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Inositol and Rhizopine Metabolism - Key Pathways for Competitive Host Nodulation in Sinorhizobium Meliloti

Petra R. A. Kohler
Western Michigan University

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INOSITOL AND RHIZOPINE METABOLISM - KEY PATHWAYS FOR COMPETITIVE HOST NODULATION IN SINO RHIZOBIIUM MELILOTI

by

Petra R. A. Kohler

Dissertation
Submitted to the Faculty of The Graduate College in partial fulfillment of the requirements for the Degree of Doctor of Philosophy Department of Biological Sciences Advisor: Silvia Rossbach. Ph.D.

Western Michigan University Kalamazoo, Michigan June 2011
INOSITOL AND RHIZOPINE METABOLISM - KEY PATHWAYS FOR COMPETITIVE HOST NODULATION IN
SINORHIZOBIUM MELILOTI

Petra R. A. Kohler, Ph.D.
Western Michigan University, 2011

The rhizopine concept suggests that rhizopines, which are inositol derivatives, function as nutritional mediators and play an important role in host-bacteria interactions. This thesis analyzes the hypothesis that not only rhizopines but also naturally occurring inositol isomers, such as myo-, D-chiro-, and scyllo -inositol, function as nutritional mediators in the Sinorhizobium meliloti - alfalfa nitrogen-fixing symbiosis. The idhA-encoded myo-inositol dehydrogenase is responsible for the first step in the degradation of myo- and D-chiro-inositol. A different dehydrogenase, IolY, was found to be essential for growth with scyllo-inositol. The iolA and iolCDEB genes were required for the further degradation of myo-, D-chiro- and scyllo-inositol. The idhA and iolA genes are monocistronically transcribed, while the iolYRCDEB genes comprise an operon. The gene product of iolR, an RpiR-like repressor, negatively regulates iol gene expression in the absence of inositol. IolR was shown to recognize a conserved binding motif (5'-GGAA_{5,11}TTCC-3') in the promoter regions of the idhA and iolY genes. Two additional IolR-binding motifs
were confirmed within the *iolYRCDEB* operon, upstream of *iolR* and *iolC*, indicating that the operon contains two internal operators and that IolR has autoregulatory function. IolR-mediated repression was antagonized by a pathway intermediate, probably 2-keto-5-deoxy-gluconic acid 6-phosphate. The *iolA* gene was not regulated by IolR, but constitutively expressed. The *iolA* gene product does not seem to be part of the central inositol catabolic pathway but rather a point of intersection of different metabolic pathways, including valine catabolism. The inositol derivative scyllo-inosamine (SIA) has been proposed to be a rhizopine. We used chemically synthesized SIA to confirm its proposed structure. The same genes essential for the degradation of crude rhizopine isolated from root nodules, namely the *idhA* and *mocABCR* genes were also found to be required for the utilization of the chemically synthesized SIA. In plant-bacteria interactions, mutants with insertions in the *idhA*, *iolA*, the individual *iolYRCDE* and *mocACR* genes could not compete with the wild type in a nodule occupancy assay on alfalfa plants. In conclusion, this work strongly supports the hypothesis that rhizopine and inositol metabolism are important nutritional and signaling factors in the *S. meliloti* – alfalfa symbiosis.
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Petra R. A. Kohler
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INTRODUCTION

Inositol – structure and occurrences

Inositol, or hexahydroxycyclohexane, is a simple sugar alcohol and occurs in nine different stereoisomers (Fig. 1.1) (2). The major naturally occurring isomers are myo-, d/-chiro-, scyllo- and neo-inositol, of which the myo-form is the most abundant (Fig. 1.1). The epi, cis, muco and allo isomers are synthetic inositols (Fig. 1.1) (2).

![Inositol Isomers](image)

Figure 1.1: Stereoisomers of inositol.
Inositols play important structural and signaling roles in mammalian systems. D-chiro-inositol (Fig. 1.1) is the product of epimerization of the C₁ hydroxyl group of myo-inositol. The myo- and D-chiro-isomers have been shown to be components of two different inositol phosphoglycan (IPGs) molecules in mammalian systems (64, 102). These IPGs are cell membrane associated and serve as putative insulin second messengers (93). In response to insulin, IPGs are released from glycosylphosphatidylinositols in the cell membranes into the cytoplasm, where they affect some of the enzymes implicated in the activity of insulin. The insulin mimetic effect of IPGs has been widely documented (87). Therefore, myo-inositol and D-chiro-inositol have great potential for the development of type II diabetes therapy (59, 102). All six hydroxyl groups of scyllo-inositol are oriented in equatorial position (Fig. 1.1). Mammalian tissues contain approximately ten-fold less scyllo-inositol than myo-inositol (2). In contrast to myo- and D-chiro-inositol, the scyllo-isomeric form does not seem to be metabolized by the mammalian system and might have a function in nervous tissues: scyllo-inositol seems to prevent the formation of amyloid beta oligomers that are the main constituent of amyloid plaques in the brains of Alzheimer's disease patients and therefore has great potential in Alzheimer's therapy (20, 26, 89).
Inositol in plants and rhizosphere soils

myo-Inositol is also commonly found in plants, where it is involved in general structural and signaling functions common to eukaryotic cells and also in the transport and storage of the plant hormone auxin (53). In addition, plants use myo-inositol for phosphate storage by converting it into inositol hexakisphosphate (myo-IP₆), also known as phytic acid (free acid form) or phytate (salt of phytic acid) (Fig. 1.2) (90). Phytic acid can comprise up to 100% of the total inositol phosphate content in important crops like legumes and cereals (45). Inositols are introduced into the environment mainly in the phosphorylated myo-isomeric form through animal and plant waste; inositol phosphates are a major player in the phosphate cycle of terrestrial and freshwater ecosystems (90).

Figure 1.2: Structure of phytic acid, inositolhexakisphosphate, and the corresponding phytate anion, that forms salts with environmentally available cations (mainly sodium).
The soil directly adjacent to plant roots is commonly referred to as the rhizosphere. Plants exude a great variety of compounds derived from their primary and secondary metabolism into the rhizosphere. This complex mixture of organic compounds presents a reservoir of nutrient sources for microorganisms that is far greater than can be found in nutrient-limited bulk soil (12). Hence microorganisms are mainly associated with the rhizosphere, because this specialized area satisfies their nutritional needs (91). In addition, plant roots provide a “comfortable” environment for microbes by attracting water, loosening up the soil and buffering pH and temperature changes (12). Inositols are mainly exuded into the rhizosphere as phytic acid, which is subjected to dephosphorylation and epimerization by indigenous microorganisms, especially *Pseudomonas* spp. (17, 18, 41, 42, 90). Inositol is an ideal carbon and energy source for microorganisms based on its chemical properties. The catabolism of *myo*-inositol has been studied in a variety of microorganisms, including some members of the *Firmicutes* (low-CG Gram positives) (46, 96, 103), *Enterobacteriaceae* (10, 11, 47, 85) and *Rhizobiaceae* (43, 70).

The *Rhizobiaceae* are a remarkable family of α-proteobacteria because many genera engage in negative or positive interactions with eukaryotic hosts. The human pathogens among the *Rhizobiaceae* are represented by *Brucella* spp. that cause brucellosis in humans and animals. This disease is difficult to diagnose and treat, because *Brucella* spp. are intracellular pathogens that invade and inhabit macrophages to avoid immune response (1). These zoonotic pathogens seem to share
evolutionary roots with plant associated *Rhizobiaceae*, agrobacteria and rhizobia (68). The agrobacteria are prominent plant pathogens that induce tumors on plant roots or at the junction between the root and the shoot. The virulence of agrobacteria depends on the presence of a tumor-inducing plasmid. A DNA segment (T-DNA) from the plasmid is introduced into the host cell via conjugative transfer and integrates into the host genome. The expression of the T-DNA in an infected host cells results in uncontrolled cell proliferation and tumor development. The infected tissue produces amino acid derivatives and other opines in high concentrations, which are consumed by the agrobacteria, which remain extracellular (for review see (15)).

The plant symbiotic genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Bradyrhizobium* induce root nodules on legumes, which they inhabit. Here they fix nitrogen for the host plant in exchange for C4 dicarboxylates (succinate, malate) and glucose (15). Interestingly, the development of the symbiotic relationship seems to be positively influenced by the presence of myo-inositol (72). It is important to note that the nitrogen-fixing symbiosis is not limited to the rhizobia and their legume host. A group of β-proteobacteria, known as the β-rhizobia, has been identified that also induce nitrogen-fixing nodules on legume roots (60). In addition, the nitrogen-fixing actinobacterium *Frankia spp.* establishes symbiotic relationships with actinorhizal plants like tropical alder trees (37).
Symbiotic nitrogen fixation

The world’s largest nitrogen reservoir is the atmosphere. Atmospheric nitrogen (N₂) is chemically inert, this means it does not readily react with other atoms or molecules that can be incorporated into living matter (16). The process of nitrogen fixation converts chemically inert nitrogen into more reactive nitrogen species such as ammonia by breaking the very stable N-N triple bond. This process is very energy costly and cannot be carried out by plants. Therefore the availability of nitrogen is a growth-limiting factor. The only organisms known to fix nitrogen are microorganisms (16, 92). The symbiotic association of nitrogen-fixing bacteria with plants generally occurs in multicellular structures known as root nodules (66). The genetic information necessary for the establishment and maintenance of the symbiosis in rhizobia is either located in a symbiotic island on the bacterial chromosome or on one or more large plasmids (32, 44, 84). The legumes possess nodulin genes that enable them to engage in symbiotic relationships (54).

The earliest and most essential events during plant-microbe interaction occur in the rhizosphere and involve a highly complex signal exchange and bacterial chemotaxis towards plant roots (9). Rhizobia possess several polar flagella (49) allowing them to move through the soil to follow a gradient of plant-derived flavonoids. Every plant species sends out a specific cocktail of flavonoids that stimulates the production of the rhizobial Nod-factors, which are species-specific
lipochito-oligosaccharides that initiate the nodulation process and stimulate the division of subepidermal root cells and inner cortex cells that will form the root nodule (33). A prominent example is the *Sinorhizobium meliloti - Medicago spp.* symbiosis. *Medicago spp.* produce mainly luteolin and a mix of other flavonoids that are hydroxylated at the C4 and C7 position. *S. meliloti* responds to this cocktail with the production of a certain sulfated Nod-factor that is received specifically as a signal by *Medicago spp.* (33). Hence a particular host plant can only be infected by certain rhizobial species, however many different strains of the same species are indigenous to the rhizosphere of the host. These strains are competing for host infection and the chance to occupy nodules (95). The rhizobial colonization process, called nodulation, was described by Hadri et al. in detail (38): The dividing cells in the root cortex, called prenodule, trigger the root hairs above to become target sites for the infection. Once the bacteria have reached a root hair tip, the cell exhibits abnormal growth in response to signals produced by the bacteria. This growth results in the entrapment of the rhizobia by curling of the root hair around them, visible as the so-called shepherd’s crook. Localized root hair wall degradation by the rhizobia leads to infection of the host. Inside the root hair the bacteria are contained in an infection thread, a membrane enclosed structure similar to a pollen tube that grows towards the developing nodule. The rhizobia within the infection thread proliferate in a fast manner. As soon as the infection thread fuses with membranes of the nodule cells the rhizobia are released into the cells, still enclosed by the so-called peribacteroid membrane (symbiosome) (38).
Soon the bacteria stop dividing and undergo a remarkable morphological and physiological change. Soupene et al. (1995) described the differentiation of rhizobia into nitrogen-fixing bacteroids (82). This differentiation is triggered by the environmental conditions in the developing nodule. The peribacteroid membrane functions as an oxygen diffusion barrier. Inside the nodule, the oxygen concentration has to be very low, due to the extreme oxygen sensitivity of the nitrogen-fixing enzyme, the nitrogenase. Low oxygen concentration is the major signal controlling the expression of the *nif* and *fix* genes responsible for bacteroid development and nitrogen fixation (82). Newly formed nodule cells are constantly infected with proliferating bacterial cells from the steadily growing infection thread (67, 94).

Legume root nodules have a stem-like anatomy with peripheral vascular bundles and infected cells in the central tissue (36). Two types of legume nodules have been defined: determinate and indeterminate nodules. The indeterminate nodules originate from cell divisions in the inner cortex and have a persistent meristem at their apex (38). Due to the continuous activity of this meristem, the nodule cells form a developmental gradient from the distal meristem to the proximal root attachment site (94). The active meristem allows the indeterminate nodule to constantly increase in size, which results in an elongated shape (33). The determinate nodules derive from cell division in the outer cortex and do not have a persistent meristem. The meristem of this nodule type ceases to divide at an early stage of development. As a result, all the nodule cells are at a rather similar developmental stage (67). The determinate nodule stops increasing in size at some point, and takes on a round shape (33).
Nitrogen fixation is very energy-costly. The rhizobia have to generate large amounts of ATP, and therefore require oxygen for respiration. To satisfy the respiratory needs of the microsymbionts, the plant cells in the nodule accumulate the protein leghemoglobin in their cytoplasm, which binds oxygen with an extremely high affinity (50). Its role is to buffer the oxygen concentration and to control the diffusion of oxygen to the actively respiring bacteroids (4). The combination of the peribacteroid membrane and leghemoglobin leads to an extremely low concentration of free oxygen around the bacteroids, allowing efficient nitrogen fixation. The bacteroids incorporate the nitrogen into glutamate, which they provide for the host plant in exchange for C4 dicarboxylates (succinate, malate) and glucose (33).

Communication is key in every good relationship. This also holds true for symbiotic nitrogen fixation. A constant signal exchange between the macro- and the microsymbiont is required at all stages of the symbiotic relationship. The signaling system is extremely complex and involves a great variety of plant- and bacteria-derived compounds are required for nodulation, including the extracellular proteins and polysaccharides of the rhizobia as well as the reactive oxygen species produced by the hosts defense mechanisms (24). An interesting group of signals are nutritional mediators. These are compounds that probably function as plant-derived signals but also serve as a nutrient source for the rhizobia. Known nutritional mediators are the rhizopines, a unique group of symbiotic compounds that is not produced by the plant itself but by the nitrogen-fixing bacteroids probably from a so far unknown plant-
derived precursor (61, 79, 88). Rhizopines have been isolated from root nodules and are structurally related to the inositols. Their suggested structures are scyllo-inosamine (SIA) and 3-O-methyl-scyllo-inosamine (3-O-MSI) (Fig. 1.3) (61, 88). The rhizopines have been linked to plant-bacteria interactions as well as to rhizobial inositol metabolism (31, 35, 39).

\[
\begin{align*}
\text{scy}llo\text{-inosamine (SIA)} & \quad \text{3-O-methyl-scyllo-inosamine (3-O-MSI)} \\
\end{align*}
\]

Figure 1.3: Structures of the rhizopines: scyllo-inosamine (SIA) and 3-O-methyl-scyllo-inosamine (3-O-MSI).

Inositol metabolism in bacteria has been investigated in a variety of organisms over the last 50 years. The studies have mainly focused on myo-inositol and, although the main pathway and genes involved have been identified, many aspects of the metabolic processes and enzymes involved are not yet understood, including the mechanisms that relate the ability to catabolize a simple sugar alcohol to successful host-bacteria interactions.
Early studies of the inositol catabolism in bacteria focused on myo-inositol and investigated the biochemistry of the catabolic pathway rather than its genetic background. Anderson, Berman and Magasanik investigated the details of the degradation of myo-inositol for the first time in the late 1960s and early 1970s (3, 10, 11). The γ-proteobacterium *Aerobacter aerogenes* (reclassified as *Klebsiella aerogenes*) was used as the model organism in these studies. The authors described the enzymes involved in the degradation of myo-inositol by performing enzyme assays and kinetic studies. They characterized the metabolic intermediates via paper chromatography, UV-VIS spectroscopy, IR spectroscopy, mass spectrometry and elementary analysis. Based on their results, the authors suggested a catabolic pathway that converts myo-inositol in six steps to three final products: dihydroxyacetone phosphate, acetyl-CoA and CO₂ (3, 10, 11). The proposed myo-inositol pathway had some major gaps. They did not fully elucidate the third and fourth step of the pathway, the conversion of 3D-(3,5/5)-trihydroxy-cyclohexane-1,2-dione into 2-deoxy-5-keto D-glucuronic acid (Fig. 1.4 B). Although Anderson, Berman and Magasanik were not able to explain the complete pathway in detail, they established a basis for investigating myo-inositol catabolism in bacteria by characterizing the enzymes and the describing possible intermediates. The investigation of the myo-inositol pathway has progressed during the last three decades. Recent studies have
focused on the biochemical aspects as well as on the genetic background of the pathway.

**Inositol catabolism in Gram-positive bacteria**

*Bacillus subtilis*

The current model organism for inositol catabolism is the Gram-positive soil bacterium *Bacillus subtilis*, which belongs to the *Firmicutes*. The transcriptional organization and regulation of the *iol* genes in *B. subtilis* strain 168 have been investigated and the role of their gene products in *myo*-inositol catabolism has been characterized (30, 97, 101, 103-105). In addition, *B. subtilis* genes involved in the degradation of *D-chiro* and *scyllo*-inositol have been described (57, 102).

Initially, an NAD$^+$ dependent enzyme with a high affinity for *myo*-inositol was identified in *B. subtilis* vegetative cells and characterized as inositol 2-dehydrogenase (EC 1.1.1.18) (30, 73). A *B. subtilis* mutant strain contained *fdpA1a* large deletion and was unable to grow with *myo*-inositol and gluconate as sole carbon sources and lacked fructose-bisphosphate activity (28, 29). Later, the gene coding for the *myo*-inositol dehydrogenase (*idh*) was located in the affected region. The *idh* gene product was overexpressed in *E. coli* and its function as a *myo*-inositol dehydrogenase confirmed (30). The DNA regions up- and downstream of the *idh* gene were systematically sequenced as part of the *Bacillus subtilis* sequencing project by Yoshida et al. (100). Ten open reading frames were identified that could represent
putative inositol catabolism (iol) genes, all transcribed in the same direction as the movement of the replication fork (100).

Initial studies of the inositol catabolism in Bacillus subtilis were conducted by Yoshida et al. (97, 105) and set out to characterize the transcriptional organization of the putative iol genes rather than the involvement of the specific iol gene products in inositol catabolism. Yoshida et al. (1997) found that the iol gene cluster consisted of 10 genes, iolABCDEFGHIJ, with iolG representing the previously identified myo-inositol dehydrogenase (idh) gene (Fig. 1.4 A) (97). An $\sigma^A$-dependent promoter, piol, is located in the 5’ region of iolA and an $\rho$-independent terminator sequence at the 3’ of iolJ, tiol. The iolABCDEFGHIJ operon is transcribed from piol to tiol, resulting in an 11.4 kb mRNA. Two genes, iolR and iolS, are located upstream of the operon. They are orientated divergently to the operon and are transcribed from a $\sigma^A$-dependent promoter, piolRS, to an $\rho$-independent terminator, tiolRS (97).

