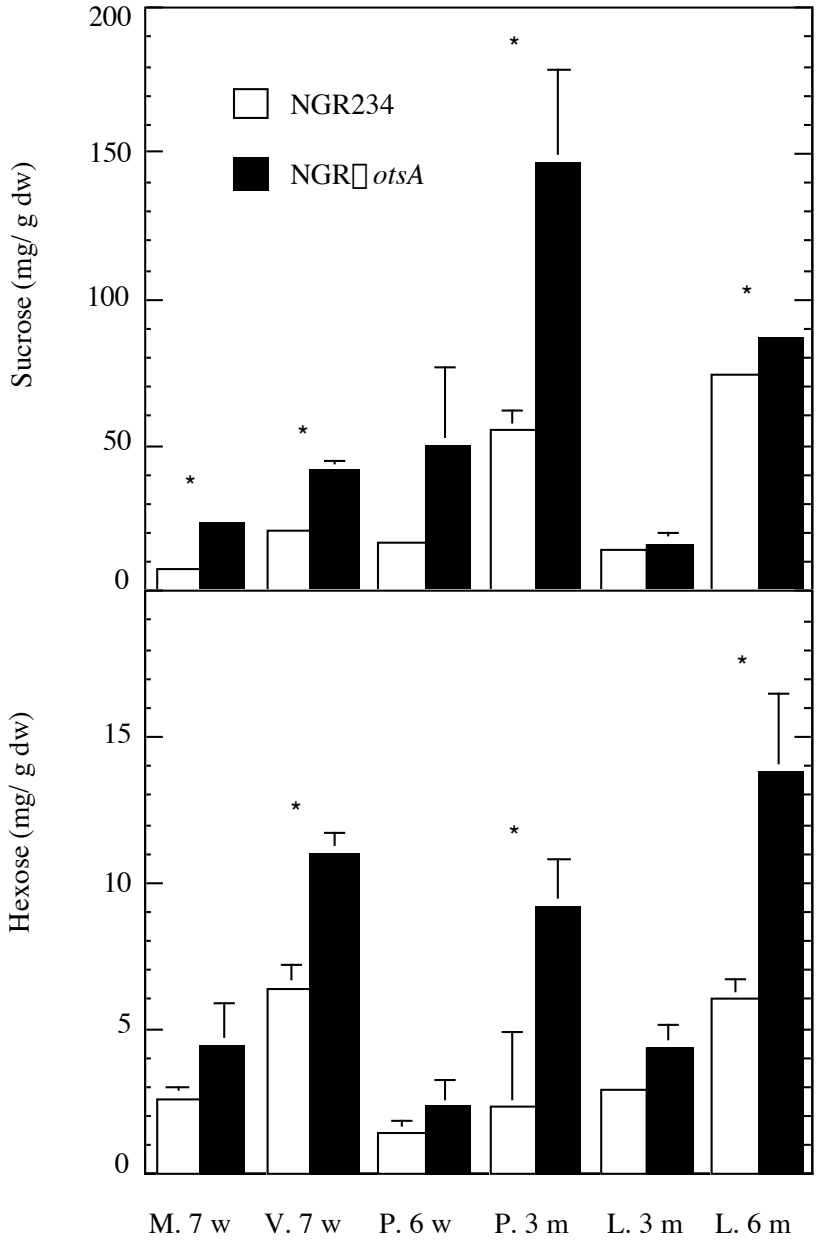


Figure 11. Sucrose and hexose contents in nodules of various legumes colonized by *Rhizobium* sp. NGR234 and its isogenic *otsA* deletion mutant (*Rhizobium* sp. NGR□ *otsA*). Mean values and SE correspond to five independent plants. Capital letters correspond to the host plant: *Macroptilium* (M.), *Vigna* (V.), *Psophocarpus* (P.), *Leucaena* (L.); small letters correspond to the age of symbiosis: week (w); month (m); *, p< 0,05

3.3.6 *Vigna unguiculata* plants infected with *Rhizobium* sp. NGRΩ*otsA* are delayed in the onset of flowering and fruiting

To study if the massive change in nodule sugar pools result in a changed ability to compete with other sink tissues for photosynthates, the flowering and fruiting capacity of *Vigna unguiculata* was examined (figure 12, table 9). Deletion of *otsA* in the microsymbiont not only increased sucrose, hexose and starch contents in nodules of *Vigna* but also delayed the onset of flowering and fruiting in 10 weeks old plants. The delayed flowering and fruiting phenotypes is supported by the decline of nitrogen fixation (ARA) in these plants. It is well established that flowering and fruiting can inhibit nitrogen fixation and leads to nodule senescence.

Figure 12. *Vigna unguiculata* 10 weeks after inoculation with *Rhizobium* sp. NGR234 and NGR□ *otsA*. White arrows are pointing to pods.

Table 9. Dry weight (dw) of shoots, number (No) of flowers, siliques and seats and nitrogenase activity (ARA) of *Vigna unguiculata*. Plants were harvested 10 week after infection with *Rhizobium* sp. NGR234 and its isogenic *otsA* deletion mutant. Mean values and SE correspond to four independent plants.

Vigna unguiculata	**inoculated with: *Rhizobium* sp. NGR234**	**inoculated with: *Rhizobium* sp. NGR *otsA***
shoot (dw, g)	3.57 ± 0.36	2.78 ± 1.46
flowers (No)	1.75 ± 1.2 *	0.2 ± 0.45 *
pods (dw, g)	0.7 ± 0.18 **	0.04 ± 0.05 **
pods (No)	2.75 ± 2.1 *	0.4 ± 0.55 *
seeds (No)	25.5 ± 5.1 *	2.8 ± 0.26 *
ARA	0.06 ± 0.07	0.29 ± 0.08

*, p< 0,05; **, p <0,001

3.3.7 *otsA* does not affect survival rates of rhizobia from nodules in reisolation experiments

In soybean nodules trehalose becomes the most abundant non-structural carbohydrate during senescence (Müller *et al*, 2001). In addition trehalose is thought to be a protectant during stress conditions such as drought and heat. In senescent nodules the structure of the plant cell is lysing and it is an ongoing debate if the bacteroids decay as well or if they redifferentiate again to free living rhizobia. Thus the conditions in senescent nodules are potentially characterized by high stress. Here we performed an electron micrographic study on senescent nodules (10 weeks old) of *Vigna unguiculata* inoculated with *Rhizobium* sp. NGR234 (figure 13). We observed a strong decay of the plant cytoplasm (figure 13A) and a decay of most granules of □-hydroxybuturate within bacteroids (figure 13B). But the membranes of the bacteroids were still fully intact. Thus the developmental stage of the bacteroids differs strongly from active nitrogen fixing nodules.

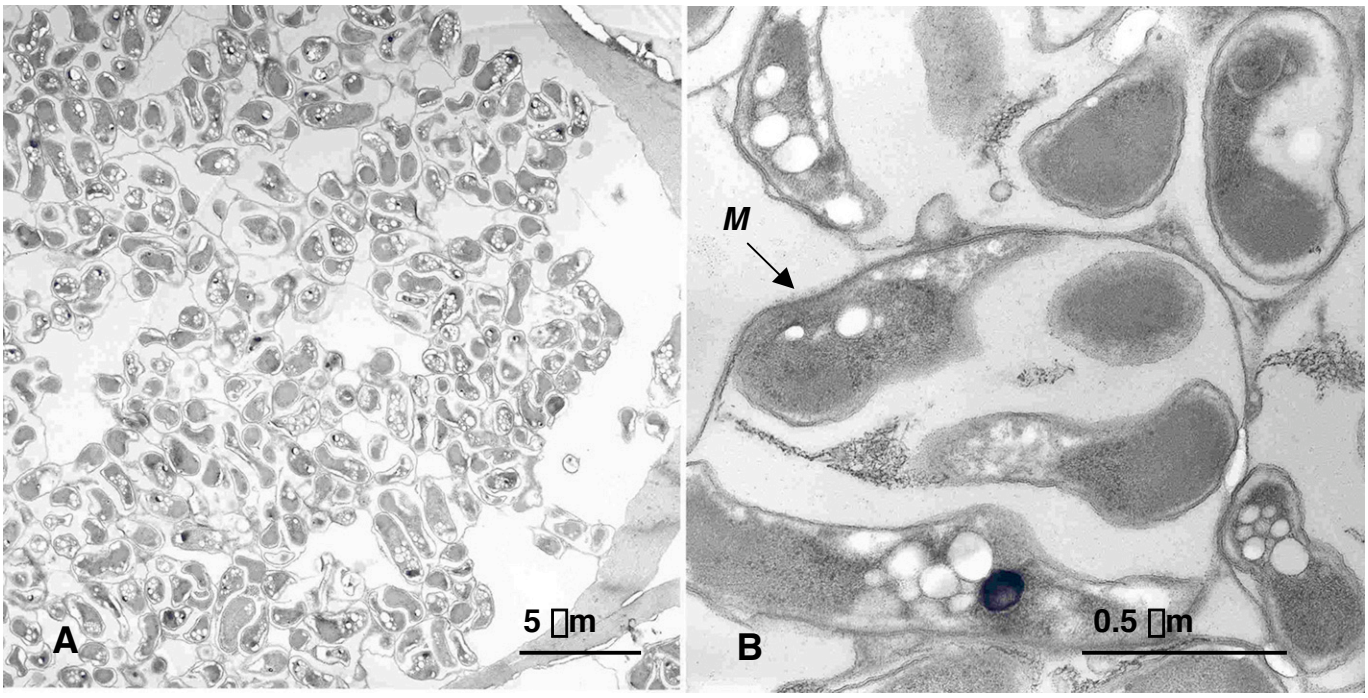


Figure 13: Electron micrographic study of an infected plant cell (A) and a bacteroid (B) that are derived from 10 weeks old *Vigna unguiculata* senescent nodules inoculated with *Rhizobium* sp. NGR234. The membrane (M) of the bacteroid is indicated.

To test the impact of trehalose on the survival rates of rhizobia from nodules of different developmental stages, reisolation experiment have been performed. These experiments included both, - young and senescent nodules. *Vigna unguiculata* nodules were collected five to ten weeks (senescent) after infection from several different planting experiments and directly subjected to the reisolation procedure. Media for dilution and enrichment have either been supplemented with an additional 200mM mannitol to osmotically stabilize them or not. Reisolation rates varied between 1.5 – 6 x 10^{9} CFU/g fresh weight of nodules (CFU, Colony Forming Unites) and were not affected by the deletion of *otsA*, nodule age (young or senescent) or the osmotic conditions during the isolation. Thus deletion of rhizobial *otsA* affects root nodule development, nodulation capacity, carbon partitioning, nitrogenase activity and the onset of flowering and fruiting. All these phenotypes concern the active stages of the symbiosis. Beyond this point at the senescent stage of the symbiosis, we could not detect any role of rhizobial *otsA* in stress protection during the process of reisolation.