The gene product of iolR negatively regulates the transcription of the iol divergon in B. subtilis (97, 105). In the absence of myo-inositol, IolR binds to the operator site within the two iol promoter regions and represses the transcription of both operons (105). The IolR protein is a repressor of the DeoR-family of bacterial regulators. Its DNA-binding properties and regulatory function were investigated by Yoshida et al. (105). The purified IolR-His$_6$ bound specifically to piol and piolRS, repressing the transcription of the iol genes and of its own gene, respectively. Nevertheless, IolR had a higher binding affinity to piol. The cis-acting IolR binding
sequences of both promoters are tandem directed repeats consisting of two relatively conserved 11-mer sequences WRAYCAADARD (where D is A, G or T; R is A or G; W is A or T; and Y is C or T) located in the +5 to +17 region of piol and the -19 and -40 of iolRS (105).

The iol divergon is inducible by myo-inositol, but neither myo-inositol 1-monophosphate, myo-inositol or 2-keto-myoinositol antagonized the IolR-mediated transcriptional repression, suggesting that a later pathway intermediate functions as the IolR antagonizing effector (105). The catabolic intermediate 2-deoxy-5-keto-D-gluconic acid 6-phosphate was identified as the inducer that binds IolR and causes the repressor to release from its target operators (103).

Two studies showed that glucose represses the transcription of the iol divergon of Bacillus subtilis via catabolite repression and inducer exclusion even if the iol genes had been induced by myo-inositol (56, 99). The iol genes are subjected to catabolite repression, mediated by CcpA and IolR in the presence of glucose (99). Two iol catabolite-responsive elements (cre) were identified involved in glucose mediated catabolite repression of the iol genes (56). The cre-iiolA element (the prefix “i” indicates a location in the intergenic region) (WTGAAARCGYTTWWN) (N is any nucleotide) involves the nucleotides +86 to +100 and the two thymines (TT) are essential for the function of cre-iiolA. The cre-iiolB element (WTGNAANCGNWWNCW) stretches from the nucleotides +2397 to +2411. Both
cre elements interact with CcpA if complexed with one of the allosteric effectors proteins, P-Ser-HP or P-Ser-Crh, that increase the DNA-binding affinity of CcpA (56).

The elucidation of the suggested *myo*-inositol catabolic pathway in *B. subtilis* was based on *myo*-inositol catabolism in *A. aerogenes* which comprises six enzymatic steps and requires a hypothetical enzyme bound transition state for the conversion of 3D-(3,5/5)-trihydroxy-cyclohexane-1,2-dione into 2-deoxy-5-keto-D-glucuronic acid (IolD and IolB reaction). Investigations of the *myo*-inositol catabolic pathway in *B. subtilis* solved the enigma of the hypothetical enzyme bound transition state and revealed that a total of seven enzymatic steps are required to convert *myo*-inositol to an equimolar mixture of dihydroxyacetone phosphate, acetyl-CoA and CO₂ (Fig. 1.4B) (103). Although the *iolRS* and *iolABCDEFGH* divergon comprises twelve genes (Fig. 1.4 A), only the gene products of *iolA, iolB, iolC, iolD, iolE, iolG (idh)* and *iolJ* are involved in *myo*-inositol catabolism (30, 101, 103). An inositol transporter is encoded by *iolF* and the *iolI* gene product plays a role in D-chiro-inositol catabolism (58, 102, 104). The function of *iolS* and *iolH* needs to be determined. The *iolS* (synonym *yxbF*) gene encodes a putative aldoketo oxidoreductase and *iolH* (synonym *yxdG*) a putative sugar phosphate isomerase (http://genolist.pasteur.fr/SubtiList).

Inositol transport in *B. subtilis* is facilitated by IolF and IolT (Fig. 1.4 B) (58, 104). Initially IolF was considered to be a minor *myo*-inositol transporter, because inactivation of *iolF* caused a weak growth defect on *myo*-inositol and the *iolF* mutant was still capable of *myo*-inositol uptake, albeit reduced (104). A more recent study revealed that IolF has a higher affinity for D-chiro-inositol, thus it seems to be
involved in D-chiro-inositol rather than in myo-inositol transport (58). The major myo-inositol transporter is encoded by the iolT gene, which is located downstream of the iolABCDEFGHJ operon (Fig. 1.4A). The iolT gene is monocitronically transcribed from a σ^A-dependent promoter that contains a cre element and belongs to the IolR regulon (58, 104). IolT has a higher affinity for myo-inositol, but also contributes to D-chiro-inositol transport (58). BLAST analysis revealed that the predicted iolF and iolT gene products belong to the major facilitator superfamily (MFS) of transmembrane transporters.

The inositol catabolic pathway of B. subtilis is summarized in Fig. 1.4B. The NAD^+ dependent myo-inositol dehydrogenase (Idh/IolG) carries out the initial oxidation of myo-inositol [I] to its corresponding ketone, 2-keto-myoinositol [II] (30, 103). In the second step, 2-keto-myoinositol is converted into 3D-(3,5/4)-trihydroxy-cyclohexane-1,2-dione [III] via the iolE encoded 2-keto-myoinositol-dehydratase (101).

The role of the iolA, iolB, iolC, iolD and iolJ gene products in the further degradation of myo-inositol was elucidated by Yoshida et al. (2008) (103). 3D-(3,5/4)-trihydroxy-cyclohexane-1,2-dione [III] is the substrate for IolD, the third enzyme of the pathway, and yields 5-deoxy-D-glucuronic acid [IV]. In the fourth step, 5-deoxy-D-glucuronic acid is isomerized into 2-deoxy-5-keto-D-gluconic acid [V] by IolB. The IolC kinase phosphorylates 2-deoxy-5-keto-D-gluconic acid resulting in 2-deoxy-5-keto-D-gluconic acid 6-phosphate [VI] (KDGP). KDGP is cleaved into dihydroxyacetone
phosphate [VII] and malonic semialdehyde [VIII] as the fifth step by the action of an enzyme Yoshida et al. refer to as IolJ aldolase (103). Finally, malonic semialdehyde is converted into acetyl-CoA [IX] and carbon dioxide by the iolA gene product, the malonic semialdehyde dehydrogenase.

The iolJ and iolA genes have been recently annotated as fbaB and mmsA in B. subtilis based on the functions of their gene products (http://genolist.pasteur.fr/SubtiList) (Fig. 1.4B). The fbaB gene encodes the glycolysis enzyme fructose-1,6-bisphosphate aldolase (http://genolist.pasteur.fr/Subti List), and the mmsA gene encodes a malonic semialdehyde dehydrogenase, which has been shown to be involved in valine metabolism in Pseudomonas spp. (7, 71, 83).

B. subtilis does not only use myo-inositol as sole carbon source but also the D-chiro- and scyllo- isomers (57, 102). D-chiro- and scyllo-inositol are potential drug candidates for type-II-diabetes and Alzheimer’s therapy, respectively (26, 51). Therefore a better understanding of how both compounds are metabolized in any organism is of immediate interest.

In B. subtilis D-chiro-inositol [X] is initially oxidized to 1-keto-chiro-inositol [XI] by the myo-inositol dehydrogenase (Fig. 1.4B). The iolI encoded 1-keto-chiro-inositol isomerase converts 1-keto-chiro-inositol [XI] into 2-keto-myoinositol [II], which is further catabolized by the enzymes of the myo-inositol catabolic pathway.
The initial oxidation of scyllo-inositol requires a different oxidoreductase, because the myo-inositol dehydrogenase cannot use scyllo-inositol as a substrate (73). The enzyme acts on the axial hydroxyl groups of myo- and D-chiro-inositol (19), but all six hydroxyl groups are equatorial in scyllo-inositol (Fig. 1.1). B. subtilis possesses two scyllo-inositol dehydrogenases, encoded by iolX and iolW (57). Nevertheless, only IolX has been shown to be essential for the growth with scyllo-inositol as sole carbon source (57). Both enzymes probably convert scyllo-inositol [XII] into 2-keto-myoinositol [II], which is further broken down to dihydroxyacetone phosphate, acetyl CoA and carbon dioxide (Fig. 1.4B). Interestingly, iolX and iolW are not regulated by IolR (57).

**Clostridium, Lactobacillus and Corynebacterium**

The myo-inositol catabolic genes have been investigated in other Firmicutes, e.g. Lactobacillus casei in the anaerobic human pathogen and Clostridium perfringens. The organization of the iol genes in all three Gram-positive organisms is highly similar to B. subtilis. The genes required for the catabolism and transport of myo-inositol are generally arranged in one cluster that is divergently oriented to the autoregulatory gene iolR, which generally encodes a putative transcriptional regulator of the DeoR family in Gram positives (Fig. 1.5) (46, 96).

The transcriptional regulation of the iol genes has been elucidated in C. perfringens and L. casei. The inositol genes of C. perfringens are part of a gene cluster that consists of 13 genes, 10 of which are predicted to be involved in inositol catabolism
Figure 1.5: Organization of inositol catabolic genes in Gram-positive and -negative bacteria. The model organism for bacterial inositol catabolism is *Bacillus subtilis*. The genes involved were designated *iol A-J* based on their occurrence in the gene cluster. The *iol* genes of other bacteria were identified based on homology to *B. subtilis*. Genes that have been shown to be required for inositol catabolism are shown in grey. Some genes carry different designations in different organisms: genes for *myo*-inositol dehydrogenase: *iolG, idh, idhA*; methyl malonate semialdehyde dehydrogenase: *iolA* and *iolK*; fructose-1,6-bisphosphate aldolase: *iolJ, fbaB, alfI*; putative *scylo*-inositol dehydrogenase in α-proeobacteria: *iolY*.

(Fig. 1.5) (46). The *iol* gene expression is inducible with *myo*-inositol and subject to catabolite repression by glucose. Interestingly, the *iol* genes are positively regulated by the global regulatory two-component system. VirR/VirS that is required for virulence (46). The *iolTABCDG1G2EJK* operon in *L. casei* contains a second methyl malonate semialdehyde dehydrogenase gene (*iolK*) (Fig. 1.5) that is not essential for the growth with *myo*-inositol as sole carbon source; however, the *iolA* gene was found to be required (96). The expression of *iol* genes is negatively regulated by IolR and inducible with *myo*-insitol. The catabolite control protein A (CcpA) protein
recognizes a cre site in the iolT promoter in the presence of glucose, demonstrating that the iol genes of L. casei are subject to glucose catabolite repression (96).

Comparing the inositol catabolic pathways of B. subtilis, C. perfringens and L. casei allows the conclusion that the organization and transcriptional regulation of the iol genes form a conserved module within the Firmicutes (Fig. 1.5).

Inositol catabolism in Gram-negative bacteria

Inositol transport in Klebsiella and Pseudomonas

The transport systems required for the import of myo- and scyllo-inositol have first been described in the γ-proteobacteria Pseudomonas spp. and in Klebsiella aerogenes (previously Aerobacter aerogenes) (for review see (23)). Investigations have focused on the biochemistry of the transport mechanisms.

K. aerogenes uses myo-, but not scyllo-inositol as sole carbon source. Nevertheless, the inositol transporter of K. aerogenes facilitates the import of both isomers (22). The inositol transporter functions as a proton symporter (74). This finding allows the conclusion that the inositol transport system of K. aerogenes belongs to the major facilitator superfamily, which was not described until later. Pseudomonas spp. use myo- and scyllo-inositol as sole carbon source (inositol hexakisphosphate) phytic acid and phosphate source (23, 75). The transport of myo- and scyllo-inositol in Pseudomonas spp. seems to involve more than one protein and a
myo-inositol specific binding protein has been purified and characterized (21, 23). Hence, inositol transport in *Pseudomonas spp.* is very likely facilitated by an ABC transporter that utilizes one or more periplasmic binding proteins.

**Regulation of inositol catabolism in *Caulobacter crescentus***

The transcriptional negative regulator of the *iol* genes in Gram-negative bacteria does not belong to the DeoR family as in Gram-positive bacteria, but is an RpiR-like repressor. The IolR of the α-proteobacterium *Caulobacter crescentus* has been described in connection with myo-inositol catabolism by Boutte et al. (13) (Fig. 1.5). Regulatory proteins of the RpiR family contain an N-terminal helix-turn-helix protein followed by a C-terminal sugar isomerase domain that is predicted to facilitate the binding of the IolR antagonizing effector molecule and while not having any catalytical function (8, 81).

The genes required for growth with myo-inositol as sole carbon source are arranged in two clusters in *C. crescentus* (13). The first cluster contains the *ibpA, iatA* and *iatP* genes, that code for typical components of an ABC transporter. All three genes are transcribed as one unit from *pibpA*. The structural genes required for myo-inositol catabolism and the regulatory gene *iolR* are located within the second cluster. The *iolR* gene is monocistronically transcribed from *pioIR* followed by the *iolCDEBA* operon that is controlled by the *iolC* promoter. The myo-inositol dehydrogenase gene is located upstream of *iolR* and divergently oriented to the other *iol* genes (Fig. 1.5). The promoter regions of *idhA, iolR, iolC* and *ibpA* contain a conserved cis-acting
regulatory motif (GGAA-N6-TTCC) that is required for IolR-mediated repression. An additional IolR-binding motif was identified within the iolB coding region (Fig. 1.5) (13).

Boutte et al. (2008) used computational predictions to identify the conserved IolR-binding motif in the putative regulatory regions of the iol genes in a few other members of the Rhizobiaceae (13): Brucella melitensis, Agrobacterium tumefaciens, Bradyrhizobium japonicum, Mesorhizobium loti and Sinorhizobium meliloti. The authors also showed that the genomic organization of the iol genes is highly conserved in these species and in C. crescentus. This suggests that the myo-inositol transport, regulatory and catabolic genes form a conserved regulon in the α-proteobacteria in general, and especially in the Rhizobiaceae, which represent important pathogenic and symbiotic species (Fig. 1.5).

**Incomplete oxidation of inositol by *Gluconobacter oxydans***

The myo-inositol dehydrogenase gene is the only inositol catabolic gene encoded in the genome of the α-proteobacterium *Gluconobacter oxydans* (www.microbesonline.org). *G. oxydans* incompletely oxidizes myo-inositol and excretes the resulting ketone, 2-keto-myoinositol into the surrounding medium (40). The myo-inositol dehydrogenase is a membrane-associated dehydrogenase that requires pyrroloquinoline quinone (PPQ) as a cofactor, that shuttles the electrons gained from incomplete oxidation of myo-inositol to the respiratory chain (40).
*G. oxydans* posesses multiple PPQ-dependent membrane associated dehydrogenases that incompletely oxidize various sugars, alcohols and polyols in the periplasm to drive oxidative phosphorylation (34, 55). Based on its unique capability to produce a great variety of aldehydes, ketones and organic acids stereo- and regio-selectively, *G. oxydans* is used in several industrial processes (for review see (14)).

**Inositol catabolism in Salmonella**

The myo-inositol catabolism of the gastrointestinal pathogen *Salmonella enterica* serovar *Typhimurium* (*S. enterica*), a γ-proteobacterium, has been described partly by Kroeger and Fuchs (47). *S. enterica* exhibits an unusually long generation time of approximately 2 hours after an initial lag phase of 55 hours if myo-inositol is offered as sole carbon source. The myo-inositol utilization island on the *S. enterica* chromosome harbors the *iol* gene cluster comprising five divergently transcribed operons (Fig. 1.5). Thus, the organization of the myo-inositol transport, regulatory and catabolic genes in *Salmonella* is quite different from the conserved module found in α-proteobacteria. However, the IolR protein of *S. enterica* also is an RpiR-like transcriptional regulator that was shown to bind the promoter regions of all five operons (47).

Two myo-inositol transporters have been identified in *S. enterica* by Kroeger et al. (48). They are encoded by two monocitronically transcribed genes in the myo-inositol utilization island *iolT1* and *iolT2*. Both genes are co-regulated with the other
*iol* genes in an IolR-dependent manner. IolT1 belongs to the major facilitator superfamily and functions as a proton symporter. IolT1 was found to be the main *myo*-inositol transporter in *S. enterica*, while IolT2 is a minor *myo*-inositol transporter (47).

**Inositol catabolism in Rhizobiaceae**

The rhizobial inositol metabolism is not very well investigated. A better understanding is great interest because of its apparent role in host-bacteria interactions. Only a few studies have been conducted focusing on the *myo*-inositol catabolic pathway in the plant symbiotic *Rhizobium leguminosarum* bv. *viciae*, *Sinorhizobium fredii* and *S. meliloti* strains (31, 43, 70). Almost nothing is known about the catabolism of other inositol isomers besides that *S. meliloti* is able to use *scyllo*-inositol as sole carbon source (31).

The possible involvement of the inositol catabolism in host-bacteria interactions was first described by Scupham et al. (1996), who discovered that the inositol locus is not symbiotically silent as previously assumed (80). An *S. meliloti* mutant strain, with an insertion in the inositol locus was less competitive as compared to the wild type during host plant infection in a three-year field study (80). The rhizobial *myo*-inositol dehydrogenase has been characterized in *R. leguminosarum* bv. *viciae*, *S. fredii* and *S. meliloti* (31, 43, 70). The involvement of the *iolE* gene product, the 2-keto-*myo*-inositol dehydratase, in *myo*-inositol
catabolism was shown for *R. leguminosarum* bv. *viciae* and *S. fredii* (70, 98). Other genes required for *myo*-inositol catabolism and transport in *R. leguminosarum* bv. *viciae* have been investigated in part by Poole and colleagues (1994, 2001 (27, 70)), who showed that the *iolD* and *iolA* genes were shown to be required for *myo*-inositol degradation. The *iolD* gene was predicted to be part of a putative operon *iolDEB*. An ABC transport system involved in *myo*-inositol import was shown to be encoded by the *int* locus (27).

The *myo*-inositol catabolism of free living *R. leguminosarum* bv. *viciae* is inducible, but a pathway intermediate seems to function as the inducer instead of *myo*-inositol itself (27, 70). The *myo*-inositol dehydrogenase (*IdhA*) and the 2-keto-*myo*-inositol dehydratase (*IolE*) in bacteroids of *R. leguminosarum* bv. *viciae* were repressed by succinate, malate and glucose, indicating that the ability to catabolize *myo*-inositol is not required for the survival and nitrogen-fixing efficiency of bacteroids (70). Poole et al. (1994) suggested that *myo*-inositol catabolism plays a role during the early stages of competition for nodule occupancy (70). This is supported by the results of a co-challenge experiment conducted by Fry et al. (2001) (27): the *iolA* and *iolD* mutants of *R. leguminosarum* bv. *viciae* nodulated and fixed nitrogen as well as the wild type, if inoculated into plants individually, but both strains were strongly impaired in their ability to compete with the wild type in the co-challenge experiment. Interestingly, the loss of a functional *int* locus did not affect the competitiveness of the corresponding mutant.
Jiang et al. (2001) found the nitrogen-fixing ability of bacteroids derived from a *S. fredii idhA* mutant was strongly reduced (43). In addition, the bacteroids were structurally altered: the symbiosome membranes were loosely arranged and contained only one senescent bacteroid instead of multiple ones (43).

These findings support the notion that the inositol catabolic pathway is involved in early stages the symbiosis. In addition, the rhizobial inositol catabolism has been linked to the catabolism of rhizopines, a group of nutritional mediators in plant-bacteria interactions, since a *S. meliloti idhA* mutant was no longer able to use rhizopine isolated from root nodules as the sole carbon and nitrogen source in contrast to the wild type (31).