4. Discussion

The symbiosis of rhizobia and legumes contributes 30 % to the total biological nitrogen fixation that is essential to the global nitrogen cycle, a major natural cycle. A key aspect of nodule symbiosis is the exchange of plant carbon against fixed nitrogen. In this respect it is interesting to consider the role of sugars and in particular trehalose and its biosynthesis. Trehalose is widespread in nature, but is normally not present in higher plants. However in plant-microbe interactions, trehalose is regularly found in plant roots interacting with antagonistic fungi, mycorrhizal fungi, and in nitrogen-fixing root nodules, probably as a microbial substance. The impact of trehalose on plant metabolism and its role in nitrogen fixing symbiosis is unclear. As one step on the way to solve these questions, - this work approaches for the first time the molecular basis of trehalose synthesis by a microsymbiont in plant microbe interactions. The focus was put on the OtsA/B pathway. In this pathway trehalose is synthesized from UDP-glucose and glucose-6-phosphate by the action of trehalose-6-phosphate synthase (OtsA) and trehalose-6-phosphate phosphatase (OtsB).

The first part of this work is an analysis of all available sequence data of OtsA-like ORF in rhizobia and we propose here that the "OtsA-like ORF" of *Mesorhizobium loti* is not functional since it is lacking one third of the overall sequence including parts of the predicted active center. For further analysis out of the remaining three known OtsA-like ORF in rhizobia, one was chosen for an Ω-cassette insertion mutagenis. *Rhizobium* sp. NGR234 was taken as a model organism for two reasons. Firstly, *Rhizobium* sp. NGR234 is providing a good tool for molecular analysis since its symbiotic plasmid pNGR234*a* is the first symbiotic plasmid that has fully been sequenced, and it is carrying homologues of the OtsA/B trehalose biosynthesis pathway (Freiberg *et al.*, 1997). Secondly, the fast-growing *Rhizobium* sp. NGR234 exhibits the broadest host-range of any known rhizobium, permitting both - studies on important crop species as well as ecological and evolutionary studies on a vast variety of legumes (Pueppke and Broughton, 1999). The Ω-cassette insertion mutagenis resulted in *Rhizobium* sp. NGRΩ*otsA* that lacks the capability to synthesize trehalose under microaerobic growth conditions. This is a first molecular – though still indirect but nevertheless important - evidence for the functionality of the OtsA/B pathway in rhizobiacea.

In a second part, the impact of *Rhizobium* sp. NGRΩ*otsA* on the symbiosis was studied. Here it is reported that in a number of hosts, average nodule size was reduced, nodule number was increased (up to 30 %) and nitrogen fixation was reduced compared to control plants infected with the wildtype strain NGR234. Analysis of the carbohydrate content of these nodules revealed significant increases in the levels of sucrose, hexoses and starch.

4.1 Trehalose pathways and regulation in free-living rhizobia

4.1.1 The OtsA/B-pathway in rhizobia

Despite a series of physiological studies of trehalose in free living or symbiotic rhizobia, data on trehalose synthesizing pathways in rhizobia remain rare and were up to now limited on non-genetic approaches. Consequently the current work was addressing the genetic basis of trehalose synthesis in rhizobia. So far in rhizobia there are first indications for the existence of two trehalose synthesizing pathways - the OtsA/B-pathway and the MOTS–pathway. The OtsA/B-pathway forms, as already stated, trehalose from UDP-glucose and glucose-6-phophate, whereas the MOTS–pathway catalyses the convention of oligo/polymaltodextrines/glycogen into trehalose in a two-step reaction by maltooligosyl-trehalose synthase and maltooligosyltrehalose trehalohydrolase.

In rhizobia the existence of the OtsA/B-pathway has been demonstrated only indirectly on a biochemical level and so far only in slow growing rhizobia: In *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* the synthesis of trehalose from UDP-glucose and glucose-6-P has been reported, which is likely to be catalyzed by the OtsA/B-pathway, since this pathway is the only pathway that has been described to perform such a conversion (Salminen and Streeter, 1986). In parallel homologues of *otsA* and *otsB* in rhizobia were discovered in the course of various sequencing projects and were described in *Bradyrhizobium japonicum*, on the symbiotic plasmid of *Rhizobium* sp. NGR234 (pNGR234*a*) (Freiberg *et al.*, 1997) and in *Mesorhizobium loti*. Homologues of *otsA* but not of *otsB* were also detected on the symbiotic plasmid of

Sinorhizobium meliloti (pSymA) (http://www.ncbi.nlm.nih.gov/Entrez/). So far in rhizobia the functionality of these homologues has not been shown in any case.

The first step to analyze these four rhizobial OtsA-like ORF was a sequence comparison with functional protein sequences of trehalose-6-phophate synthases and we propose here that one of these OtsA-like ORF is not functional. Our alignment analysis revealed that the OtsA-like ORF of rhizobia can vary in the highly conserved regions and the active centre of this protein.

In the case of the OtsA-like ORF of *Mesorhizobium loti* we even propose that this ORF does not function as *otsA* but potentially encodes for a different glycosyltransferase for the following reasons: It is missing 43% of the average overall sequence length of functional OtsA and is thereby missing 33% of the active center and highly conserved regions of OtsA in *Escherichia coli*. The missing region includes three of four amino acids that are directly involved in the binding of the substrate glucose-6-phophate. However the OtsA-like ORF of *Mesorhizobium loti* is highly conserved in the region around amino acids corresponding to Wable and Grishin's "glycogen phophorylase glycosyltransferase" motif position 1 and 2 (Wable and Grishin, 2001). The glycosyltransferase superfamily that is characterized by this motif includes trehalose-6-phophate synthases, but also numerous other glycosyltransferases ranging from mannosyltranseferase in *Escherichia coli* to human muscle glycogen synthase. Thus the "OtsA-like ORF" of *Mesorhizobium loti* might belong to this glycosyltransferase superfamily but possess a different protein activity than the one of trehalose-6-phophate synthase.

The remaining three rhizobial OtsA-like ORFs exhibit 14% variations within the active center of OtsA of *Escherichia coli* and conserved regions of functional trehalose-6-phophate synthases. Since *Sinorhizobium meliloti* and *Rhizobium* sp. NGR234 are nearly 100% identical within the highly conserved regions, it is likely that the variations in comparison to active center and highly conserved regions of functional OtsA are not due to sequencing errors, but can be explained by a different evolution of this region. The variations of rhizobial sequences are nearly all situated at the same positions. These positions often belong to highly conserved loci, which lie in the exposed surface regions of the protein and are thought to be involved in the binding of potential regulators. Thus it is not likely that the rhizobial variations cause a different

protein activity but that these regions are rather involved in the binding of potentially different regulators. Consistently in section 4.1.2 of this work it is proposed on the basis of our phenotypic results that the OtsA/B-pathway - at least in *Rhizobium* sp. NGR234 – might be regulated by low oxygen, which represents a novel mode of induction of *otsA*.

Since previous efforts to obtain transposon insertion mutants of rhizobia unable to produce trehalose had failed (Streeter and Bhagwat, 1999), a targeted approach was chosen in this work to disrupt trehalose synthesis via the OtsA/B-pathway. In *Rhizobium* sp. NGR234 an Ω-cassette insertion mutagenesis of OtsA-like ORF was performed revealing *Rhizobium* sp. NGRΩ*otsA*. The phenotype of this mutant has been tested in growth conditions that induce trehalose accumulation in some fast growing rhizobia and slow growing rhizobia. Some fast growing rhizobia accumulate trehalose in response to salt stress (Breedveld *et al.*, 1993) and in microaerobic culture conditions (1% oxygen) (Hoelzle and Streeter, 1990). Slow growing rhizobia constitutively accumulate trehalose. Based on these findings trehalose accumulation was tested in *Rhizobium* sp. NGR234 and *Rhizobium* sp. NGRΩ*otsA* upon atmospheric, microaerobic or high salt (0.2 M NaCl) culture conditions. Just like all other previously tested fast growing rhizobia and in contrast to the slow growing bradyrhizobia, we demonstrate that *Rhizobium* sp. NGR234 and sp. NGRΩ*otsA* also do not accumulate trehalose under atmospheric exponential growth conditions. Thus these culture conditions represent an appropriate negative control for trehalose accumulation in *Rhizobium* sp. NGR234. Microaerobic or high salt culture conditions were both tested positive in the wildtype of *Rhizobium* sp. NGR234 for the first time for trehalose accumulation und could thus act as a positive control. The deletion of the rhizobial *otsA*-homologue impaired trehalose synthesis under microaerobic growth conditions. This is a very strong indication that trehalose synthesis induced in hypoxic milieu is directed by the symbiotic plasmid encoded *otsA*-homologue. Taken together with previous biochemical results these first molecular - however still indirect - results they are very strong indications for the functionality of *otsA* in rhizobia and in □-proteobacterium in general. For the surely missing direct prove the consequent deletion of the second gen of the pathway *otsB* was disapproved, since phosphatase is regularly complement by other unspecific phosphatase. The attempt to complement a trehalose-6-phosphate synthase minus mutant in yeast failed, since the yeast stain that was lacking one of its stress protection system was, at least in our hands, impossible to transform. Our last approach to

directly prove the functionality of rhizobial *otsA* was the attempt to measure activity of trehalose-6-phosphate synthase *in vitro*. For this a recombinant glutathione-S-transferase tagged rhizobial OtsA had been constructed. This recombinant protein was shown to be insoluble and inactive. Other expression systems might serve as a more suitable tools for activity measurements.