**The rhizopine concept**

The ability to synthesize rhizopines is a rare trait among the rhizobia, which only 11% of *S. meliloti* and 12% of *R. leguminosarum* bv. *viciae* strains possess (61, 79, 88, 95). These strains became known as rhizopine strains in order to distinguish them from non-rhizopine producing strains.

A hypothesis known as the rhizopine concept claims that rhizopine strains have an advantage over non-rhizopine strains during the competition process for nodule occupancy. The rhizopines were predicted to function as nutritional mediators and to play an important role in plant-bacteria interactions (62, 95).
The genes required for rhizopine synthesis and catabolism have been identified in *S. meliloti* strain L5-30 and comprise the *mos* and the *moc* gene clusters, respectively. Both loci are located on the large symbiotic plasmid pSymA and are separated by 4.5 kb (61). The pSymA also harbors the nitrogen fixation and nodulation genes (*nif*, *fix* and *nod* genes) (32). The rhizopine, 3-O-MSI (Fig. 1.3), is synthesized by the gene products of the *mos* cluster in *S. meliloti* strain L5-30 under symbiotic conditions (62). The *mos* locus contains four open reading frames: ORF1, *mosA*, *mosB* and *mosC* (63). The *mos* genes are co-regulated with the nitrogen fixation genes and expressed in nitrogen-fixing bacteroids only. They were shown to be expressed from a NtrA (sigma 54)-dependent promoter and the expression was shown to be controlled by the NifA regulatory protein (63), which acts in concert with NtrA (78). The NifA/NtrA transcription regulatory system is expressed under microaerophilic conditions in the nodule and activates genes required during the symbiosis, including the *nif* and *fix* genes as well as the C4 dicarboxylate transporter genes *dct* (76).

The gene product of *ORF1* is not required for rhizopine synthesis, but the gene itself might be necessary for the regulation of the *mosABC* genes, since it exhibits extensive homology with the promoter region of *nifH* (63). The genes *mosA*, *mosB* and *mosC* genes are involved in the synthesis of 3-O-methyl-scyllo-inosamine. Loss-of-function mutants with insertions in all three genes did not produce the rhizopine under symbiotic conditions (62). MosA displays similarity with the
dihydrodipicolinate synthase (DapA) of *E. coli* (63). The function of MosA as dihydrodipicolinate synthase was confirmed by Tam et al. (86). In addition, Phenix et al. (2008) showed that MosA not involved in the synthesis of 3-*O*-MSI (69). Certain domains of MosB share extensive similarities with several diverse proteins involved in antibiotic or outer cell wall synthesis as well as with regulatory proteins. The *mosC* gene product shows similarities with transmembrane transporters involved in sugar transport (62, 63).

The *moc* genes are essential for the catabolism of the rhizopines. The non-rhizopine strain, *S. meliloti* 1021 was able to use the rhizopines extracted from nodules as nutrient source after it had been transformed with a cosmid that carried the *mocABC* genes of the rhizopine catabolizing strain *S. meliloti* L5-30 (61). Saint et al. (1993) showed that the *moc* genes are regulated independently of NifA/NtrA and suggested that the *moc* genes are expressed in the free-living bacteria but not in the nitrogen-fixing bacteroids (79).

The *moc* gene cluster of *S. meliloti* L5-30 and *Rhizobium leguminosarum* bv. *viciae* comprises the *mocC*, ORF334, ORF293, *mocA*, *mocB*, *mocR*, *mocD*, *mocE* and *mocF* genes, but only the gene products of *mocABCDEFR* are required for the catabolism of crude rhizopine isolated from root nodules (5, 6, 77). Hypothetical functions of the *moc* gene products predicted were predicted based on homology to other proteins (5, 6, 77). The gene product of *mocC* seems to be a dehydratase related
to the keto-inositol dehydratase encoded by *iolE*. The ORF334 might be translated into an oxidoreductase of the GFO/IDH/MocA family. No significant homologies were found for the predicted product of ORF293. The hypothetical protein derived from *mocA* also belongs to the GFO/IDH/MocA family and is highly homologous to the *myo*-inositol dehydrogenase of *S. meliloti* and *B. subtilis*. A periplasmic binding protein of the ABC transporter family is encoded by *mocB*. The N-terminus of the *mocR* gene product contains a helix-turn-helix motif and belongs to the GntR-like bacterial regulatory protein family, whereas the C-terminus shares similarities with aminotransferases. The *mocD* gene product has similarities to fatty acid dehydrogenases/oxygenases, *mocE* to Rieske-like ferredoxins and the *mocF* gene product to ferredoxin reductases (5, 6, 77).

Based on the similarities, Bahar et al. (1998) proposed a model for the catabolism of 3-O-MSI (6): MocB is involved in 3-O-MSI transport. The initial demethylation of 3-O-MSI to SIA is carried out by the *mocDEF* encoded ferredoxin dependent oxygenase system. SIA is probably the first pathway intermediate of 3-O-MSI catabolism, but also seems to function as a nutritional mediator on its own (6, 79). SIA is catabolized by the NAD(H) dependent dehydrogenase MocA and the gene product of MocC. The unidentified endproduct of the Rhizopine degradation is probably catabolized by the inositol catabolic pathway and converted into dihydroxyacetone phosphate, acetyl-CoA and CO₂. So far only the requirement of the *idhA* encoded *myo*-inositol dehydrogenase has been demonstrated (31).
The hypothesis that rhizopines play a role as nutritional mediators in plant-bacteria interactions has been supported by the results of several co-challenge experiments for host plant nodulation. The ability of a *S. meliloti* L5-30 *moc* mutant to nodulate the host was strongly diminished when competing with the wild type, indicating that the ability to catabolize rhizopine is required for competitiveness in plant-bacteria interactions (77).

A long-term greenhouse study was conducted by Murphy’s group with *S. meliloti* L5-30 wild-type and *moc* and *mos* mutants (35, 39). The mutants nodulated and fixed nitrogen with the same efficiency as the wild type when inoculated onto plants as single inocula. The *mos* mutants were as competitive as the wild type in a co-challenge experiment, showing that the ability to synthesis rhizopine is not required for competition during nodulation. In contrast, the *moc* mutants were outcompeted by the wild-type after 10 months (35). The experiment was continued over a time period of four years with practically the same results (39).

The requirement of inositol and rhizopine catabolism for successfully infecting the host does not seem to be limited to the plant symbiotic species in the *Rhizobiaceae*. *Brucella spp.* cause brucellosis in humans and animals (1). A *mocC/iolE* and a *mosA* homolog were found to be required for survival inside macrophages in *B. abortus B.* and *melitensis*, respectively (25, 52).
Hypothesis

Not only the inositol-derived rhizopines, but also naturally-occurring inositol isomers, such as myo-, scyllo-, and chiro-inositol, function as nutritional mediators during host-bacteria interactions and that the role of inositol extends beyond being a simple nutrient source for the Rhizobiaceae.

The primary goal of this study is a detailed investigation of the rhizobial inositol and rhizopine metabolism and how both pathways relate to host-bacteria interactions using the *S. meliloti – M. sativa* (alfalfa) model. This work will improve understanding of the beneficial relationships between rhizobia and legumes in general and may lead to applications that will use nutritional mediators to create so-called “biased rhizosphere”. A biased rhizosphere was achieved through transgenic plants that were engineered to exude compounds that selectively support beneficial microorganisms that promote plant growth and health (65). Such an environment could increase the chances for rhizobia to nodulate their hosts and will ensure sufficient crop yields through natural nitrogen fixation, without the use of inorganic fertilizers. In addition, information derived from studies in *S. meliloti* are relevant to understanding the inositol and rhizopine metabolism in other *Rhizobiaceae* and their possible requirement for symbiosis or virulence.
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CHAPTER 2

INOSITOL CATABOLISM IN *SINORHIZOBIUM MELILOTI*, A KEY PATHWAY FOR COMPETITIVE HOST NODULATION

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Abstract

The nitrogen-fixing symbiont of alfalfa, *Sinorhizobium meliloti*, is able to use myo-inositol as the sole carbon source. Putative inositol catabolism genes (*iolA, iolRCDEB*) have been identified in the *S. meliloti* genome based on their similarities with the *Bacillus subtilis iol* genes. In this study, functional mutational analysis revealed that the *iolA* and *iolCDEB* genes were required for growth not only with the *myo*-isomer, but also for growth with *scyllo-* and *d-chiro-*inositol as the sole carbon source. An additional, hypothetical dehydrogenase of the *IdhA/MocA/GFO* family encoded by the *smc01163* gene was found to be essential for growth with *scyllo-*inositol, whereas the *idhA*-encoded *myo*-inositol dehydrogenase was responsible for the oxidation of *d-chiro-*inositol. The putative regulatory *iolR* gene, located upstream of *iolCDEB*, encodes a repressor of the *iol* genes, negatively regulating the activity of the *myo-* and the *scyllo-*inositol dehydrogenases. Mutants with insertions in the *iolA, smc01163*, and individual *iolRCDE* genes could not compete against the wild type in a nodule occupancy assay on alfalfa plants. Thus, a functional inositol catabolic
pathway and its proper regulation are important nutritional or signaling factors in the
*S. meliloti* – alfalfa symbiosis.

### Introduction

The sugar alcohol inositol, or cyclohexanehexol, occurs in several different stereoisomers, of which the *myo*-form (1 in Fig. 2.1) is the most abundant (1). *myo*-Inositol plays important structural and signaling roles in animal and plant cells (22).

![Figure 2.1: The proposed myo-inositol catabolic pathway](http://www.genome.jp/kegg/). Compounds: 1, *myo*-inositol (MI); 2, 2-keto-*myo*-inositol (2KMI); 3, 3D-(3,4/5) trihydroxycyclohexane-1,2-dione (THcHDO); 4, 5-deoxy glucuronic acid (5DG); 5, 2-deoxy-5-keto-D-gluconic acid (DKG); 6, 2-deoxy-5-keto-D-gluconic acid 6-phosphate (DKGP); 7, dihydroxyacetone phosphate (DHAP); 8, malonic semialdehyde (MSA); 9, acetyl-CoA. Enzymes: IdhA, *myo*-inositol dehydrogenase; IolE, 2KMI dehydratase; IolD, THcHDO hydrolase; IolB, 5DG isomerase; IolC, DKG kinase; IolJ aldolase (not yet identified in *S. meliloti*); IolA, MSA dehydrogenase.
In the environment, myo-inositol mainly occurs in the phosphorylated form and is involved in the phosphate cycle of terrestrial and freshwater ecosystems (41). The stereoisomers D-chiro- and scy/lo-inositol have recently attracted attention, because they have shown therapeutic potential for diabetes and Alzheimer’s disease, respectively (11, 21). Although there is only limited knowledge about the metabolism of D-chiro- and scy/lo-inositol (25, 50), the catabolism of myo-inositol has been studied in a variety of microorganisms, including some members of the Firmicutes (17, 46, 51), Enterobacteriaceae (4, 19, 40), and Rhizobiaceae (16, 29). The myo-inositol catabolic pathway and its regulation are best understood in the Gram-positive bacterium Bacillus subtilis. The B. subtilis iol genes are organized in a divergon comprising iolABCDEFGHIJ and iolRS (47-49). In the proposed inositol catabolic pathway, the myo-inositol dehydrogenase oxidizes myo-inositol to its corresponding ketone 2-keto-myo-inositol (2KMI), which is then further catabolized by the actions of IolE, D, B, C, J, and A (Fig. 2.1). The inducer of the inositol catabolic pathway in B. subtilis is the product of the IolC reaction, 2-deoxy-5-keto-D-gluconic acid 6-phosphate (DKGP, 6 in Fig. 2.1), which antagonizes the binding of the IolR repressor to the iol promoter region (51).

Sinorhizobium meliloti, the nitrogen-fixing symbiont of alfalfa, can use myo-inositol as the sole carbon source (15). The idhA-encoded myo-inositol dehydrogenase had been shown to be required for myo-inositol catabolism (15), and more recently, an S. meliloti iolA mutant was reported that could not grow with myo-
inositol as the sole carbon source (5). Based on comparisons with *B. subtilis*, a cluster of genes (iolRCDEB) was identified in the genome of *S. meliloti* (http://sequence.toulouse.inra.fr/S.meliloti), but their functional role has not been described. Rhizobial inositol metabolism is of special interest because of its link to the catabolism of a group of nutritional mediators in plant-bacteria interactions known as the rhizopines. Rhizopines, produced by several symbiotic *S. meliloti* and *R. leguminosarum* bv. *viciae* strains, are inositol derivatives, namely scyllo-inosamine and 1-3-O-methyl-scyllo-inosamine (26, 36, 38, 43). The ability to catabolize these inositol derivatives (rhizopines) has been shown to play a role in competition for nodule occupancy (35), but it seems that the ability to catabolize myo-inositol itself may also play a role in plant-bacteria interactions. For example, *R. leguminosarum* bv. *viciae* iolA and iolD mutants were reported to be strongly impaired in their ability to compete with the wild type during the nodulation process (14), and a *S. fredii* idhA mutant induced nodules with aberrant ultrastructure and showed reduced nitrogen-fixing ability (16). In contrast, an *S. meliloti* idhA mutant was not affected in the ability to nodulate its host plant or to fix nitrogen, but results from competition experiments have not been reported for *S. meliloti* (15). Here, we present a detailed analysis of the *S. meliloti* smc01163, iolA and iolRCDEB genes and elucidate their role in the catabolism of different inositol isomers and in plant-bacteria interactions.
Materials and Methods

Microbiological methods

The bacterial strains and plasmids used in this study are listed in Table 2.1. *Escherichia coli* strains were grown at 37°C in LB medium (37). Antibiotic concentrations for *E. coli* were 50 µg/ml ampicillin (Ap), 30 µg/ml chloramphenicol (Cm), 15 µg/ml gentamycin (Gm), 25 µg/ml kanamycin (Km), 25 µg/ml spectinomycin (Sp) and 10 µg/ml tetracycline (Tc). *S. meliloti* cultures were grown at 28°C. Rich media for *S. meliloti* were tryptone yeast (TY) (3) or LB medium supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LBMC); minimal media were minimal M medium (34), with 0.1% KNO₃ or 0.1% NH₄Cl as sole nitrogen (N-) source for the catabolism studies and enzyme assays, and GTS minimal medium (18) for the selection of exconjugants. Carbon (C-) sources were added to the minimal media at a final concentration of 0.2% unless otherwise indicated. Antibiotic concentrations for *S. meliloti* were 15 µg/ml Gm, 200 µg/ml Km, 250 µg/ml streptomycin (Sm), 200 µg/ml Sp, and 10 µg/ml Tc. For the catabolism studies, *S. meliloti* strains were inoculated 1:100 from TY precultures into liquid minimal M medium. Cultures were grown on a shaking incubator and the growth was determined spectrophotometrically at 600 nm after 3, 5 and 7 days. Catabolism studies were carried out in duplicate, and values represent the average of two independent experiments ± SEM.
Preparation of 2-keto-myoinositol

The 2-keto-myoinositol (2KMI) used in this study was synthesized using Gluconobacter oxydans (Acetobacter suboxydans ATCC621) according to Carter et al. (7) with the following adaptations: G. oxydans was grown on sorbitol agar containing 2.5% sorbitol, 0.5% yeast extract, 0.3% peptone and 1.5% agar or in sorbitol broth containing 10% sorbitol and 0.5% yeast extract. G. oxydans was inoculated 1:100 from an overnight preculture into oxidation medium containing 3% myo-inositol, 0.5% yeast extract and 0.1% sorbitol. The oxidation was carried out at 28°C for 4 days. Bacteria were removed from the medium via centrifugation at 6,000 g. The crude product was concentrated and re-crystallized from a water-methanol mixture to afford the clean 2KMI.

2-keto-myoinositol. m.p. 199° C (lit. m.p. 201° C); ¹H NMR (400 MHz, D₂O): δ 4.38 (d, J = 10.2 Hz, 2 H), 3.78 (t, J = 9.5 Hz, 1 H), 3.45-3.34 (m, 2 H). The melting point was determined in open capillaries using a Thomas-Hoover Unimelt instrument. The NMR spectrum was recorded using a 400 Mhz Jeol Eclipse nuclear magnetic resonance instrument.
Table 2.1: Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strains, plasmids</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>Escherichia coli</em></td>
<td></td>
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</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>(37)</td>
</tr>
<tr>
<td>HB101</td>
<td>supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</td>
<td>(37)</td>
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<tr>
<td><strong>Sinorhizobium meliloti</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1021</td>
<td>wild type, SmR derivative of SU47</td>
<td>(23)</td>
</tr>
<tr>
<td>2011</td>
<td>wild-type, SmR derivative of SU47</td>
<td>(24)</td>
</tr>
<tr>
<td>TIDHA</td>
<td>1021 idhA::Tn5#56, SmR, KmR</td>
<td>(15)</td>
</tr>
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<td>T63</td>
<td>1021 smc01163::Ω, SmR, SpR</td>
<td>This study</td>
</tr>
<tr>
<td>TIOLC</td>
<td>1021 iolC::pVO155, SmR, KmR</td>
<td>This study</td>
</tr>
<tr>
<td>TIOLD</td>
<td>1021 iolD::pVO155, SmR, KmR</td>
<td>This study</td>
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<tr>
<td>TIOLE</td>
<td>1021 iolE::pVO155, SmR, KmR</td>
<td>This study</td>
</tr>
<tr>
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<td>1021 iolB::pVO155, SmR, KmR</td>
<td>This study</td>
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<td>WIOLD</td>
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<td>WGLYA</td>
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<td><strong>Plasmids</strong></td>
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<td>pJQ200SK</td>
<td>Suicide vector with sacB gene, GmR</td>
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<td>pGEM-T</td>
<td>Cloning vector, ApR</td>
<td>Promega</td>
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<tr>
<td>Plasmids</td>
<td>Relevant characteristics</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------</td>
<td>------------------------------------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>pCR®-2.1-TOPO</td>
<td>Cloning vector, Ap&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<td>pHp45Ω</td>
<td>Source for Ω fragment, Ap&lt;sup&gt;R&lt;/sup&gt;, Sm&lt;sup&gt;R&lt;/sup&gt;, Sp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(30)</td>
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<tr>
<td>pRK2013</td>
<td>mob, tra, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(12)</td>
</tr>
<tr>
<td>pRK600</td>
<td>mob, tra, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(13)</td>
</tr>
<tr>
<td>pJZ1</td>
<td>pVO155 containing 301 bp fragment of iolC, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<td>pJZ2</td>
<td>pVO155 containing 270 bp fragment of iolD, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pJZ3</td>
<td>pVO155 containing 226 bp fragment of iolE, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pJZ4</td>
<td>pVO155 containing 399 bp fragment of iolB, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pPK63-1</td>
<td>pJQ200SK containing 1121 bp fragment of smc01163, Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pPK63Ω</td>
<td>pPK63-1 containing Ω; Gm&lt;sup&gt;R&lt;/sup&gt;, Sm&lt;sup&gt;R&lt;/sup&gt;, Sp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pTE3</td>
<td>broad host range expression vector, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(10)</td>
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<tr>
<td>pIOLC</td>
<td>pTE3 containing 2412 bp fragment of iolC</td>
<td>This study</td>
</tr>
<tr>
<td>pIOLD</td>
<td>pTE3 containing 2776 bp fragment of iolD</td>
<td>This study</td>
</tr>
<tr>
<td>pIOLE</td>
<td>pTE3 containing 945 bp fragment of iolE</td>
<td>This study</td>
</tr>
<tr>
<td>pIOLB</td>
<td>pTE3 containing 881 bp fragment of iolB</td>
<td>This study</td>
</tr>
</tbody>
</table>
DNA manipulations and microbiological methods

Preparation of plasmid DNA, DNA digests, agarose gel electrophoresis, cloning and transformation of E. coli cells were performed following established protocols (37). Di- and tri-parental conjugations were performed according to Rossbach and de Bruijn (33).