4.1.2 Regulation of the potential rhizobial *otsA* by low oxygen

Parallel to these indirect evidence of the functionality of *otsA* in rhizobia, the induction of *otsA* by low oxygen conditions would represents a novel mode of induction of *otsA*. So far *otsA* has only been described to be induced by osmotic stresses leading to osmotic trehalose synthesis ("ots"). In addition, in yeast *tps1* a homologue of *otsA* is induced by heat shock. Here it is proposed that *otsA* can also be induced by other environmental stimuli than stresses. In active nodules the environment is characterized by reduced internal O_2 tension, enabling activity of the oxygen sensitive enzyme nitrogenase. Hoelzle and Streeter (1990) have demonstrated trehalose accumulation in four different fast growing rhizobia cultured under low oxygen. Now the list can be extended to *Rhizobium* sp. NGR234. A very low free oxygen concentration in the Rhizobium-legume symbiosis has been recognized as the regulatory trigger for the expression of many essential symbiotic genes, like the nitrogen fixation *nif*-genes (Hoover, 2000). These genes are mediated by *nifA* an alternative sigma factor (σ^{54}) dependent regulator. Interestingly a homologue of a σ^{54} dependent helix-turn-helix regulator is situated upstream of *otsA*, indicating that the OtsA/B-pathway might be regulated by a similar mechanism. Taking this into account, it is intriguing to speculate that the rhizobial OtsA/B-pathway is symbiosis specific: In fast growing rhizobia trehalose is accumulated upon low oxygen stimuli that reflect the environmental condition of the nodules. This accumulation is directed by the OtsA/B-pathway, which is situated on the symbiotic plasmid and is therefore acquired during the evolution of the symbiosis. Therefore *otsA* and *otsB* are most probably not essential house keeping genes of free-living bacteria. Parallel to the symbiotic plasmid encoded OtsA/B-pathway, *Rhizobium* sp. NGR234 must exhibit a second trehalose-synthesizing pathway for trehalose accumulation triggered by high salt.

4.1.3 Trehalose accumulation in high salt and the MOTS–pathway

Under high salt culture conditions, the deletion of rhizobial *otsA* did not affect trehalose synthesis. Therefore, it is likely that *Rhizobium* sp. NGR234 possesses a second trehalose-synthesizing pathway. Streeter and Bhagwat (1999) have analyzed a variety of rhizobia for their ability to synthesize trehalose from maltooligosaccharides and reported that this activity is present in most of them including *Rhizobium* sp. NGR234. In these experiments trehalose synthesis from maltooligosaccharides is induced by high osmotic potential. This osmotic potential is induced by the addition of 100mM gluconate. Therefore, these two experiments cannot be directly correlated. However, high salt also provides high potential. Even though salt and organic solutes often operate by different mechanisms, here it is suggested that high salt and organic solutes both induce trehalose synthesis via the MOTS – pathway since the *otsA* pathway is not involved in high salt induced trehalose synthesis. The ability to cope with osmotic stresses might not only be essential for rhizobia as a free-living saprophyte in the soil but also in a symbiotic relationship with leguminous plants since all steps of plant microbe interaction may be influenced by osmotic pressure (Østerås *et al*., 1998). *Rhizobium* sp. NGR234 provides the first model to study the OtsA/B-pathway, triggered by low oxygen trehalose accumulation and the MOTS – pathway, that supposedly is induced by high salt trehalose accumulation in one system. Are both pathways involved in the development of the symbiosis or is the MOTS – pathway specific to the free–living state of rhizobia? A double mutant would be a suitable tool to investigate these topics. For this reason a co-operation with John Streeter (Ohio State University, USA) has been initiated and *Rhizobium* sp. NGRΩ*otsA* has been made available to him.

To test the hypothesis that the low oxygen regulated OtsA/B-pathway is symbiosis specific, the impact of the deletion of *otsA* on the symbiosis was tested.

4.2 The impact of *Rhizobium* sp. NGR□*otsA* on the symbiosis

The symbiotic properties of *Rhizobium* sp. NGRΩ*otsA* were tested on various legumes. Although all plants could be colonized by the mutant, the phenotype of the symbiosis was strongly changes in comparison to the wild type symbiosis. In a number of hosts, average nodule size was reduced, nodule number was increased and nitrogenase activity was reduced compared to control plants infected with the wildtype strain NGR234. Analysis of carbohydrate contents of these nodules revealed significant increases in the levels of sucrose, hexoses and starch. In addition *Vigna unguiculata* plants infected with *Rhizobium* sp. NGRΩ*otsA* were delayed in the onset of flowering and fruiting. Most of these changes were independent of the host plant. Therefore the mutation of the *otsA*-homologue has general impact on the symbiosis. This is consistent with the finding that *otsA* is induced by lox oxygen, which reflect the environmental conditions inside the nodules. It is tempting to speculate that the OtsA/B-pathway in rhizobia is symbiosis specific under natural circumstances and might not be essential for rhizobia as free-living saprophytes in the soil. This hypothesis is underlined by the fact that *otsA* and *otsB* are located on the symbiotic plasmid and have been gained only during the evolution of the symbiosis.

In this comparative study between the wildtype phenotype and mutant phenotype a general involvement and importance of the OtsA/B-pathway in the symbiosis is proposed. Assuming that the OtsA/B-pathway will prove functional in rhizobia this study can give insights into the contexts in which the OtsA/B-pathway could be involved in the symbiosis. But it cannot be specified in which case the OtsA/B-pathway would be directly involved in phenotypic changes, or in which case only secondary. This is demonstrated in figure 1. A mutation of rhizobial *otsA* leads to a loss of the trehalose-6-phophate synthase that in turn leads to a depletion of trehalose-6-phosphate and trehalose. All these molecules are in a cellular compartment different from the compartments, where most measured effects on the carbon pools in the nodules occurred. Trehalose-6-phophate synthase, trehalose-6-phosphate and trehalose are located in the microsymbiotic compartment (bacteroid) and are divided from the plant cytoplasm by the microsymbiont derived bacteriod membrane and the plant derived peribacteroid membrane. If trehalose-6-phophate synthase, trehalose-6-phosphate or trehalose are transported across this barriers to provoke changes in carbon pools or if the depletion of these molecules leads indirectly

to the changes is not yet clear. However, our results are in a line with evidence of previous physiological studies and permit a more detailed interpretation. It also has to be taken into consideration that the mutant phenotype had an effect on the plant trehalose synthesis. But since we know very little about plant trehalose synthesis and expression patterns this aspect is highly speculative.