Construction of S. meliloti mutants

Internal DNA fragments of the individual iolCDEB genes and an 1121 bp DNA fragment that contained smc01163 were PCR-amplified from cultures of S. meliloti 1021 with primers listed in Table 2.2. The iolCDEB and the smc01163 PCR products were initially cloned into the pGEM-T or pCR®2.1-TOPO vectors (Table 2.1). The individual iolCDEB inserts were re-cloned into the insertion vector pV0155 (Table 2.1) and smc01163 into the sacB containing suicide vector pJQ200SK (Table 2.1). An Ω Sm/Sp fragment replaced the 341 bp NruI fragment of smc01163 resulting in plasmid pPK63::Ω. The pVO155 vectors carrying the internal fragments of the iolBCDE genes and pPK63::Ω were conjugated into S. meliloti 1021 with the helper plasmids pRK600 and pRK2013 (Table 2.1), respectively. The insertion events of the pVO155 derivatives were selected for by plating the conjugation mixture onto LBMC Sm Km. The double homologous recombination event with pPK63::Ω was selected for by plating on GTS Sm Sp, followed by counterselection on TY Sm Sp containing 5% sucrose and screening for the absence of the suicide vector on TY Sm Gm. The correct insertions of pVO155 in the individual iolCDEB mutants, as well as of the Ω
fragment in the smc01163 deletion mutant were confirmed by PCR. The transposon (mTn5-STM) mutants of *S. meliloti* strain 2011 were provided by Dr. Anke Becker (28).

**Construction of plasmids for the complementation analysis**

DNA-fragments containing the complete ORFs of the wild-type *iolC*, *iolD*, *IolE*, and *iolB* genes including their ribosomal binding sites were PCR-amplified from a liquid *S. meliloti* 2011 culture with primers that were engineered to contain either PstI or NsII at their 5’ and BamHI or BglII sites at their 3’ ends (Table 2.2). The PCR products were cloned into the broad host range expression vector pTE3 (Table 2.1), bringing the PCR products in correct orientation under the control of the *Salmonella trp* promoter, which allows constitutive expression in *S. meliloti* (10). The resulting plasmids carrying the individual *iolCDEB* genes (Table 2.1) as well as pTE3 as empty vector control were introduced into the individual *S. meliloti* 1021 and 2011 *iolC*, *iolD*, *iolE* and *iolB* mutants via triparental mating (33). The presence of the wild-type and of the mutated *iol* genes in the mutant strains was confirmed with PCR.
Table 2.2: Primers used

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>IolB1</td>
<td>5’ GCG GCA AGG CGA AGA TTT CC 3’</td>
</tr>
<tr>
<td>IolB2</td>
<td>5’ GAG GCG GTG ATA ATA GGT CTC 3’</td>
</tr>
<tr>
<td>IolC1</td>
<td>5’ GCA GCC GCC TTG TCG ACT GGC CG 3’</td>
</tr>
<tr>
<td>IolC2</td>
<td>5’ GAT CAC GGC GTC GAT CGC AGC 3’</td>
</tr>
<tr>
<td>IolD3</td>
<td>5’ GCT GAC GGA TCC TGC CGA TTG C 3’</td>
</tr>
<tr>
<td>IolD4</td>
<td>5’ CGG CCT GCG TCT CGA GGA CCG G 3’</td>
</tr>
<tr>
<td>IolE3</td>
<td>5’ GCT GGC AGT CGA CCA ACC TCC TG 3’</td>
</tr>
<tr>
<td>IolE4</td>
<td>5’ GTG GTG GTG GTA GAC GAG ATC GAC 3’</td>
</tr>
<tr>
<td>63Nru1F</td>
<td>5’ CGA CTA GTC ATT GCA GCG ATA GAA CGG 3’</td>
</tr>
<tr>
<td>63Nru1R</td>
<td>5’ CTA TTT CGT CGA GCA CAG AGG GCC TG 3’</td>
</tr>
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<td>PKiolCFPstI</td>
<td>5’ CGA ATC CTG CAG GGT CTG GTT CGA AC 3’</td>
</tr>
<tr>
<td>PKiolCRBamHI</td>
<td>5’ CGG TAG GTC GGG ATC CTG TCG CGA AC 3’</td>
</tr>
<tr>
<td>PKiolDFNsII</td>
<td>5’ CGG TCG ACC AAT GCA TCA AGG TGC TTT C 3’</td>
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<td>PKiolDFBglII</td>
<td>5’ CGA GAC GAA GAC GAG ATC AGA TCT TGA GAG C 3’</td>
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<td>FiolE-Nsil</td>
<td>5’ GCA ACA GAG CGA TGC ATC CGC CTT AAG 3’</td>
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<td>RiolE-BamHI</td>
<td>5’ GCA GCC GGA TCC GGG GGC AAT TAC G 3’</td>
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<tr>
<td>FioB-Nsil</td>
<td>5’ CGG GGA GAA TGC ATA CCA CAG AAA CCC 3’</td>
</tr>
<tr>
<td>2PKiolBRBglII</td>
<td>5’ CGG CAA GAT CTG ATA GGG CGA AGG CTC 3’</td>
</tr>
</tbody>
</table>

Underlined bases were modified.

β-Glucuronidase assays

The β-glucuronidase assays were optimized based on Wilson et al. (44). Precultures of S. meliloti strains were inoculated 1:100 in 5 ml minimal M medium containing NH₄Cl as N-source and either myo-inositol, 2KMI, glycerol, glucose or succinate as C-sources. Three hundred fifty μl of mid-exponential-phase cultures (OD₆₀₀ = 0.7-1) were harvested by centrifugation at 4,500 g for 10 min. The pellet was resuspended in 350 μl GEB Buffer (50 mM sodium phosphate buffer pH 7, 0.6% β-mercaptoethanol, 10 mM EDTA, 1% Triton X100, 0.1% sodium lauryl sarcosine). After an initial equilibration period of 15 min at 37°C, 35 μl of 20 mM 4-nitrophenyl-β-D-glucuronide (PNPG) was added to the cell lysate. One hundred μl of the reaction
mix were transferred into 800 μl of a 400 mM NaCO₃ stop solution after 5, 10, and 15 min. Cell debris was removed by centrifugation at 16,000 g for 30 seconds and the OD₄₀₅ was determined spectrophotometrically. The reaction rate was expressed in nmol p-nitrophenol produced per min per OD₆₀₀ unit ± SEM (nmol min⁻¹ OD₆₀₀ unit⁻¹). The values represent the mean of two independent experiments and each assay was carried out in duplicate.

**NAD(H)-dependent dehydrogenase assays**

*S. meliloti* precultures were inoculated 1:100 into 500 ml Erlenmeyer flasks containing 100 ml minimal M medium with NH₄Cl as N-source, glycerol as C-source, and either 0.02% myo-inositol or 2KMI as inducers. Late-exponential phase cultures (OD₆₀₀ = 1-1.25) were harvested via centrifugation at 6,000 g, washed with 40 mM HEPES buffer (pH 7) containing 10 mM β-mercaptoethanol, and resuspended in 5 ml 40 mM HEPES buffer (pH 7). Cell extracts were prepared with a sonicator at 50 W with three 30 s sonication periods (Misonix XL-2020, Farmingdale, NY). The myo-, scyllo- and d-chiro-inositol dehydrogenase activities were determined at room temperature (21-23°C). Each reaction mix (1 ml) contained 50 mM NH₄Cl, 50 mM Na₂CO₃, 100 μl cell-free extract, and 0.4 mM NAD⁺. A baseline of background reduction of NAD⁺ in the absence of substrate was established at a wavelength of 340 nm for slope correction. The increase in absorbance (A₃₄₀) in the presence 25 mM myo-, scyllo- or d-chiro-inositol was monitored for 3 min. The protein content of the cell extracts was determined with a Bradford assay (Pierce Coomassie Plus The
Better Bradford™ Assay Kit, Thermo Fisher Scientific, Rockford, IL). The specific myo-, scyllo-, and d-chiro-inositol dehydrogenase activities were expressed as nmol NAD$^+$ reduced min$^{-1}$ mg protein$^{-1} \pm$ SEM. The values represent the mean of two independent experiments, each of them performed in duplicate, if not otherwise indicated.

**Competition assay for nodule occupancy**

Axenic alfalfa (*Medicago sativa*) plants were prepared by germination from surface sterilized seeds on folded Whatman filter paper in 20 ml nitrogen-free B&D growth medium (6) in 25 mm diameter tubes as described previously (36). Plants were grown at room temperature under a cycle of 16 h light and 8 h dark. Before inoculation, rhizobial cultures were pelleted, washed, and resuspended in sterile deionized H$_2$O and their optical density at 600 nm was determined spectrophotometrically. Mixed cultures of the *S. meliloti* 2011 wild type and individual mutant strains were prepared in a 1:1 ratio based on the OD$_{600}$ values. In addition, the 1:1 input ratio was verified via serial dilution on TY Sm and TY Sm Km. Seven day-old alfalfa seedlings were inoculated with 1 ml of the mixed cultures or 1 ml of the wild type or the individual mutant strains. One ml of sterile deionized H$_2$O was added to control plants. The total number of nodules per plant was determined and after 20 weeks, the nodules were harvested and surface-sterilized with 70% ethanol. Plant fresh and dry weights were determined and averaged from six plants of each treatment. Rhizobia were re-isolated by
homogenizing the nodules in sterile H₂O and serial dilutions were prepared. The wild type versus the mutant strain output ratio was determined by selective plating on TY Sm and TY Sm Km. The values represent the average of two independent studies ± SEM with six plants each.

Results

Structure and organization of the inositol catabolism genes in *S. meliloti*

In contrast to the organization of the *iol* genes in *B. subtilis*, the inositol catabolism genes of *S. meliloti* are not arranged in a single gene cluster. The *idhA* gene is located on the pSymB plasmid, whereas the putative *iolR, iolC, iolD, iolE,* and *iolB* genes are organized in one cluster on the chromosome, all oriented in the same direction (Fig. 2.2). The *iolA* gene is located a further 400 kb away on the chromosome. An additional gene of interest, *smc01163*, encoding a putative dehydrogenase of the IdhA/MocA/GFO family, is located directly upstream of *iolR* (Fig. 2.2). The *S. meliloti* transcriptional regulator encoded by *iolR* belongs to the RpiR repressor family and is a homologue of the IolR regulator in *Caulobacter crescentus* (5). The *iolCDEB* genes are predicted to comprise an operon, whereas the *smc01163* and *iolR* genes seem to be transcribed separately (www.microbesonline.org; Fig. 2.3).
Figure 2.2: Ability of *S. meliloti* wild-type and the *idhA, smc01163* (1163), *iolR, iolC*, *iolD, iolE, iolA* and *iolB* mutant strains to grow with 0.2% *myo*-inositol, *D-chiro*-inositol, *scylo*-inositol, or 2-keto-*myo*-inositol (2KMI) as the sole carbon source in minimal medium. Open reading frames are depicted as open arrows. The locations of the mini-Tn5 insertions in the *S. meliloti* 2011 mutants are marked by vertical arrowheads. The star indicates the position of the plasmid insertion in the *S. meliloti* *iolB* mutant. Horizontal arrows above the genes indicate predicted transcriptional units. Each mutant’s ability (+) or inability (-) to use inositol compounds as the sole carbon source is indicated.

**Catabolism studies**

To conduct a complete study of the roles of the predicted *iol* genes in the catabolism of different inositol stereoisomers, mutants of *S. meliloti* strain 2011 were obtained that contained mTn5-STM transposon insertions in the *idhA, iolA* and the individual *iolRCDE* genes, as well as two mutant strains with different insertions in the *smc01163* gene (28). As part of a comparative study, mutants were also constructed in the *S. meliloti* 1021 strain, specifically, insertions in *smc01163* and the individual *iolCDEB* genes. The 1021 and 2011 strains are both streptomycin-resistant.
derivatives of the \textit{S. meliloti} SU47 wild type (23, 24), but years of culturing in different laboratories have resulted in minor differences between these two strains (20, 42).

Figure 2.3: Growth of \textit{S. meliloti} wild-type strain 2011 and the corresponding \textit{idhA} (WIDHA), \textit{smc01163} (W63-1), \textit{iolR} (WIOLR), \textit{iolC} (WIOLC), \textit{iolD} (WIOLD), \textit{iolE} (WIOLE) and \textit{iolA} (WIOLA) mutants, as well as the \textit{S. meliloti} 1021 derived \textit{iolB} (TIOLB) mutant in minimal medium with 0.2\% \textit{myo}-inositol (A), \textit{scylo}-inositol (B), \textit{d-chiro}-inositol (C), and 2-keto-\textit{myo}-inositol (2KMI; D) as sole carbon sources. The optical density was determined spectrophotometrically at 600 nm after 72 hours. Bars represent the average of two independent experiments, each performed in duplicate, Error bars denote ± SEM.

When grown in minimal medium with \textit{myo}-inositol as the sole C-source, both wild-type strains grew to an OD\textsubscript{600} of around 1.2, while their corresponding \textit{idhA}, \textit{iolC}, \textit{iolD}, \textit{iolE}, \textit{iolB} and \textit{iolA} mutants did not grow (Fig. 2.2; Fig. 2.3 A). The \textit{iolR} and the three different \textit{smc01163} mutants grew to similar optical densities as the wild
type (shown for W63-1 and W63-2 in Fig. 2.2 and for W63-1 in Fig. 2.3 A). In a control experiment, all strains were able to grow with glucose as the sole C-source (data not shown). Hence, the idhA, iolA and iolCDEB genes are essential for myo-inositol catabolism in \emph{S. meliloti}. Since the iolCDEB genes are predicted to form an operon, the mTn5-STM transposon and the plasmid insertions in the iolC, iolD and iolE genes could have polar effects. We cloned the individual iolCDEB genes under the control of a constitutive promoter in the broad host range expression vector pTE3 (Table 2.1). The resulting plasmids were conjugated into the respective \emph{S. meliloti} 1021 iolC, iolD, iolE, and iolB mutants as well as into the 2011 iolC, iolD and iolE mutants. The mutant strains containing the plasmids with the individual iolC, iolD, iolE and iolB genes were able to grow with myo-inositol as the sole C-source, whereas the same strains carrying the empty vector as control could not (Fig. 2.4). The growth of the mutant strains containing the plasmid with the iolC, iolD, and iolE genes was delayed as compared to the wild types or the iolB mutant strain containing the iolB-carrying plasmid (Fig. 2.4). Thus, we conclude that the mTn5-STM transposon and the plasmid insertions seem to allow a low level of expression of the downstream iol genes, probably due to a weak read-through from the integrated kanamycin resistance genes.
We investigated which other inositol isomers can be catabolized by *S. meliloti*. Strains 1021 and 2011 were grown with the commercially available *scylo*, *muco*, *allo*, D-*chiro* and L-*chiro*-inositol as sole C-sources (structures shown in Fig. 5). Both wild-type strains could grow with *scylo* or D-*chiro*-inositol (shown for strain 2011 in Fig. 2.3), but they were not able to use L-*chiro*, *muco*, nor *allo*
inositol (data not shown). The role of the iol genes in the catabolism of scyllo- and D-chiro-inositol was further investigated. The idhA and the iolR mutants could grow with scyllo-inositol, but the iolC, iolD, iolE, iolB, and iolA mutants could not (Fig. 2.2, Fig. 2.3B). Interestingly, all three smc01163 mutants were unable to grow with scyllo-inositol as the sole C-source (W63-1 shown in Fig. 2.3B). Thus, the smc01163 gene product appears to be essential for the catabolism of scyllo-inositol. D-chiro-inositol was used as the sole C-source by the iolR and the smc01163 mutants, but not by the idhA, iolC, iolD, iolE, iolB, and iolA mutants (Fig. 2.2, Fig. 2.3C), suggesting that the idhA-encoded myo-inositol dehydrogenase facilitates the oxidation of both, myo- and D-chiro-inositol.

We also tested the first proposed intermediate in the myo-inositol catabolic pathway, 2-keto-myoinositol (2KMI), which was synthesized in our laboratory (see Materials and Methods). The iolC, iolD, iolE, iolB, and iolA mutants did not catabolize 2KMI, but the idhA, iolR and smc01163 mutants could (Fig. 2.2, Fig. 68).
2.3D). The fact that the \textit{idhA} mutant grew on 2KMI as the sole C-source while the \textit{iolA} and \textit{iolCDEB} mutants failed to do so, strongly supports the notion that also in \textit{S. meliloti} 2KMI is the product of the \textit{myo}-inositol dehydrogenase (IdhA) reaction.

**Regulation of \textit{S. meliloti} inositol catabolism**

The 2011 \textit{idhA}, \textit{smc01163}, \textit{iolR}, \textit{iolD}, and \textit{iolE} mutants contain the mTn5-STM::\textit{gusA} transposon in the same orientation as the respective genes, creating a transcriptional fusion and therefore allowing to investigate the regulation of the inositol genes by measuring the \textit{\beta}-glucuronidase activity (28). Mutant strains were grown in minimal medium either with \textit{myo}-inositol, 2KMI, glycerol, glucose and succinate as carbon sources or in combinations to analyze the effect of different carbon sources. Cells were harvested, solubilized and their \textit{\beta}-glucuronidase activities were determined. The wild-type strain, which does not contain a \textit{gusA} gene, served as negative control and did not exhibit any detectable \textit{\beta}-glucuronidase activity (data not shown). The expression of the \textit{idhA} gene in its corresponding mutant was not inducible by \textit{myo}-inositol, but was induced in the presence of 2KMI with all carbon sources tested (Fig. 2.6A). This finding indicates that not \textit{myo}-inositol itself, but either 2KMI or a later pathway intermediate functions as an inducer in \textit{S. meliloti}, as has been shown for \textit{B. subtilis} (51). The expression of \textit{smc01163} in its corresponding mutant was induced by \textit{myo}-inositol or 2KMI with all carbon sources tested (Fig. 2.3B). We did not notice any major catabolite repression effect, when glycerol, glucose, or succinate were present in the medium (Fig. 2.6A and B). The \textit{iolR} gene,
encoding the RpiR-like repressor, was constitutively expressed in its corresponding mutant, since high β-glucuronidase activities were displayed under all growth conditions, even when grown without inducer (Fig. 2.6C). It is interesting to note, that only very low β-glucuronidase activities were observed in the *iolD* and *iolE* mutants in the presence or absence of the inducers *myo*-inositol or 2KMI (between 2 and 3 nmol min⁻¹ OD₆₀₀ unit⁻¹). This further supports the notion that neither *myo*-inositol nor 2KMI, but a later pathway intermediate, which cannot be synthesized by the *iolD* or *iolE* mutant, serves as the true inducer in *S. meliloti*.