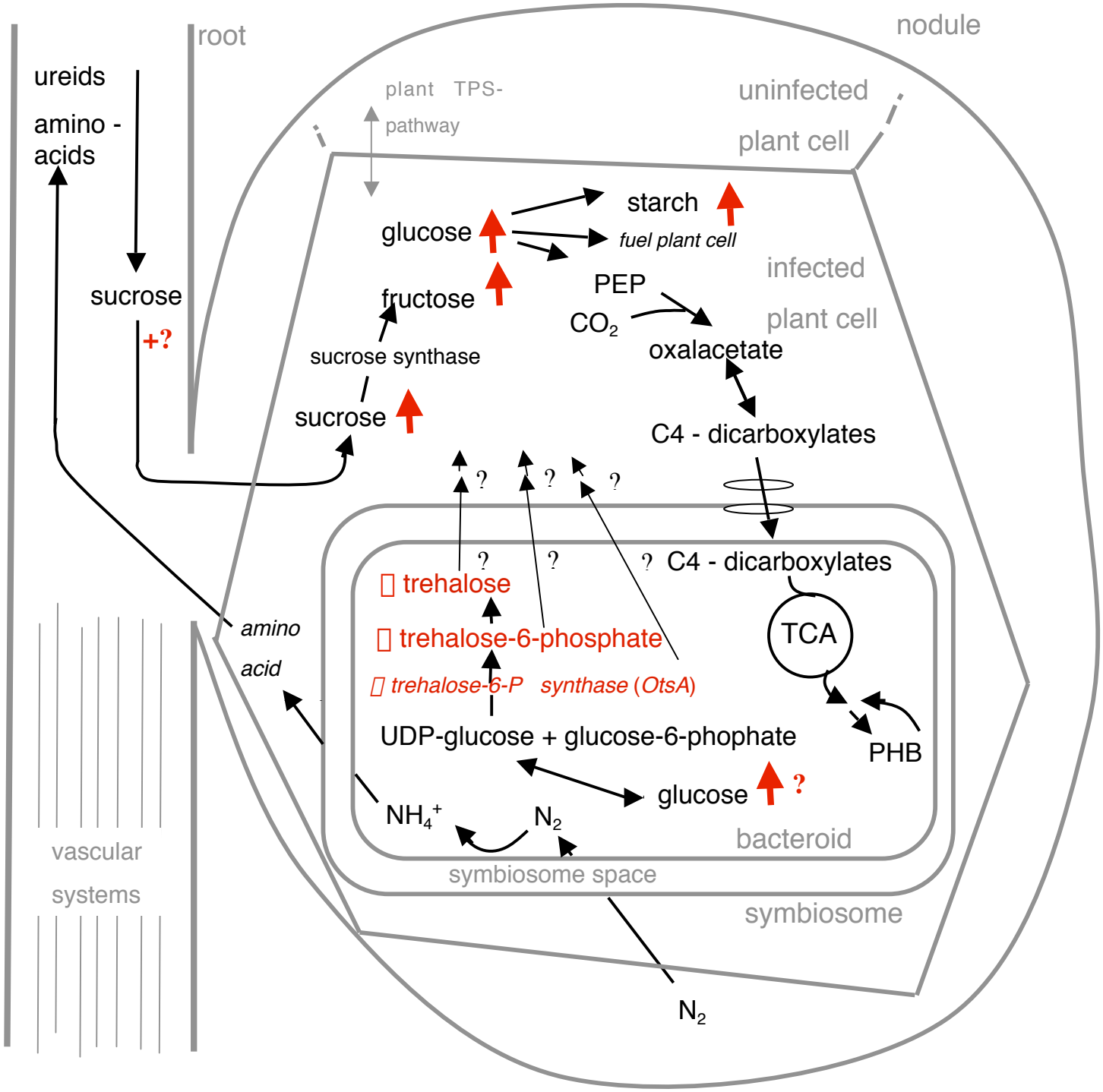


Figure 1: Scheme of the effects (marked in red) upon the deletion of rhizobial *OtsA-like ORF* on the nodule metabolism. Red arrows indicate an increase of the corresponding compound. PEP (phophoenolpyruvate); TCA-cycle; PHB (polyhydroxybutyrate)