**Determination of *myo*-Inositol dehydrogenase activity**

For the determination of the *myo*-inositol dehydrogenase activity an NAD(H)-dependent dehydrogenase assay was used. Cultures were grown in minimal medium containing glycerol as C-source and either with *myo*-inositol or 2KMI as inducer. The specific *myo*-inositol dehydrogenase activities of the wild types, the *idhA*, the *smc01163* and the individual *iolCDEB* mutant strains were low without prior induction (0.2 to 21 nmol min⁻¹ mg protein⁻¹; Table 2.3). Upon induction with *myo*-inositol, the 2011 and 1021 wild types displayed *myo*-inositol dehydrogenase activities of 103 and 131 nmol min⁻¹ mg protein⁻¹, respectively (Table 2.3). When grown with 2KMI as inducer, the wild-type cells exhibited ~30% lower *myo*-inositol
Figure 2.6: β-Glucuronidase activities of the *S. meliloti* *idhA* (A), *smc01163* (B), and *iolR* (C) *gusA* reporter gene fusions in the respective mutant strains. The reaction rate is expressed in nmol *p*-nitrophenol produced per minute per OD$_{600}$. Cultures were grown in minimal medium containing 0.2% of the following carbon sources: *myo*-inositol (MI), 2-keto-*myo*-inositol (2KMI), glycerol (Gly), glucose (Glu), succinate (Suc) or combinations thereof. Bars represent the average of two independent experiments and error bars denote ± SEM. MI* indicates that the *idhA* mutant did not grow with *myo*-inositol as the sole carbon source in minimal medium, but the residual β-glucuronidase activity is probably due to the carry-over of cells from the TY preculture.
dehydrogenase activities (Table 2.3). Regardless, whether the idhA mutant was induced with myo-inositol or 2KMI, its myo-inositol dehydrogenase activity was basically abolished (Table 2.3), which confirmed the results of Galbraith et al. (15) that the idhA gene, located on the pSymB plasmid, encodes the myo-inositol dehydrogenase. All three mutants with insertions in smc01163 exhibited myo-inositol dehydrogenase activity comparable to the wild type (W63-1 shown in Table 2.3). The iolR mutant displayed a four- to five-fold higher myo-inositol dehydrogenase activity than the wild type, even when not induced (Table 2.3). The iolC, iolD, iolE and iolB mutants, however, exhibited very low myo-inositol dehydrogenase activities (Table 2.3), regardless whether they were grown with or without myo-inositol or 2KMI as inducers, indicating that all four gene products (IolC, IolD, IolE, and IolB) are needed for inducer production.

**Determination of scyllo-inositol dehydrogenase activity**

The results of the catabolism studies suggest that the dehydrogenase encoded by smc01163 functions as a scyllo-inositol dehydrogenase. Thus, the scyllo-inositol dehydrogenase activities of the wild type and the smc01163 mutants were investigated. Based on our findings that the smc01163 gene was inducible by myo-inositol and 2KMI (see above), wild-type and mutant strains were grown in minimal medium with glycerol as C-source and with myo-inositol or 2KMI as inducer. The specific scyllo-inositol dehydrogenase activities of the uninduced wild type and the smc01163 mutants were very low (10 and 5 nmol min⁻¹ mg protein⁻¹, respectively).
### Table 2.3: Specific myo-inositol dehydrogenase activities of *S. meliloti* wild-type and mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>myo-inositol dehydrogenase activity (nmol min(^{-1}) mg protein(^{-1}))</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>uninduced</td>
<td>2KMI induced</td>
<td>myo-inositol induced</td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>wild type</td>
<td>5 ± 0.3</td>
<td>65 ± 9</td>
<td>103 ± 4</td>
<td></td>
</tr>
<tr>
<td>1021</td>
<td>wild type</td>
<td>21 ± 4</td>
<td>79 ± 10</td>
<td>131 ± 7</td>
<td></td>
</tr>
<tr>
<td>WIDHA</td>
<td><em>idhA</em></td>
<td>2 ± 0.3</td>
<td>0.6 ± 0.3</td>
<td>0.5 ± 0</td>
<td></td>
</tr>
<tr>
<td>W63-1</td>
<td><em>smc01163</em></td>
<td>10 ± 2</td>
<td>55 ± 5</td>
<td>103 ± 42</td>
<td></td>
</tr>
<tr>
<td>WIOLR</td>
<td><em>iolR</em></td>
<td>522 ± 2</td>
<td>337 ± 6</td>
<td>410 ± 24</td>
<td></td>
</tr>
<tr>
<td>WIOLC</td>
<td><em>iolC</em></td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>8 ± 0</td>
<td></td>
</tr>
<tr>
<td>WIOLD</td>
<td><em>iolD</em></td>
<td>2 ± 2</td>
<td>5 ± 1</td>
<td>5 ± 0</td>
<td></td>
</tr>
<tr>
<td>WIOLE</td>
<td><em>iolE</em></td>
<td>0.4 ± 0.2</td>
<td>0 ± 0</td>
<td>5 ± 0</td>
<td></td>
</tr>
<tr>
<td>TIOLB</td>
<td><em>iolB</em></td>
<td>5 ± 1</td>
<td>2 ± 0.3</td>
<td>0.4 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.4: Specific scylo-inositol dehydrogenase activity of *S. meliloti* wild-type and mutant strains

| Strain  | Relevant genotype | scylo-inositol dehydrogenase activity (nmol min\(^{-1}\) mg protein\(^{-1}\)) |  |  |  |
|---------|------------------|--------------------------------------------------------------------------------||--|--|--|
|         |                  | 2KMI induced | myo-inositol induced |  |
| 2011    | wild type        | 74 ± 2       | 69 ± 1                 |  |
| 1021    | wild type        | n.d.         | 91 ± 2                 |  |
| WIOLR   | *iolR*           | n.d.         | 477\(^a\)              |  |
| W63-1   | *smc01163*       | 50 ± 5       | 40 ± 1                 |  |
| W63-2   | *smc01163*       | 39\(^a\)     | 52\(^a\)               |  |
| T63     | *smc01163*       | n.d.         | 62 ± 1                 |  |
| WIOLE   | *iolE*           | n.d.         | 3 ± 0.5                |  |

\(^a\) Value based on one experiment
n.d.: not done
Upon induction with myo-inositol, the 2011 and 1021 wild types displayed a scyllo-inositol dehydrogenase activity of 69 and 91 nmol min$^{-1}$ mg protein$^{-1}$, respectively, while the three smc01163 mutants showed between 58% and 75% of the wild type scyllo-inositol dehydrogenase activity (Table 2.4). The results of the scyllo-inositol dehydrogenase assay were unexpected. The smc01163 mutants were unable to use scyllo-inositol as sole C-source, suggesting that the smc01163 gene encodes the scyllo-inositol dehydrogenase and the mutants would not exhibit any scyllo-inositol dehydrogenase activity. Nevertheless, we obtained the same results for all three smc01163 mutants, which were constructed independently in two different genetic backgrounds. The scyllo-inositol dehydrogenase activities of the iolR and iolE mutants were also determined. The iolR mutant displayed an almost seven-fold higher scyllo-inositol dehydrogenase activity than the wild type (Table 2.4). The scyllo-inositol dehydrogenase activity of the iolE mutant was very low (Table 2.4). When the scyllo-inositol dehydrogenase activity was determined from cultures grown with 2KMI as inducer, similar results were obtained (Table 2.4).

**Determination of D-chiro-inositol dehydrogenase activity**

The 2011 wild type and the corresponding idhA and iolR mutants were subjected to a D-chiro-inositol dehydrogenase assay. The wild type displayed a D-chiro-inositol dehydrogenase activity of $52 \pm 0.2$ nmol min$^{-1}$ mg protein$^{-1}$, while the idhA mutant showed only marginal dehydrogenase activity in the presence of D-chiro-inositol ($0.3 \pm 0.1$ nmol min$^{-1}$ mg protein$^{-1}$). The D-chiro-inositol
dehydrogenase activity of the WIOLR mutant was four-fold increased (235 ± 10 nmol min⁻¹ mg protein⁻¹).

**Competition assay for nodule occupancy**

To investigate the role of *S. meliloti* inositol catabolism during symbiotic interactions, a competition assay for nodule occupancy was performed with the 2011 wild type and each of the *idhA, smc01163, iolR, iolC, iolD, iolE* and *iolA* mutants. The wild type and the individual mutant strains were inoculated onto axenic alfalfa plants in a 1:1 ratio and as single inoculants as controls. All strains nodulated the host plants successfully when inoculated individually. Plants developed six nodules on average, and there was no difference between the average fresh and dry weight of the plants nodulated by the wild type (avg. fresh weight 131 ± 12 g, avg. dry weight 14 ± 2 g) or by the individual mutant strains (avg. fresh weight 138 ± 16 g, avg. dry weight 14 ± 2 g). Twenty weeks post inoculation, the nodules were harvested and rhizobia were re-isolated from surface-sterilized nodules. On average, 10⁶ bacteria were re-isolated from the nodules of one plant and the output ratio of kanamycin-resistant (mutant) to kanamycin-sensitive rhizobia (wild type) was determined by selective plating. If a gene does not play a role in the competition for nodule occupancy, the same output as input ratio (50:50) is expected. This was true for a control mutant (WGLYA) that carried a mTn5-STM insertion in an unrelated gene, since WGLYA was re-isolated from the nodules with a frequency of 60%, which was similar to the input ratio of 50% (Fig. 2.7). In contrast, the mutants with insertions in the *idhA,*
smc01163, iolR, iolC, iolD, iolE and iolA genes represented only a small fraction of the rhizobia re-isolated from the nodules. The values varied between 0.15% for the iolA and 25% for the smc01163 mutant (Fig. 2.7). Thus, the idhA, smc01163, and the iol mutants were out-competed by the wild type in all cases, showing that a functional inositol catabolic pathway, the transcriptional regulator IolR and the dehydrogenase encoded by smc01163 are all required for successful competition during alfalfa nodulation. We also determined the wild type/mutant output ratios after 10 and 15 weeks, with essentially the same result.

Figure 2.7: Competition assay for nodule occupancy. The S. meliloti 2011 idhA (WIDHA), smc01163 (W63-1, W63-2), iolR (WIOLR), iolC (WIOLC), iolD (WIOLD), iolE (WIOLE), iolA (WIOLA), and glyA2 (WGLYA) mutant strains were inoculated on alfalfa plants in a 1:1 ratio with the wild type. After 20 weeks nodules were harvested, surface sterilized and rhizobia were re-isolated from the nodules. The wild type (white) versus the mutant (black) output ratio of the re-isolated rhizobia was determined via selective plating. Bars represent the average of two independent experiments representing nodules from six plants each. Error bars denote ± SEM.
Discussion

We have shown that the *iolA* and *iolCDEB* genes are not only essential for *myo*-, but also for *scyllo-* and D-*chiro*-inositol catabolism in *S. meliloti*. It is interesting to note that *myo-* and especially *scyllo*-inositol serve as excellent carbon sources for *S. meliloti*, since the wild types grew to optical densities (OD$_{600}$) $>$1 in minimal medium, which is comparable to the growth with other C-sources such as glucose, glycerol or succinate. This is in contrast to *B. subtilis*, which does not grow as efficiently with *myo-* or *scyllo*-inositol as compared to glucose (25). Similarly, a lag time of 60 hours has been reported for *Salmonella enterica* when grown with *myo*-inositol (19).

The *idhA*-encoded *myo*-inositol dehydrogenase acts on *myo-* and D-*chiro*-inositol

The *idhA* mutant could not grow with *myo-* or with D-*chiro*-inositol as the sole C-source. As confirmation, cell extracts of the *idhA* mutant did not display any detectable dehydrogenase activity when *myo-* or D-*chiro*-inositol was offered as substrate in the enzyme assay. Thus, we conclude that the initial dehydrogenation of *myo-* as well as of D-*chiro*-inositol is carried out by the *idhA*-encoded dehydrogenase. This is not without precedent; the purified *myo*-inositol dehydrogenase of *B. subtilis* was shown to oxidize both, *myo-* and D-*chiro*-inositol (8). The *S. meliloti idhA* mutant was able to use *scyllo*-inositol as the sole C-source, indicating that there is at least one other dehydrogenase involved in the oxidation of *scyllo*-inositol and that *scyllo*
inositol is probably not a substrate for the myo-inositol dehydrogenase. In fact, scyllo-inositol could not react with the purified myo-inositol dehydrogenase of *B. subtilis* (32), and our preliminary data revealed that scyllo-inositol is not a substrate for the *idhA* gene product overexpressed in *E. coli* (P. Kohler and S. Rossbach; unpublished observation).

**Inositol catabolism in *S. meliloti* requires induction through a pathway intermediate**

The results from the *b*-glucuronidase and NAD(H)-dependent dehydrogenase assays clearly demonstrated that functional *idhA, iolC, iolD, iolE,* and *iolB* genes are required for induction of the *iol* genes. Thus, the inositol catabolism genes are not necessarily induced by inositol, but by a later pathway intermediate. This is comparable to *B. subtilis*, in which the binding of 2-deoxy-5-keto-D-gluconic acid 6-phosphate (DKGP) to the negative regulator IolR antagonizes the transcriptional repression of the *iol* genes (51). DKGP is the fifth intermediate in the pathway and the product of the IolC reaction (Fig. 2.2.1). Usually, myo-, scyllo-, and D-chiro-inositol occur together in soil (41), and that may explain the advantage a common pathway intermediate would have as an inducer over a specific inositol isomer.

**IolR negatively regulates the activities of the myo- and scyllo-inositol dehydrogenases**

The *iolR* mutant was able to grow with myo-, scyllo-, D-chiro-inositol and 2KMI as sole C-sources, demonstrating that the *iolR* gene is not a structural gene in
the inositol catabolic pathway. Nevertheless, the \textit{iolR} gene is required for the regulation of the \textit{myo-} and \textit{scyllo-}dehydrogenase activity in \textit{S. meliloti}, since the activity of both enzymes was 4-7 fold higher in the \textit{iolR} mutant than in the wild type (Tables 3 and 4). The \textit{S. meliloti} IolR, like IolR from \textit{C. crescentus}, belongs to the RpiR repressor family (5). In \textit{C. crescentus}, a conserved DNA sequence, \texttt{GGAAANATNCGTTCCA}, was identified in the promoter region of the \textit{iol} genes as probable IolR-binding site (5). Computational predictions revealed related motifs in \textit{S. meliloti} upstream of the \textit{idha}, \textit{iolR}, and \textit{iolC} genes (5). Interestingly, we detected a similar sequence \texttt{~80 bp upstream of the start codon of smc01163 (CGAAATAATATTTTCA).} Our biological data confirm that IolR represses the \textit{myo-} and \textit{scyllo-}inositol dehydrogenase activities. The presence of a putative IolR binding site upstream of \textit{iolR} and the constitutive expression of the \textit{iolR-gusA} fusion in the \textit{iolR} mutant indicate that IolR negatively regulates its own expression.

**The \textit{smc01163} gene is essential for \textit{scyllo-}inositol metabolism**

None of the three different \textit{smc01163} mutants constructed in two different strains was able to grow with \textit{scyllo-}inositol as the sole C-source. Thus, the putative dehydrogenase encoded by the \textit{smc01163} gene is essential for \textit{scyllo-}inositol catabolism. Nevertheless, the \textit{smc01163} mutants displayed 58 to 75\% of the wild-type dehydrogenase activity when \textit{scyllo-}inositol was offered as substrate in the enzyme assay. We can exclude unspecific \textit{NAD}\(^+\) reduction through background activity, because the background \textit{NAD}\(^+\) reducing activity was determined for each
cell extract in the absence of the substrate for slope correction. All strains showed little NAD$^+$ reducing background activity (1 to 5% of the myo- and scyllo-inositol dehydrogenase activities). In addition, we determined the enzyme activity in the presence of various substrate concentrations to ensure substrate specificity (data not shown).

Although smc01163 encodes a dehydrogenase essential for scyllo-inositol catabolism, this enzyme may not be the only dehydrogenase that interacts with scyllo-inositol. Recently, two scyllo-inositol dehydrogenases were identified in B. subtilis, IolX and IolW (25). Both purified enzymes reacted with scyllo-inositol, but only the iolX mutant showed impaired growth with scyllo-inositol as the sole C-source (25). Interestingly, our computational analysis of the smc01163-deduced protein predicts the presence of an N-terminal signal peptide. Thus, a periplasmic location of SMc01163 is probable. We conclude that the catabolism of scyllo-inositol in S. meliloti may require at least one additional, probable cytoplasmic enzyme. This might explain the results of the growth studies as compared to the results from the scyllo-dehydrogenase assays using crude cell extracts. SMc01163 seems to be essential for the initial interaction with scyllo-inositol in the periplasm, but this reaction is bypassed when the cell is lysed by sonication for the dehydrogenase assay. Another enzyme, not the one encoded by smc01163, oxidizes scyllo-inositol in the cytoplasm of S. meliloti, detectable as scyllo-inositol dehydrogenase activity in the enzyme assay with the crude cell extract. Further work will be necessary to experimentally verify
the periplasmic location of the \textit{smc01163}-encoded dehydrogenase and to identify the substrate(s) and product(s) of the purified enzyme.

\textbf{The inositol catabolism genes and their regulation are required for successful competition during alfalfa nodulation}

The \textit{idhA} mutants of \textit{S. meliloti} and \textit{R. leguminosarum} \textit{bv. viciae} nodulated their host plants and fixed nitrogen at the same levels as the wild-type strains (14, 15, 29). Nevertheless, the \textit{iolA} and \textit{iolD} mutants of \textit{R. leguminosarum} \textit{bv. viciae} could not compete with the wild type in a competition assay (14). Our results showed that the \textit{idhA}, \textit{smc01163}, \textit{iolA} and the \textit{iolRCDEB} mutants of \textit{S. meliloti} nodulated alfalfa successfully when inoculated onto plants individually, but in co-challenge experiments the mutants were out-competed by the wild type. This demonstrates that a functional inositol catabolism is required for \textit{S. meliloti} to successfully compete during the process of host nodulation and colonization of nodules. It is worth emphasizing that the \textit{iolR} mutant also could not compete against the wild type for nodule occupancy. The inositol catabolic genes are derepressed in the \textit{iolR} mutant, which should allow an even higher rate of inositol catabolism. Thus, also the correct regulation of the inositol catabolism genes appears to be required for \textit{S. meliloti} to successfully compete for nodule occupancy, suggesting that the role of inositol isomers extends beyond being a nutrient source.

Recently, some inositol isomers have become of great interest in the medical field because of their therapeutic potential (11, 21). Our study contributes a better
understanding of inositol metabolism and emphasizes its role in symbiotic nitrogen fixation and agriculture. Since legume crops such as alfalfa plants contain inositol (9), the presence of inositol compounds may act as one of the signals for rhizobia communicating the presence of potential host plants and may also sustain the rhizobia while they are moving towards the plant, during root hair attachment or multiplication in the infection thread or nodule. In fact, in soybean nodules induced by *Bradyrhizobium japonicum*, the second and third most abundant carbohydrates after sucrose are myo- and D-chiro-inositol with 1-2 mg per g of nodule fresh weight (39). Also, inositol has been found to be exuded into soil by legume plants (45). Other inositol derivatives, the rhizopines scyllo-inosamine and L-3-O-methyl-scyllo-inosamine, have been termed nutritional mediators, because they represent exclusive carbon and nitrogen sources for the rhizobial strains that carry the rhizopine catabolism genes (26, 36, 38, 43). Rhizopine and inositol catabolism are interrelated, because a functional inositol catabolic pathway was shown to be required for the catabolism of rhizopines (2, 15, 38). Clearly, inositol compounds and the ability to catabolize them play important nutritional or signaling roles in the symbiotic relationship between rhizobia and legume plants.
References


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CHAPTER 3

THE RPIR-LIKE REPRESSOR IOLR REGULATES INOSITOL CATABOLISM IN SINORHIZOBIUM MELILOTI

Manuscript in preparation

Abstract

*Sinorhizobium meliloti*, the nitrogen fixing symbiont of alfalfa, has the ability to catabolize *myo*-*, scyllo*- and *D-chiro*-inositol. Functional inositol catabolism (*iol*) genes are required for growth on these inositol isomers and they play a role during plant bacteria interactions. The inositol catabolism genes comprise the chromosomally encoded *iolA* and the *iolY(smc01163)RCDEB* genes, as well as the *idhA* gene located on the pSymB plasmid. Reverse transcriptase assays showed that the *iolYRCDEB* genes are transcribed as one operon. The *iol* genes were weakly constitutively expressed, but their expression was also strongly induced by *myo*-inositol. The putative transcriptional regulator of the *iol* genes, IolR, belongs to the RpiR-like repressor family. Electrophoretic mobility shift assays demonstrated that IolR recognized a conserved palindromic sequence (5'-GGAA5_{11}TTCC-3') in the upstream regions of the *idhA*, *iolY*, *iolR* and *iolC* genes. The autoregulatory function of IolR was confirmed using β-glucuronidase reporter gene assays. Further expression studies indicated that the late pathway intermediate 2-keto-5-deoxy-D-gluconic acid 6-phosphate (KDGP) functions as the true inducer of the *iol* genes. The *iolA* gene

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encoding methylmalonate semialdehyde dehydrogenase was not regulated by IolR and expressed constitutively. The iolA gene product does not seem part of the core inositol catabolic pathway, but rather plays a more general role in the metabolism of *S. meliloti*, including valine catabolism.

**Introduction**

Inositol compounds are present in plants and their rhizospheres (7, 36). Especially high concentrations have been found in legume plants (7, 34). *Sinorhizobium meliloti*, the nitrogen fixing symbiont of alfalfa, is able to catabolize myo-, scyllo- and D-chiro-inositol and requires a functional inositol catabolic pathway for successfully competing during host plant nodulation (18). Catabolism of myo-inositol has been linked to successful plant-bacteria interactions in other members of the *Rhizobiaceae* as well (10, 14).

The inositol catabolic (*iol*) genes are organized in single clusters in various species of the *Firmicutes* and *Enterobacteriaceae* (3, 16, 19, 35, 38). In contrast, the inositol catabolic genes are dispersed at three different loci in *S. meliloti*. Specifically, the *idhA* gene is located on the pSymB plasmid, while the *smc01163* and *iolRCDEB* genes are clustered together on the chromosome, and the *iolA* gene is located a further 400 kb away from the *iol* cluster (http://iant.toulouse.inra.fr/S.meliloti).
The proposed inositol catabolic pathway in *S. meliloti* is similar to the pathway in *Bacillus subtilis* (39) (Fig. 3.1). The initial oxidation of D-chiro- [I] and myo-inositol [II] in *S. meliloti* is carried out by the idhA-encoded myo-inositol dehydrogenase, yielding 2-keto-myoinositol [2KMI, IV] (11, 18). Growth with scyllo-inositol [III] as sole carbon source requires the dehydrogenase encoded by the smc01163 gene (18). The deduced gene product of smc01163 displays an N-terminal signal peptide suggesting a periplasmic location. So far, only two other scyllo-inositol dehydrogenases have been described from any organisms, namely IolW and IolX in *B. subtilis* (24). IolW and IolX are probable cytoplasmic enzymes and share limited identity with Smc01163 (26 and 27%, respectively). Based on these differences we suggest annotating smc01163 as iolY. The product of the IolY reaction is probably also 2KMI (18). The further degradation of 2KMI requires the iolE, iolD, iolB, iolC, and iolA gene products (18) (Fig. 3.1). The iolA gene encodes the methylmalonate semialdehyde dehydrogenase, which oxidizes both methylmalonate semialdehyde or malonate semialdehyde, and therefore iolA has been renamed mmsA in *B. subtilis* (http://genolist.pasteur.fr/SubtiList).

Our previous work indicated that the iolR gene product is involved in the regulation of the structural inositol catabolic genes. The myo-, as well as the scyllo-inositol dehydrogenase activities were highly upregulated in an *S. meliloti* IolR mutant, even under non-inducing conditions (18). Also, plasmid-borne gene fusions
to the idhA and iolC genes were no longer repressed in an iolR mutant of *Rhizobium leguminosarum* bv. *viciae* (15). The deduced IolR protein belongs to the RpiR-like transcriptional regulator family, and contains a predicted DNA-binding helix-turn-helix motif at its N-terminus and a predicted phosphosugar binding site at its C-terminus. Conserved putative IolR-binding motifs were identified in the regions upstream of the *S. meliloti* idhA, iolY, iolR and iolC genes (4, 18). These findings, together with our result that iolR mutants cannot compete with the wild type for nodule occupancy (18), prompted us to intensify our studies analyzing the role of IolR in *iol* gene expression.
Here, we report the transcriptional organization of the *iol* genes and their regulation through IolR based on results from reverse transcriptase (RT)-PCR and electrophoretic mobility shift assays (EMSA). Gene expression studies and enzyme assays were used to elucidate the nature of the inducer of the *iol* genes. Moreover, a new role was found for the *S. meliloti* iolA gene in valine catabolism.

**Materials and Methods**

**Microbiological methods**

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria Bertani (LB) medium (31). Antibiotics for *E. coli* were ampicillin (Ap; 100 μg/ml) and tetracycline (Tc; 10 μg/ml). *S. meliloti* cultures were grown at 28°C. Rich medium for *S. meliloti* was tryptone yeast (TY) (2); minimal media were minimal M medium (29), with 0.1% (v/v) NH₄Cl as sole nitrogen (N-) source for the catabolism and enzyme assays or GTS minimal medium for the selection of exconjugants (17). Carbon (C-) sources were added to the minimal media at a final concentration of 0.2% (v/v) unless otherwise indicated. Antibiotics for *S. meliloti* were streptomycin (Sm; 250 μg/ml), kanamycin (Km; 200 μg/ml), and Tc (10 μg/ml). For the catabolism study with valine as sole C-source, *S. meliloti* precultures were inoculated 1:100 into liquid minimal M medium. Cultures were grown on a shaking incubator and the growth was determined spectrophotometrically at 600 nm every 48 hours for 10 days. Triparental
conjugations were performed according to the methods described by Rossbach and de Bruijn (28).

**DNA manipulations**

Preparation of plasmid DNA, DNA digests, agarose gel electrophoresis, cloning and transformation of *E. coli* cells were performed following established protocols (31).

**Expression of iolR and purification of IolR-His<sub>6</sub>**

The *iolR* gene was PCR-amplified from a liquid *S. meliloti* 2011 culture. Primers were engineered to contain an NdeI site at the 5’, and an EcoRI site at the 3’ end without the stop codon (Table 3.2). The purified PCR product was cloned into the expression vector pET21a creating a C-terminal His-tag fusion. DNA-sequencing confirmed the correct sequence of the IolR-fusion (Cornell, Life Sciences Core Laboratory Center, Ithaca, NY). *E. coli* BL21 Star<sup>TM</sup> (DE3) (Invitrogen, Carlsbad, CA) containing the resulting plasmid pPK64 (Table 3.1) was cultured in 5 ml LB (Ap) overnight and inoculated 1:100 in 250 ml flask containing 50 ml LB (Ap) and grown to an optical density of 0.6 (OD<sub>600</sub>). Expression of *iolR* was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 4 hours of incubation, the cells were harvested via centrifugation at 5,000 g and resuspended in 4 ml EW Buffer (50 mM sodium phosphate, pH 7, 300 mM NaCl). The cells were sonicated at 50 W with three 30 s periods (Misonix XL-2020, Farmingdale, NY) and a cell-free extract
was prepared by centrifugation at 20,000 g for 30 min at 4°C. A cobalt-based TALONspin™ column (Clontech Laboratories, Mountain View, CA) was equilibrated with 5 ml EW buffer. The cell-free extract was applied three times to the column using gravity flow. The resin was washed with 5 ml of EW Buffer. IolR-His₆ was eluted with 150 mM imidazol in EW buffer and four fractions were collected. The total protein concentrations of the eluted fractions were determined with the Coomassie Plus Protein Assay Kit (Pierce Biotechnology, Rockford, IL). The purity of the overexpressed IolR-His₆ was verified via SDS-PAGE and Coomassie staining (Fig. 3.2).

Fig. 3.2: SDS-PAGE of crude BL-21/(pPK64) extract and IolR₆₆₆₆ purification fractions. Lane 1: Protein-Marker; lane 2: crude BL-21 (pPK64) extract after lysis; lane 3: IolR₆₆₆₆ purification fraction 1; lane 4: IolR₆₆₆₆ purification fraction 2; lane 5: IolR₆₆₆₆ purification fraction 3; lane 6: IolR₆₆₆₆ purification fraction 5; lane 7: crude BL-21 (pPK64) extract after IolR₆₆₆₆ purification.
Table 3.1: Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strains, plasmids</th>
<th>Relevant characteristics, Phenotype</th>
<th>Reference</th>
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<tr>
<td><em>Escherichia coli</em></td>
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</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyr96 thi-1 relA1</td>
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<tr>
<td>BL21 Star™ (DE3)</td>
<td>F⁻ ompT hsdSB (rB' mB') gal dcm rnc131 (DE3)</td>
<td>Invitrogen</td>
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<td><em>Sinorhizobium meliloti</em></td>
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<td>1021</td>
<td>Wild type, SmR derivative of SU47</td>
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<tr>
<td>2011</td>
<td>Wild-type, SmR derivative of SU47</td>
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<td>TIOLB</td>
<td>1021 smc00432::pVO55, SmR, KmR</td>
<td>(26)</td>
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<td>WIDHA</td>
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<td>pRK2013</td>
<td>mob, tra, KmR</td>
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<td>pET21a(+)</td>
<td>Protein expression vector, ApR</td>
<td>Invitrogen</td>
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<td>pPK64</td>
<td>pET21a containing 857 bp fragment of iolR</td>
<td>This study</td>
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<td>pTE3</td>
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<td>This study</td>
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<td>pIOLD</td>
<td>pTE3 containing 2776 bp fragment of iolD</td>
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<td>pIOLE</td>
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<td>pIOLB</td>
<td>pTE3 containing 881 bp fragment of iolB</td>
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**Table 3.2: Primer used in this study**

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<tr>
<td>pet64EcoRIR</td>
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<td>2PKRiolRFNsi</td>
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<tr>
<td>RiolR-BamH1</td>
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<td>RTidhARev</td>
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<tr>
<td>RTiolYRFor</td>
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<tr>
<td>RTiolRCFor</td>
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<td>RTiolIDEFor</td>
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<td>C2iolYRev</td>
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<td>PiolRFor</td>
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</tr>
<tr>
<td>PnodD1Rev</td>
<td>CCGATGATCGTTATC</td>
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Underlined bases were modified.

Bold bases represent the first and last two bases of the IolR-binding motif (5'-GGAA_{11}TTCC-3').
Electrophoretic mobility shift assay (EMSA)

PCR fragments that contained the putative promoter sequences of the *idhA*, *iolY, iolR, iolC* and *iolA* genes as well as the known promoter of the *nodD1* gene were PCR-amplified from a liquid *S. meliloti* 2011 culture. The PCR fragments with sizes between 90 to 108 bp were used as substrates in the EMSA. Ten ng of DNA were mixed with increasing concentrations of IolR-His6 (0 - 0.5 μM) in binding buffer (25 mM HEPES, ph 7.5, 5 mM sodium acetate, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 200 ng/ml bovine serum albumine and 10% glycerol) in a total volume of 10 μl and incubated at 16°C for 1 h. The samples were loaded with 2 μl 6 X loading buffer (3 X Tris borate-EDTA buffer, 18% glycerol, 0.3% xylene cyanol, 0.03% bromphenol blue) on a native 5% polyacrylamide gel. Initially, 80 volts for 15 min were applied with ice-cold 0.5 X Tris borate-EDTA buffer at room temperature followed by 25 volts for 6 h at 4°C. The DNA was stained with ethidium bromide and visualized by UV.

Reverse transcriptase PCR of the *idhA, iolYRCDEB* and *iolA* genes

*S. meliloti* 2011 TY precultures were diluted 1:100 in 5 ml minimal M medium with 0.2 % glycerol or *myo*-inositol as sole C-sources. Five hundred μl of mid-exponential-phase cultures (OD₆₀₀ = 0.5) were harvested via centrifugation at 4,500 g for 10 min. Extraction of total RNA was performed using the Quick-RNA™ MiniPrep kit (Zymo, Irvine, CA) according to manufacturer’s instructions. One μl of the total RNA served as template in reverse transcriptase PCR using the One-Step-RT
PCR kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The primers used (Table 3.2) flank each of the intergenic regions in the *iolYRCDEB* gene cluster or are homologous to the intragenic regions of the *idhA* and *iolA* genes. The cytochrome C oxidase gene (*smc01981*) as control. To ensure that the RNA is DNA-free, a control PCR with the RNA as template was conducted. Initial generation of cDNA was performed at 55°C for 30 min. Heat inactivation of RT and activation of the Taq-polymerase was accomplished by heating at 95°C for 15 min. Amplification of cDNA was carried out in 30 cycles of, denaturation at 94°C for 1 min, primer annealing 59°C for 1 min, primer extension at 72°C for 1.5 min, followed by a final elongation step at 72°C for 10 min. The PCR-products were separated by electrophoresis on a 1% agarose gel.

**Complementation of the *S. meliloti* 2011 *iolR* mutant**

A DNA-fragment containing the *iolR* wild-type gene including its ribosomal binding site was PCR-amplified from a liquid *S. meliloti* culture with primers engineered with a NsiI at the 5’ and a BglII site at the 3’ end (Table 3.2). The PCR product was cloned into the broad host range expression vector pTE3 (Table 3.1) under the control of the *Salmonella trp* promoter, which is constitutively expressed in *S. meliloti* (8). The resulting plasmid pIOLR (Table 3.1) and pTE3 as empty vector control were introduced into *S. meliloti* *iolR* mutant via triparental mating. The presence of the wild-type and of the mutated *iolR* gene were confirmed via PCR.
**β-Glucuronidase assays**

The β-glucuronidase assays were carried out as described previously (18). Briefly, precultures of *S. meliloti* strains were diluted 1:100 in 5 ml minimal M medium containing *myo*-inositol, glycerol, glucose or succinate as sole C-sources or combinations thereof (final concentration 0.2%). The reaction rate was expressed in nmol *p*-nitrophenol produced min⁻¹ OD₆₀₀⁻¹ ± SEM. The values represent the mean of two independent experiments and each assay was carried out in duplicate. As control, the wild-type strain did not exhibit any detectable β-glucuronidase activity due to the absence of the *gusA* gene.

**NAD(H)-dependent *myo*-inositol dehydrogenase assays**

NAD(H)-dependent *myo*-inositol dehydrogenase assays were conducted as described previously (18). Briefly, *S. meliloti* precultures were diluted 1:100 into 500 ml Erlenmeyer flasks containing 100 ml minimal M medium with 0.1% NH₄Cl as N-source and 0.2 % glycerol or *myo*-inositol as C-source. The specific *myo*-inositol dehydrogenase activities were expressed as nmol NAD⁺ reduced min⁻¹ mg of protein⁻¹ ± SEM. The values represent the mean of two independent experiments, each of them performed in duplicate.
Results

**IolR binds upstream of the idhA, iolY, iolR and iolC genes**

The *S. meliloti* *iolR* gene encodes a protein of 284 amino acids. It displays a DNA-binding domain with a helix-turn-helix (HTH) motif at its N-terminus (residues 19 – 88, PFAM 01418), followed by a predicted C-terminal sugar isomerase (SIS) domain (residue 146 – 271, PFAM 01380). To verify the DNA-binding properties of IolR, the *iolR* gene was cloned in the pET21a vector, expressed in *E. coli* and IolR-His$_6$ was purified from the soluble fraction. The purified recombinant IolR-His$_6$ had an apparent molecular weight of 32 kDa on an SDS-PAGE (Fig. 3.2), which correlates well with its calculated molecular weight (31.99 kDa).

The upstream regions of the *idhA, iolY, iolR* and *iolC* genes each contain variations of a putative IolR-binding motif (5’-GGAA$_5$.11TTCC-3’) (Fig. 3.3). We conducted an electrophoretic mobility shift assay to investigate the IolR-DNA interactions *in vitro*. Increasing concentrations of the purified IolR-His$_6$ protein (0 – 0.5 μM) were added to 10 ng of eight individual DNA fragments. Five different fragments represented the upstream regions of the *idhA, iolY, iolR, iolC* and *iolA* genes. DNA shifts were observed for the *idhA, iolY, iolR* and *iolC* fragments (Fig. 3.3A). The binding of IolR to the upstream region of its own gene confirms its function as an autoregulator. Retardation of the fragments was achieved by IolR concentrations as low as 0.03 μM and increased with increasing protein concentration.
(Fig. 3.3A). The upstream region of *iolA* lacks the predicted IolR-binding motif and no DNA shift was visible, indicating that the *iolA* gene is regulated independently of IolR (Fig. 3.3A). The promoter region of the *nodD1* gene served as an IolR-independent negative control and no DNA retardation occurred, as expected (Fig. 3.3A). Two additional control fragments were included to verify the requirement of the putative IolR-binding site for the IolR-DNA interaction. Control fragment 1 contained the DNA sequence of the *iolY* regulatory region upstream of the predicted IolR-binding motif including the first two bases of the motif, while control fragment 2 contained the sequence downstream of the predicted motif including its last two bases. No DNA retardation was visible for controls 1 and 2 (Fig. 3.3B). Thus, IolR did not bind to the sequences flanking the predicted binding motif in the putative *iolY* promoter, confirming the requirement of the conserved consensus sequence for IolR-binding.