4.2.1 Trehalose or trehalose-6-phosphate as signal molecules in carbon allocation

For a long time trehalose or trehalose-6-phosphate have been proposed to act as a regulator of sugar sensing and partitioning in plants for several reasons. Trehalose and sucrose share not only similar structures but also their main metabolic pathways: trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase have their counterparts in sucrose-P-synthase and sucrose-P-phosphatase and trehalase is analogue to invertase. Consequentially trehalose could mimic sucrose in certain regulatory circumstances. In addition, yeast mutants that lack a functional *tps*-gene are sensitive to added glucose due to an uncontrolled influx of glucose into glycolysis. Trehalose-6-phosphate is able to inhibit hexokinase activity *in vitro* (Blázquez *et al*., 1993) and possibly controls the influx of glucose by inhibiting hexokinase *in vivo* (Thevelein and Hohmann, 1995). Since hexokinase acts as a sugar sensor in yeast and possibly also in plants, it was suggested that trehalose-6-phosphate might also to be involved in the regulation of plant metabolism (Goddijn and Smeekens, 1998). In fact studies on transgenic tobacco expressing *otsA* and *otsB* from *E. coli* showed an altered photosynthetic capacity (Paul *et al*., 2001). In addition, Eastmond *et al*. (2002) reported that an *Arabidopsis* mutant with an insertion in the *tps1* gene is impaired in the embryo maturation in the phase of storage reserve accumulation. Parallel to these effects of trehalose-6-phosphate on the carbon metabolism additional, developmental alterations have been described in transgenic plants expressing *E. coli otsA* or yeast *tps1* (Goddijn *et al*., 1997). But the precise mechanisms of the action of trehalose-6-phosphate remains largely unresolved (Wingler, 2002). Trehalose-6-phosphate might be targeted by an endogenous plant 14-3-3 protein. It was extracted from cauliflower and binds *in vitro* to trehalose-6-phosphate synthase (Moorhead *et al*., 1999). In *Arabidopsis* 14-3-3 proteins have been described to act as negative regulators in starch biosynthesis (Sehnke *et al*., 2001). Just as trehalose-6-phosphate, trehalose has been discussed to act as a regulator of sugar sensing and partitioning in plants. In barley, externally supplied trehalose induces the activity of sucrose:fructan-6-fructosyltransferase, an enzyme of fructan biosynthesis (Müller *et al*., 2000). Feeding trehalose to *Arabidopsis* seedlings strongly induced starch biosynthesis in cotyledons and leaves (Wingler *et al*., 2000). In addition, a starch biosynthetic mutant of *Arabidopsis* was complemented when fed with trehalose (Fritzius *et al*., 2001). Another line of evidence for the potential role of trehalose

or trehalose-6-phosphate as endogenous signaling molecule in higher plants is the discovery of genes for trehalose synthesis in *Arabidopsis* and in a range of crop plants. In plant-microbe interactions two trehalose metabolic pathways exists side by side: the one of the plant and the one of the microsymbiont. The resulting intersection between these two systems might represent an important network of signals that determines the carbon flux between the two partners.

For a better understanding of this interplay one of the two systems is likely to be disrupted in the current work. It is the first molecular approach to investigate the impact of trehalose production by the microsymbiont. Here propose that changes in the carbon flux to the nodules are linked to the rhizobial OtsA/B-pathway. Deletion of OtsA-like ORF in *Rhizobium* sp. NGR234 affects sucrose, hexose and starch contents in nodules of *Vigna unguiculata*, *Macroptilium atropurpureum*, *Psophocarpus tetragonolobus* and *Leucaena leucocephala.* Sucrose, hexose and starch pools are highly increased and can reach up to three times of the pool size in nodules that were infected with the wildtype. This results fit in a line of evidences that trehalose or trehalose-6-phosphate could act as a regulator of carbon allocation into the nodules. In previous work it has been shown that the addition of a trehalase inhibitor to soybean and cowpea nodules causes an increase in trehalose and a decrease in sucrose and starch pools (Müller *et al.*, 1995). Soybean nodules with naturally occurring high levels of trehalose have significantly higher levels of sucrose synthase and alkaline invertase and significantly lower levels of sucrose than nodules with low levels of trehalose (Müller *et al.*, 1997).

There are three possible ways of interpreting these data. Several authors suggested that trehalose from the microsymbiont is a microbial contamination and toxic for the plant metabolism due to interference with the endogenous plant trehalose metabolism. In this concept a nodule enhanced and plant derived trehalase “detoxifies” the nodules from trehalose (Mellor, 1992; Wingler, 2002). Our results do not fit into that concept unless the “detoxification” would be rather leaky. If trehalase effectively removes all microbial trehalose there should be no trehalose left to interfere with the endogenous plant trehalose metabolism. But in this case the disruption of rhizobial *otsA* should not have had any effect on the plant metabolism. In addition, in that concept one would expect trehalase to be substrate induced. This is not the case for nodules: there trehalase is constitutively expressed and not induced by its substrate (Müller *et al.*, 1995).

Here, it is suggested that microbial trehalose is not a contaminant to the plant partner but rather acts as a signal for carbon allocation to the nodules. Our results give strong indications that rhizobial *otsA* is induced by the nodule environment and is most likely symbiosis specific. The counterpart of the rhizobial trehalose the plant-derived trehalase is specifically induced in the nodules as well. Trehalose concentration might be dependent on the concentration of bacteroids in the nodules, since trehalase activity is independent of its substrate. Previously the general ability of trehalose to interfere with the regulation of carbohydrate metabolism was demonstrated. In this study this ability is confirmed since the deletion of trehalose synthesis caused sever shifts in the sugar pools of the nodules. Since trehalose might represent the concentration of bacteroids and in addition, has the ability to interfere with the carbon metabolism, it might be a suitable signal to tune the carbon flux into the nodules in order to minimize the costs of nitrogen fixation. Thus trehalose and trehalase in nodules might interfere with the regulatory circuits of carbon flux to nodules and sink strength. Although nodules infected by the wildtype rhizobium did not accumulate detectable amounts of trehalose at the timepoint of harvest, it is proposed that the concentration of trehalose in nodules is very low, but sufficient to act as a signal. In *Arabidopsis* only after inhibition of trehalase, trehalose synthesis can be detected (Vogel *et al.*, 2001).