**Transcriptional organization of the *iol* genes**

An operon prediction program (www.microbesonline.org) suggests that the *idhA, iolA, iolY* and *iolR* genes are monocistronically transcribed while *iolCDEB* comprise an operon. This prediction is supported by the finding that IolR binds to the upstream regions of *idhA, iolY, iolR* and *iolC* (Fig. 3.3A). To elucidate the *iol* operon structure, RT-PCR was performed using the total RNA isolated from *S. meliloti* 2011 grown in minimal medium containing *myo*-inositol or glycerol as sole C-source. cDNA was successfully amplified from all five intergenic regions of the *iolYRCDEB*
Figure 3.3: IolR – DNA binding assay. Increasing concentrations of purified IolR-His$_6$ protein (indicated at the top of each lane and the figure) were incubated with 10 ng of 8 different PCR products. The positions of free DNA (open arrowheads) and IolR-DNA complexes are indicated (solid arrowheads). As control 0.3 μM of protein without DNA in binding buffer were loaded. (A) EMSA with the upstream regions of idhA, iolY, iolR, iolC, iolA and nodD1 (IolR-independent control). (B) The identified IolR-binding motifs of the idhA, iolY, iolR and iolC genes; controls 1 and 2 DNA fragment containing the sequences up- and downstream of the IolR-binding motif of iolY. M denotes the 100 bp ladder
cluster, confirming that these genes are transcribed in one single mRNA (Fig. 3.4). Quantitative differences were found in the amount of the amplified cDNA. In all instances, the PCR fragments showed greater intensity when they were amplified from the RNA of cells grown with myo-inositol than with glycerol as sole C-source. Thus, we conclude that the \textit{iolYRCDEB} operon is expressed at low levels when \textit{S. meliloti} is grown on glycerol but its expression increased when cells are grown with myo-inositol (Fig. 3.4). We also conducted RT-PCR to investigate the expression of the separately located \textit{idhA} and \textit{iolA} genes. The cDNA was amplified from the intragenic regions of both genes when grown on glycerol and expression significantly increased if grown with myo-inositol (Fig. 3.4). The \textit{smc01981} gene, encoding a putative cytochrome C oxidase, served as control. The amount of cDNA produced from its intragenic region was the same, regardless if \textit{S. meliloti} was grown with glycerol or myo-inositol (Fig 3.4). The size of the cDNA fragments corresponded to the size of the fragments obtained from the \textit{S. meliloti} genomic DNA (between 106 and 627 bp, compare with Fig. 3.5). The isolated RNA was free of DNA, as no cDNA was produced in the no RT control reactions (Fig 3.4, lanes indicated with “no RT”).
Figure 3.4: Transcriptional organization of *S. meliloti* inositol catabolic genes. The 2011 wild type was grown in minimal medium with *myo-*inositol (MI) or glycerol (Gly) as sole carbon source. The total RNA was purified and used as a template in RT-PCR (RT). cDNA was amplified with primers flanking the indicated intergenic regions of the *iolYRCDEB* cluster and the intragenic regions of the *idhA*, *iolA* and *smc01981* (cytochrome C, cC) genes. Boxes represent the individual inositol genes and arrows represent transcriptional units. As controls, PCR was performed with RNA samples (no RT) and no template (H₂O).

Figure 3.5: Control PCR for the primers used in the RT-PCR with an *S. meliloti* liquid culture. Lane 1: *idhA*; lane 2: *iolY-iolR*; lane 3: *iolR-iolC*; lane 4: *iolC-iolD*; lane 5: *iolD-iolE*; lane 6: *iolE-iolB*; lane 7: *iolA*; Lane 8: *cycC*.
Regulation of *iol* gene expression

The *iolR, iolD* and *iolE* mutants of *S. meliloti* 2011 contain mTn5-STM::*gusA* insertions in the same orientation as the respective genes, creating transcriptional fusions that allow the investigation of *iolRDE* gene expression by determining the β-glucuronidase activity (26). (The mTn5-STM in the *iolC* mutant is oriented in the opposite direction as the *iolC* gene, and no transposon induced *iolB* mutant is available in *S. meliloti* strain 2011).

It was previously shown in growth studies that cloned fragments carrying the individual *iol* genes complement the *iolR, iolC, iolD, iolE* and *iolB* mutants (18). Here we used the same mutant strains expressing the corresponding *iol* genes from the constitutive promoter of the pTE3 vector (8) to study *iol* gene induction. The *iolR, iolD*, and *iolE* mutants containing the empty pTE3 vector served as control. All strains were grown in minimal medium with glycerol or myo-inositol as sole C-source. The WIOLR/pTE3 mutant displayed high β-glucuronidase activities under both growth conditions, hence *iolR* is constitutively expressed in its corresponding mutant (Fig. 3.6). The *iolR* gene was not expressed in the complemented WIOLR/pIOLR strain when grown with glycerol, but *iolR* expression was induced in the presence of myo-inositol (Fig. 3.6).

The WIOLD/pTE3 and WIOLE/pTE3 mutants displayed very low β-glucuronidase activities regardless if grown with glycerol or myo-inositol. Nevertheless, the *iolD* and *iolE* gene expression was induced in the complemented WIOLD/pIOLD and
WIOLE/pIOLE mutants when grown with myo-inositol but not with glycerol (Fig. 3.6). This shows that functional iolD and iolE genes are required for inducer production, supporting the notion that not myo-inositol itself, but a pathway intermediate, functions as the inducer of the iol genes in S. meliloti.

![Graph showing glucuronidase activities of the S. meliloti iolR-, iolD- and iolE-gusA reporter gene fusions in the respective mutant strains.](image)

**Fig. 3.6:** \(\beta\)-Glucuronidase activities of the S. meliloti iolR-, iolD- and iolE-gusA reporter gene fusions in the respective mutant strains. The WIOLR/pTE3, WIOLD/pTE3 and WIOLE/pTE3 strains harbor the empty vector pTE3 as control, while the WIOLR/pIOLR, WIOLD/pIOLD and WIOLE/pIOLE strains are expressing the corresponding wild type genes from pTE3. The reaction rate is expressed in nmol p-nitrophenol produced per minute per OD\(_{600}\). Bars represent the average of two independent experiments and error bars denote ± SEM. Cultures were grown in minimal medium containing glycerol (Gly) or myo-inositol (MI) as sole carbon source.

**Regulation of the myo-inositol dehydrogenase activity**

To identify the metabolic step that produces the inducer of the iol genes, we carried out myo-inositol dehydrogenase assays with the S. meliloti wild type and the complemented iol mutant strains. Previously no myo-inositol dehydrogenase activity
was detectable in the individual \textit{iolCDEB} mutants even in the presence of \textit{myo}-inositol (18). No \textit{iolB} mutant was available in the \textit{S. meliloti} strain 2011; therefore an \textit{iolB} vector insertion mutant of \textit{S. meliloti} 1021 was used (18). The \textit{iolR}, \textit{iolC}, \textit{iolD}, \textit{iolE} and \textit{iolB} mutant strains carrying the empty pTE3 vector served as control. Bacterial cultures were grown in minimal medium containing glycerol or \textit{myo}-inositol as sole C-sources. The specific \textit{myo}-inositol dehydrogenase activities of the wild types 2011 and 1021, carrying the empty pTE3 vector as control, were low in the absence of \textit{myo}-inositol (11 and 2 nmol min$^{-1}$ mg of protein$^{-1}$, respectively; Fig. 3.7). When grown with \textit{myo}-inositol, the wild type strains displayed \textit{myo}-inositol dehydrogenase activities of 142 and 169 nmol min$^{-1}$ mg of protein$^{-1}$, respectively (Fig. 3.7). The WIOLR/pTE3 strain displayed a four- to five-fold higher \textit{myo}-inositol dehydrogenase activity than the wild type, even when grown with glycerol as sole C-source (Fig. 3.7). The \textit{myo}-inositol dehydrogenase activity of the complemented WIOLR/pIolR strain was comparable to wild-type activity when grown with \textit{myo}-inositol and only low \textit{myo}-inositol dehydrogenase activity was detectable when grown with glycerol (Fig. 3.7). The WIOLC/pTE3, WIOLD/pTE3, WIOLE/pTE3 and TIOLB/pTE3 strains exhibited very low \textit{myo}-dehydrogenase activities when grown with glycerol (Fig. 3.7). These strains do not grow with \textit{myo}-inositol as sole C-source therefore enzyme activities could not be determined. Previously \textit{myo}-inositol dehydrogenase activities were not detectable in the individual \textit{iolCDEB} mutants even in the presence of \textit{myo}-inositol (18). The \textit{myo}-inositol dehydrogenase activities were low in the WIOLC/pIOLC, WIOLD/pIOLD, WIOLE/pIOLE and TIOLB/pIOLB
mutants when grown with glycerol as sole C-source. The complemented strains displayed myo-inositol dehydrogenase activities comparable to the wild type activity when grown with myo-inositol (Fig. 3.7). The iolA mutant is affected in the final step of the proposed inositol catabolic pathway (Fig. 3.1). Interestingly, the myo-inositol dehydrogenase activity of this mutant was two-fold increase, 268 nmol min$^{-1}$ mg of protein$^{-1}$, as compared to the wild type. The increased activity could be due to inducer accumulation in this mutant strain. These findings allow the conclusion that the iolCDEB gene products, but not the iolA gene product, are required for the production of the inducer.

Figure 3.7: NAD(H)-dependent myo-inositol dehydrogenase assay with crude cell extracts obtained from S. meliloti wild-type and mutant strains grown in minimal medium containing 0.2% glycerol (Gly) or myo-inositol (MI) as sole carbon. The reaction rate is expressed in nmol NAD$^+$ reduced per minute per mg protein. Bars represent the average of two independent experiments and error bars denote ± SEM. The WIOLC/pTE3, WIOLD/pTE3, WIOLE/pTE3 and TIOLB/pTE3 strains did not grow in minimal medium with myo-inositol as sole carbon source.
The *iolA* gene is constitutively expressed

The 2011 *iolA* mutant contains a transcriptional *gusA* fusion, allowing the investigation of *iolA* expression (26). Since the *iolA* gene is required for the growth with inositol, the respective mutant cannot grow if inositol is the only C-source (4, 18). Therefore, the *iolA* mutant was grown in minimal medium with either glycerol, glucose or succinate as sole C-sources or in combination with myo-inositol for the analysis of gene expression with the β-glucuronidase assay. The *iolA-gusA* fusion was constitutively expressed at high levels in the corresponding mutant and the expression increased approximately two-fold in the presence of myo-inositol, when grown with glycerol or glucose, but remained the same if grown with succinate and myo-inositol (Fig. 3.8). In general, the *iolA::gusA* fusion expressed at much higher levels than the expression of *iolR*, *iolD* and *iolE* fusions (Fig. 3.7 and 3.8).

![Figure 3.8: β-Glucuronidase activities of the *S. meliloti iolA-gusA* reporter gene fusion in the *iolA* mutant strain. The WIOLA mutant was grown in minimal medium containing the following carbon sources: myo-inositol (MI), glycerol (Gly), glucose (Glu), succinate (Suc) or combinations thereof at a final concentration of 0.2%. Bars represent the average of two independent experiments and error bars denote ± SEM.](image-url)
The *iolA* gene is required for valine catabolism

The finding that the *iolA* gene is constitutively expressed and not co-regulated with the other inositol catabolic genes indicates that its gene product is not part of the central inositol catabolic pathway, but might play a more general role in the metabolism of *S. meliloti*. The *iolA*-encoded methyl malonate semialdehyde dehydrogenase (MmsA) is known to be required for valine metabolism in *Pseudomonas spp.* (1, 27, 33). Therefore, a possible role of IolA in valine metabolism of *S. meliloti* was investigated by conducting a growth study in minimal medium with 0.2% valine as sole C-source. The *iolA* mutant did not grow with valine as sole C-source, whereas the wild type reached an OD$_{600}$ of 1.3 after 10 days (Fig. 3.9).

![Figure 3.9: Growth of the *S. meliloti* wild-type strain 2011 (open circles) and the corresponding *iolA* mutant (solid squares) with 0.2% valine as sole carbon source in minimal medium. The optical density was determined spectrophotometrically at 600 nm. Bars represent the average of two independent experiments, each performed in duplicate. Error bars denote ± SEM.](image-url)
Discussion

The transcriptional repressors of the inositol catabolic pathways in *Firmicutes* and Gram-negative bacteria belong to different families of regulatory proteins, although all are designated as IolR based on their function. The *iolR* gene-products in *Firmicutes* belong to the DeoR family, while the IolR proteins in Gram-negative bacteria including *S. meliloti* are RpiR-like transcriptional regulators (4, 16, 38, 41). Structural analysis of the predicted IolR protein revealed a putative DNA-binding HTH motif at the N-terminus followed by a SIS domain that is predicted to bind phosphosugars. Members of the RpiR-family have been shown to function as positive and negative transcriptional regulators. In *B. subtilis*, maltose metabolism is regulated by an RpiR-like transcriptional activator (37). RpiR repressors control ribose and N-acetylmuramic acid catabolism in *Escherichia coli* as well as glucose metabolism in *Pseudomonas putida* (6, 13, 32). The inositol catabolism genes of *Caulobacter crescentus* and *Salmonella enterica* are negatively regulated by functional homologs of the *S. meliloti* RpiR-like IolR (4, 19).

BLAST analysis of the predicted IolR protein showed that this putative RpiR-like regulator is well conserved in all *Rhizobiaceae* that possess *iol* genes. These include the symbiotic nitrogen-fixing genera *Sinorhizobium*, *Mesorhizobium* and *Rhizobium* as well as the genera containing pathogenic *Agrobacteria* and *Brucella* strains that infect plants, animals and humans. Therefore information derived from
studies in *S. meliloti* is relevant to understanding the regulation of inositol catabolism in other *Rhizobiaceae* and its possible requirement for host-bacteria interactions.

**A conserved IolR-binding motif is required for IolR-DNA interactions**

The purified IolR-His<sub>6</sub> bound to the regulatory sequences of the *idhA*, *iolY*, *iolR* and *iolC* genes, which contain variations of the conserved IolR-binding motif 5′-GGAA<sub>5-11</sub>TTCC-3′ (Fig. 3.3), but it did not interact with the upstream region of *iolA*, which lacks the motif (Fig. 3.3). IolR is regulating its own expression as well as the expression of the inositol catabolism genes at at least two different loci: the *idhA* gene on pSymB and the *iolYRCDEB* operon on the chromosome. In addition, IolR did not bind to the fragments containing the sequences up- and downstream of the conserved I motif of the *iolY* gene, indicating that it was required for IolR-DNA-interactions (Fig. 3.3).

**Induction of inositol catabolism**

IolR does not completely repress *idhA* and *iolYRCDEB* expression in the absence of inositol. The reverse transcriptase PCR revealed that the *iol* genes are weakly constitutively expressed (Fig. 3.4), suggesting that the inositol catabolic enzymes are always present in the cytoplasm at low concentrations. This is confirmed by the results of the *myo*-inositol dehydrogenase assays with an uninduced *S. meliloti* wild-type and its *idhA* mutant. The *myo*-inositol dehydrogenase activity of the wild-type cell extract was significantly higher than the activity of the *idhA* mutant and the
NAD\textsuperscript{+} reducing background activity of both strains, even if grown without inositol (11, 18). The constant presence of the inositol catabolic enzymes is probably required for the production of the IolR-antagonizing effector. We have shown previously that the myo-inositol dehydrogenase activity absent in the \textit{iolC}, \textit{iolD}, \textit{iolE} and \textit{iolB} mutants (18). Here, we demonstrated that the \textit{iolC}, \textit{iolD}, \textit{iolE} and \textit{iolB} genes and their gene products are required for induction of \textit{iol} gene expression, exemplified by the results of the \textbeta-glucuronidase and myo-inositol dehydrogenase assays (Figs. 3.6 and 3.7). It is important to note that the myo-inositol dehydrogenase activity of the \textit{iolA} mutant was two-fold increased as compared to the wild type. The \textit{iolA} gene product, the methyl malonate semialdehyde dehydrogenase, is proposed to be the last enzyme of the inositol catabolic pathway (Fig. 3.1). The increased myo-inositol dehydrogenase activity of the \textit{iolA} mutant supports that the inducer of the \textit{iol} genes is produced by the action of IolCDEB. The increased myo-insositol dehydrogenase activity could be due to some kind of feedback regulation or even inducer accumulation.

The IolC reaction is the only step in the inositol pathway that yields a phosphosugar, 2-keto-5-deoxy-D-gluconic acid 6-phosphate (KDGP), which is likely to bind to the C-terminal SIS domain of IolR. Although \textit{iolC} is an early gene in the \textit{iolYRCD} operon, its gene product catalyzes the last step of the inositol catabolic enzyme (Fig 3.1). KDGP has been identified as the inducer of inositol catabolism in \textit{B. subtilis} (40). It is interesting to note that the Entner-Doudoroff pathway
intermediate, 2-keto-3-deoxy-D-gluconic acid 6-phosphate, a structurally similar phosphosugar, binds to the RpiR-like HexR repressor and induces glucose metabolism in *P. putida* (6).

KDGP is the substrate for the IolJ aldolase in the proposed inositol catabolic pathway of *S. meliloti* and *B. subtilis* (Fig. 3.1, (39)). The *myo*-inositol dehydrogenase activity of an *iolJ* mutant would be expected to be strongly increased, because of inducer accumulation. Unfortunately, a gene coding for the IolJ aldolase has not yet been identified in *S. meliloti*. The *iolJ* gene has been annotated as *fbaB* in *B. subtilis* ([http://genolist.pasteur.fr/SubtiList](http://genolist.pasteur.fr/SubtiList)), since it functions as the fructose-1,6-bisphosphate aldolase in the glycolytic pathway. An *S. meliloti* mTn5-STM *fbaB* mutant was not affected in its ability to grow with *myo*-inositol as the sole carbon source (P. Kohler and S. Rossbach; unpublished observation). The *S. meliloti* genome contains two additional genes coding for putative aldolase (*cbbA1* and *cbbA2*). KDGP might be a substrate for FbaB, CbbA1 and CbbA2. The role of these putative aldolases in *S. meliloti* inositol catabolism remains to be determined.

*S. meliloti iol gene regulation is fine-tuned*

The occurrence of three regulatory regions in the *iolYRCDEB* operon suggests that the operon is under the control of the *piolY* and contains two additional operators. For example, three operators have been identified in the prototypical *E. coli lac* operon, in which DNA looping is a feature of regulation by the Lac repressor (25, 30). Interestingly, the sequences of the conserved IolR-binding motifs in the
The structure of $\sigma^{70}$-dependent promoters in *S. meliloti* has been elucidated as 5'-CTTGAC-N$_{17}$-CTATAT-3' (20). The -35 region is fairly well conserved and can start with a C, a G or an A, while the -10 region is poorly conserved and very diverse (20). We screened the upstream sequences of the IolR-dependent *idhA* and *iolYRCDEB* genes for known promoter structures in *S. meliloti*. No obvious promoter sequences were found upstream of the the *iolR* and *iolC* genes. However, the upstream region of the *idhA* and *iolY* genes contain possible -35 and -10 regions of $\sigma^{70}$-dependent promoters, 5'-CTTGAC- N$_{17}$-AATAAA-3' and 5'-ATTGAC- N$_{17}$-TTTCAT-3', respectively. The IolR-binding motif of the *idhA* gene overlaps with the predicted -10 region of *pidhA* and involves the nucleotides -17 to -5, while the IolR-binding element of *piolY* is located directly upstream of the -35 region and stretches between the nucleotides -38 and -51 (Fig. 3.10).
Our current model of the IolR-mediated regulation of the *S. meliloti* *iol* genes is summarized in Fig. 3.11. Upon entering the cell, inositol is catabolized and converted into KDGP, which is either further metabolized (Fig. 3.1), or binds to IolR and releases the repressor from its target promoters and operators, including its own. Therefore, not only the inositol catabolic enzymes but also IolR are being produced upon induction, creating a balance in the concentrations of the repressor and its effector KDGP as long as inositol is present. *S. meliloti* does not seem to catabolize inositol at a maximum rate. This notion is supported by the finding that the *myo-* and *scyllo-*inositol dehydrogenase activities were 4- to 5-fold higher in the *iolR* mutant as compared to the wild type (18).