There is a third aspect that has to be taken into account, when interpreting the data of changed sugar pools in nodules. The deletion of OtsA-like ORF has changed nitrogenase activity in some hosts at the timepoint of harvest. Since nodules are a powerful sink for carbohydrates and, when nitrogenase activity is low, carbohydrates tend to accumulate - presumably from lack of consumption. This explanation works for some of the tested host but not for others – *Vigna unguiculata* in particular. Thus it is questionable if this is a sufficient explanation. In addition, it is improbable that the nitrogen fixation is impaired during a long period of the symbiosis, since the plants infected with the mutant did not show any symptoms of nitrogen starvation. Changes of nitrogenase activity in certain hosts are probably temporary and secondary effects, due to a different physiology of the nodules. In contrast changes of sugar pools were constant and independent of the host plant or the timepoint of harvest.

4.2.2 Plant systemic effects due to the deletion of the microbial OtsA-like ORF

Vigna unguiculata plants infected with *Rhizobium* sp. NGRΩ*otsA* are delayed in the onset of flowering and fruiting. Thus the deletion had a systemic effect on the plant partner. Flowers and nodules are both sink organs. With the removal of rhizobial trehalose the competiveness of nodules for carbohydrates became possibly superior to the sink strength of flowers. Enlarged sugar pools in the nodules infected with *Rhizobium* sp. NGRΩotsA support this theory. In *Arabidopsis* the induction of flowering is not only mediated by light and temperature but also by the availability of carbon compounds (Corbesier *et al*., 2002). Thus we suggest that the lack of trehalose in the nodules leads to an uncontrolled metabolic flux into the nodules that delays the flowering and fruiting of the host plant. Once flowers and fruits are developed the nodules senescent and do not exhibit nitrogenase activity any more. But in the plants infected with *Rhizobium* sp. NGRΩotsA nitrogenase is still active. It does not seem to be a general change in the development of the whole plant, since the dry weight of the shoots are the same.

4.2.3 The impact of rhizobial *otsA* on nodule development

A number of transgenic plants expressing the trehalose biosynthetic pathway are severely disturbed in growth and development even if only minute amounts of trehalose are formed in the transgenic plant (reviewed by Goddijn and Van Dun, 1999). Here we demonstrate that in a number of different hosts, nodule number is increased (up to 30%) and average nodule size is reduced when the rhizobial OtsA-like ORF is mutated. But the total nodule mass per plant did not change. The nodule architecture did not show any abnormal structures on the electron microscopic level, too. In addition, the poor nodulation host *Pachyrhizus tuberosus* becomes nodulating when infected with *Rhizobium* sp. NGRΩotsA. Thus rhizobial OtsA-like ORF has a clear effect on nodule size development and total nodule number regulation and in *Pachyrhizus* it even serves as a negative regulator of nodulation. These effects are supposed to be independent from the changes in sugar pools and nitrogen fixation because *Macroptilium atropurpureum* does not show any changes in nodule development but exhibits the other effects.

4.3 Outlook

In a network of self and non self-signals in plant-microbe interactions both partners have to identify und integrate the signals of the partner in order to manage an effective symbiotic interaction. Trehalose is one component of such a network. It is a fascinating task to further investigate the exact signaling cascades at the intersection between a prokaryote and an eukaryote. Transcriptomic and proteomic investigations might provide expression maps of when and where certain genes are activated. To understand the metabolic fluxes between plant and bacteroids these findings have to be combined with metabolic studies and in the long run metabolomic profiles have to be built up. A better understanding of regulation of carbon flux in nodules could also improve our understanding of nitrogen fixation in grasses that are important crop plants. Nitrogen fixing symbiosis not only plays an exceedingly influential part in natural ecosystems but may also be of great importance in man made biological systems or in the recovery of an increasing area of eroded landscapes. This work has demonstrated that the molecular approach is suitable tool to examine the role of trehalose in plant-microbe interactions. After a start is made further research should continue in this direction.

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Abbreviations

ARA	acetylene reduction assay
bp	base pair
DA	Dalton
20E	20E-medium
EDTA	ethylenedinitro-N,N,N′,N′-tetaacetic acid
EtOH	ethanol
GC	gas chromatography
GST	glutathione-S-transferase
HPLC	high pressure liquid chromatography
IEM	Interdivisional Electron Microscopy
IPTG	isopropyl-beta-D-thiogalactopyranoside
LB	Luria-Bertani medium
MES	2-Morpholinoethanesulfonic acid Monohydrate
OD	optic density
ORF	open reading frames
PAGE	polyacylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
R	resistance
RMM	Rhizobium Minimal Medium
rpm	rounds per minute
SDS	sodium dodecyl sulfate
Tris	tris[hydroxymethyl]aminomethan
U	Unite
v/v	volume per volume
wt	wildtype
w/v	weight per volume

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