Four putative inositol transporter genes are located in the genome of *S. meliloti* (http://iant.toulouse.inra.fr/S.meliloti, (21)). We identified a putative IolR-binding motif (5′-GGAA$_{5,11}$TTCC-3′) upstream of the *smb20072* gene that encodes a periplasmic soluble protein. The *ibpA* (*smc20712* previously *mocB*), *iatA* (*smc20713*),

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**Figure 3.10:** Predicted promoter structures and regulatory elements of the *iolY* (A) and *idhA* (B) genes.
iatP (smc20714) genes are arranged in one cluster and encode the three components of a typical ABC-transporter. The ibpA promoter region is predicted to be a σ^{70}-dependent promoter (20) and also contains a putative IolR-binding motif (5’-AGAA₅₆TTCC-3’) (4), but no IolR-binding motif could be identified upstream of iatA and iatP, suggesting that ibpA, iatA and iatP comprise an operon. Inositol transport has yet to be investigated in S. meliloti but our findings suggest that the transport genes are also IolR-regulated.

Figure 3.11: Model for IolR-mediated iol gene regulation. IolR binds to the conserved binding motif GGAAANATNCGGTCCCA in the regulatory region of the idhA gene and in the three regulatory regions in the iolYRCDEB operon in the absence of inositol, but does not repress transcription completely. Inositol is being catabolized as soon as it is imported into the cell and a late pathway intermediate, 2-deoxy-5-keto-D-gluconic acid 6-phosphate antagonizes the IolR-mediated transcriptional repression. The mmsA (iolA) gene is constitutively expressed and not subjected to IolR regulation, but iolA expression is nevertheless increased in the presence of inositol.
The *iolA* gene product is not part of the core inositol catabolic pathway

The *iolA* gene encodes a methyl malonate semialdehyde dehydrogenase, the last enzyme in the proposed inositol catabolic pathway, and was shown to be required for the catabolism of *myo*, *scyllo*-, and D-*chiro*-inositol in *S. meliloti* (4, 18). The expression of *iolA* is dependent of IolR in *B. subtilis* and *S. enterica* (19, 40). This is different in *S. meliloti*, since we found that the *iolA* gene is not only constitutively expressed in the corresponding mutant, but expressed at higher levels than the other inositol catabolism genes and subject to catabolite repression by succinate (Fig. 3.8). We have shown earlier that succinate does not affect *iol* gene expression (18). Most importantly, the *iolA* promoter does not contain an IolR-binding motif and no DNA retardation was visible in the EMSA of *pioL* by the purified IolR protein. Analysis of genes functionally related to *iolA* indicates that its expression could possibly be regulated by a transcriptional regulator of the LysR family, Smc00780, encoded directly upstream of *iolA* (www.microbesonline.org).

We found that the *iolA*-encoded methyl malonate semialdehyde dehydrogenase (MmsA) is not only involved in the catabolism of inositol isomers, but it is also required for the catabolism of valine (Fig. 3.9). In addition, it is interesting to note that the initial growth of the *S. meliloti* 2011 *iolA* mutant strain in full medium and minimal medium containing glycerol, glucose or succinate as sole C-source was delayed within the first 24h as compared to the wild type strain (P. Kohler and S. Rossbach; unpublished observation). Our findings that the *iolA* gene is constitutively expressed, not regulated by IolR and also plays a role in valine metabolism, support
the notion that in *S. meliloti* the methyl malonate semialdehyde dehydrogenase is not part of the central inositol catabolic pathway, which comprises the *idhA*, *iolY*, and *iolCDEB* gene products. Based on these findings we suggest annotating *iolA* as *mmsA*, as it has been done with its homologs in *Pseudomonas spp.* and *B. subtilis* ((1, 27, 33), http://genolist.pasteur.fr/SubtiList).

**Additional regulatory factors are involved in *iol* gene regulation**

In this study we have confirmed the role of IolR as the transcriptional repressor of *S. meliloti myo-, scyllo-, and d-chiro*-inositol catabolism. Although IolR-independent, the level of *mmsA* gene expression is still inducible by inositol (Fig. 3.8). This indicates the presence of additional regulatory mechanisms that modulate *mmsA* gene expression and possible the expression of the inositol catabolism genes. In fact, the *S. meliloti iol* genes are co-regulated with signaling pathways important for the establishment of the nitrogen-fixing symbiosis. For example, the IdhA protein was found to accumulate in response to the activation by the SinI/ExpR quorum sensing system, which contributes to the efficiency of nodule initiation (12). Also, the transcription of the *iolY* and the *iolCDEB* genes was found to be positively regulated by the ExoS/ChvI two-component system, which is required for symbiotic development (5). Interestingly, not only the mutants with insertions in the structural inositol catabolic genes, but also the *iolR* mutant were outcompeted by the *S. meliloti* wild type in a co-challenge experiment for nodule occupancy (18). Thus, the regulatory mechanisms of the inositol catabolism play an important role during plant-
bacteria interactions, supporting the hypothesis that inositols are part of the complex signaling circuit required for an efficient *Rhizobium*-legume symbiosis.

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CHAPTER 4

THE RHIZOPINE SCYLLO-INOSAMINE IS A NUTRITIONAL MEDIATOR IN THE SINORHIZOBIUM MELiloti L5-30 – ALFALFA NITROGEN-FIXING SYMBIOSIS

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Abstract

Rhizopines, scyllo-inosamine (SIA) and L-3-O-methyl-scyllo-inosamine (3-O-MSI), have been proposed to play a role as nutritional mediators during the establishment of the symbiotic relationship between legumes and rhizobia. The genes involved in the synthesis (mos genes) and catabolism (moc genes) of rhizopines were originally identified in Sinorhizobium meliloti L5-30. The nitrogen-fixing bacteroids of S. meliloti L5-30 produce the rhizopines under symbiotic conditions in the nodule. The rhizopines are being exuded into the rhizosphere, where they are predicted to serve as a proprietary carbon source for the free-living kin of the bacteroids. The mechanisms of rhizopine nutritional mediation is not well understood. So far, studies of rhizopine metabolism have been limited to the use of crude rhizopine isolated from the root nodules. Here we confirmed SIA as a rhizopine, its chemical structure and biological activity in the S. meliloti L5-30 – alfalfa nitrogen-fixing symbiosis. A catabolism assay in minimal medium with chemically synthesized SIA as sole carbon and nitrogen source showed that the moc genes previously shown to be essential for the
degradation of the crude rhizopines are also essential for the utilization of the chemically synthesized SIA. In addition, a co-challenge experiment revealed that mutants with insertions in the *mocA*, *mocC*, and *mocR* genes could no longer compete with the wild type for host plant nodulation.

**Introduction**

Beneficial soil bacteria of the *Rhizbiaceae* family induce nodules on the roots of legume plants, which they inhabit and fix atmospheric nitrogen symbiotically (8). The rhizobia incorporate the nitrogen into glutamate, which they provide for the host plant in exchange for C4 dicarboxylates (succinate, malate) and glucose (8). A constant signal exchange between the macro- and the microsymbiont is required at all stages of the symbiotic relationship. The signaling system is extremely complex and involves a great variety of plant- and bacteria-derived compounds (8).

An interesting group of signals are nutritional mediators, compounds that are proposed to function as plant-derived signals, but also serve as a nutrient source for the rhizobia in the rhizosphere. The concept of nutritional mediation in symbiotic nitrogen fixation was introduced after the discovery of a unique group of symbiotic compounds, the rhizopines. The nitrogen-fixing bacteroids produce the rhizopine probably from a so far unknown plant-derived precursor (18, 25, 27). Rhizopines have been isolated from the root nodules and are considered to be inositol derivatives.
Their suggested structures are scyllo-inosamine (SIA) and 3-O-methyl-scyllo-inosamine (3-O-MSI) (Fig. 4.1) (18, 27).

The ability to synthesize rhizopine is a rare trait among the rhizobia, that only 11% of the *S. meliloti* and 12% of *R. leguminosarum* bv. *viciae* strains possess (18, 25, 27, 29). These strains became known as rhizopine strains in order to distinguish them from non rhizopine producing strains. Rhizopine strains are thought to have an advantage over non-rhizopine strains during the competition process for nodule occupancy, because the rhizopines serve as a proprietary carbon source for the rhizopine strains (18, 29). This hypothesis has been widely publicized (5, 6, 15, 16, 20), although few biological data are available confirming the rhizopine concept. Nevertheless, it is necessary to mention that many of the *S. meliloti* and *R. leguminosarum* bv. *viciae* strains described as highly competitive and persistent in the literature are rhizopine strains (13, 23, 29).

![Figure 4.1: The proposed structures of the rhizopines: scyllo-inosamine (SIA) and 3-O-methyl-scyllo-inosamine (3-O-MSI).](image)

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The genes required for rhizopine synthesis and catabolism have first been identified in \textit{S. meliloti} L5-30 and comprise the \textit{mos} and the \textit{moc} gene cluster, respectively. Both loci are adjacent and located on the large symbiotic plasmids pSymA (17). The rhizopines are synthesized by the gene products of the \textit{mos} cluster under symbiotic conditions (18).

Rhizopine catabolism has been studied in \textit{S. meliloti} L5-30 and \textit{Rhizobium leguminosarum} \textit{bv. viciae} using crude rhizopine isolated from root nodules (2, 24). The \textit{moc} gene cluster comprises the \textit{mocC}, ORF334, ORF293, \textit{mocA}, \textit{mocB}, \textit{mocR}, \textit{mocD}, \textit{mocE} and \textit{mocF} genes, but only the gene products of \textit{mocABCDEFR} are required for the catabolism of the rhizopine (2, 24). The authors predicted the hypothetical functions of the \textit{moc} gene products based on homology. The gene product of \textit{mocC} seems to be a dehydratase related to the keto-inositol dehydratase encoded by \textit{iolE}. The ORF334 translates into an oxidoreductase of the GFO/IDH/MocA Family. No significant similarities were found for the ORF293. The hypothetical protein derived from \textit{mocA} also belongs to the GFO/IDH/MocA family and are high similar to the \textit{myo}-inositol dehydrogenase of \textit{S. meliloti} and \textit{B. subtilis}. A periplasmic binding protein of an ABC transporter is encoded by \textit{mocB}. The N-terminus of the \textit{mocR} gene product contains a helix-turn-helix motif and belongs to the GntR-like bacterial regulatory proteins, whereas the C-terminus has similarities to an aminotransferase. The \textit{mocD} gene product shows similarities with fatty acid
dehydrogenases/oxygenases, mocE encodes a Rieske-like ferredoxin and the mocF gene product share high similarities to ferredoxin reductases (2, 24).

Based on the suggested functions, Bahar et al. (1998) proposed a model for the catabolism of 3-O-MSI (2). MocB is involved in 3-O-MSI transport. The initial demethylation of 3-O-MSI to SIA is carried out by MocDEF that form a ferredoxin dependent oxygenase system. SIA should be further catabolized by the NAD(H)-dependent dehydrogenase MocA and the gene product of MocC. SIA is probably the first pathway intermediate of 3-O-MSI catabolism, but also seems to function as a nutritional mediator on its own (2, 25). The degradation of the rhizopines probably requires the inositol catabolic pathway and results in the endproducts dihydroxyacetone phosphate, acetyl-CoA and CO₂. This was confirmed by the finding that the myo-inositol dehydrogenase was required for rhizopine catabolism (7).

The hypothesis that rhizopines play a role as nutritional mediators in plant-bacteria interactions has been investigated by two independent co-challenge experiments for host plant nodulation using the S. meliloti L5-30 wild type and transposon insertion mutants affected in the moc and mos locus. The mutants nodulated and fixed nitrogen with the same efficiency as the wild type when inoculated onto plants as single inoculants (9, 12). The mos mutants were as competitive as the wild type in a co-challenge experiment, showing that the ability to synthesis rhizopine is not required for competition during nodulation (9). In contrast,
the *moc* mutants’ ability to nodulate the host was strongly diminished when competing with the wild type (9, 12, 24).

The biological data obtained to support the rhizopine concept were conducted with crude rhizopine isolated from root nodules and can therefore not confirm 3-*O-*MSI and SIA as the actual rhizopines. In addition, the location of the Tn5 transposons in the mutant strains used for the catabolism assays and co-challenge experiments had been mapped previously only by restriction enzyme analysis (1, 2, 22). Under best circumstances, mapping can determine the location of transposon insertions with the accuracy of ±50 base pairs. For this study, the exact locations of the Tn5 insertions in the *idhA* gene and the *moc* locus of the *S. meliloti* L5-30 mutants have been determined via DNA sequencing. Here we show that the same genes that are required for the degradation of the rhizopine isolated from root nodules, *mocABCDER*, are also essential for the utilization of the chemically synthesized SIA. A co-challenge experiment revealed that mutant strains affected in *mocA, mocC, mocD mocE* and *mocR* could not compete with the wild type during nodulation.

**Materials and Methods**

**Microbiological methods**

Bacterial strains used are listed in Table 4.1. *S. meliloti* cultures were grown at 28°C. Rich medium for *S. meliloti* was tryptone yeast (TY) (4) and minimal medium
was minimal M medium (22). Antibiotic concentrations for \textit{S. meliloti} were streptomycin (Sm), 250 \(\mu\)g/ml and kanamycin (Km), 200 \(\mu\)g/ml. For the catabolism studies, \textit{S. meliloti} strains were inoculated 1:100 from TY precultures into liquid minimal M medium with either 0.2\% myo-inositol as sole carbon (C-) and 0.1\% KNO\(_3\) as nitrogen (N-) source or 0.2\% proline or 0.2\% \textit{scyllo}-inosamine (SIA) as sole C- and N-source. The cultures were shaken (200 rpm) at 28 °C. After 72 h, the bacterial growth was determined by measuring the absorbance at 600 nm (OD\(_{600}\)) with a Beckman DU 640 spectrophotometer. Catabolism studies were carried out in duplicate, and values represent the average of two independent experiments ± SEM.

\textbf{Synthesis of \textit{scyllo}-inosamine}

The SIA for growth studies was provided by our collaborators from the Department of Chemistry at Western Michigan University, Dr. Elke Schoffers and Sing Raj Gurung, who prepared SIA in seven steps with an improved method and 32\% overall yield from readily available \textit{myo}-inositol (26).

\textbf{Determination of transposon insertion sites by PCR and DNA sequencing}

The exact insertion site of the Tn5 insertions in the \textit{S. meliloti} L5-30 mutant strains was determined via PCR followed by DNA sequencing. As template, 1 \(\mu\)l of a liquid bacterial culture was used in 50 \(\mu\)l reaction mixtures containing (as final concentrations): Red-Taq PCR Buffer according to the manufacture’s instructions (Sigma-Aldrich Corp., St. Louis, MO), 0.2 mM of each deoxyribonucleotide, 50 pmol
of each forward and the Tn5Ext primer (see Table 4.2), and 1.5 U of Red-Taq DNA polymerase (Sigma–Aldrich Corp., St. Louis, MO). PCR amplification was carried out in a Mastercycler gradient DNA thermal cycler (Eppendorf, Westbury, NY), with an initial denaturation step for 5 min at 95 °C; 30 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min for elongation; and a final elongation step at 74°C for 7 min. The PCR products were purified using the Promega Wizard SV Gel and PCR Clean-up System (Promega, Madison, Wisconsin). DNA sequencing was performed by the Cornell Biotechnology Resource Center (Cornell Univerity Ithaca, NY) using the Tn5Ext Primer.

**Competition assay for nodule occupancy**

The competition assay for nodule occupancy was conducted as described previously (Chapter I) (14). Briefly, axenic alfalfa (*Medicago sativa*) plants were grown at room temperature under of 16 hours light and 8 hours dark cycle. Seven day-old alfalfa seedlings were inoculated with 1 ml of mixed cultures of the *S. meliloti* L5-30 wild type and the individual mutant strains (1:1 ratio) or 1 ml of the wild type or the individual mutant strains as controls.
Table 4.1: Sinorhizobium meliloti wild-type and mutant strains, shown with the exact locations of the transposon insertions in the inositol dehydrogenase (idhA) and the rhizopine catabolism (moc) genes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Tn5 insertion n bp after start/total gene length in bp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1021</td>
<td>Wild-type derivative of SU47 Sm^R Ino^+ Mos^- Moc^-</td>
<td>none</td>
<td>(17)</td>
</tr>
<tr>
<td>L5-30</td>
<td>Wild-type Sm^R Ino^+ Mos^+ Moc^+</td>
<td>none</td>
<td>(14)</td>
</tr>
<tr>
<td>LIDHA</td>
<td>S. meliloti L5-30 #56 idhA::Tn5 Sm^R, Km^R Ino^- Mos^+ Moc^-</td>
<td>387/993</td>
<td>(7)</td>
</tr>
<tr>
<td>MOCC</td>
<td>S. meliloti L5-30 M11 mocC::Tn5 Sm^R Km^R Ino^+ Mos^+ Moc^-</td>
<td>841/978</td>
<td>(23)</td>
</tr>
<tr>
<td>ORF334</td>
<td>S. meliloti L5-30 M13 ORF334::Tn5 Sm^R Km^R Ino^+ Mos^+ Moc^-</td>
<td>633/1005</td>
<td>(23)</td>
</tr>
<tr>
<td>ORF293</td>
<td>S. meliloti L5-30 M16 ORF293::Tn5 Sm^R Km^R Ino^+ Mos^+ Moc^-</td>
<td>541/882</td>
<td>(23)</td>
</tr>
<tr>
<td>MOCA</td>
<td>S. meliloti L5-30 M20 mocA::Tn5 Sm^R Km^R Ino^+ Mos^+ Moc^-</td>
<td>418/954</td>
<td>(23)</td>
</tr>
<tr>
<td>MOCB</td>
<td>S. meliloti L5-30 M21 mocB::Tn5 Sm^R Km^R Ino^+ Mos^+ Moc^-</td>
<td>539/930</td>
<td>(23)</td>
</tr>
<tr>
<td>MOCR1</td>
<td>S. meliloti L5-30 M22 mocR1::Tn5 Sm^R Km^R Ino^+ Mos^+ Moc^-</td>
<td>542/1482</td>
<td>(23)</td>
</tr>
<tr>
<td>MOCR2</td>
<td>S. meliloti L5-30 M23 mocR2::Tn5 Sm^R Km^R Ino^+ Mos^+ Moc^-</td>
<td>910/1482</td>
<td>(23)</td>
</tr>
<tr>
<td>MOCD1</td>
<td>S. meliloti L5-30 M24 mocD1::Tn5 Sm^R Km^R Ino^+ Mos^+ Moc^-</td>
<td>263/1086</td>
<td>(23)</td>
</tr>
<tr>
<td>MOCD2</td>
<td>S. meliloti L5-30 M24 mocD2::Tn5 Sm^R Km^R Ino^+ Mos^+ Moc^-</td>
<td>667/1086</td>
<td>(23)</td>
</tr>
<tr>
<td>MOCE</td>
<td>S. meliloti L5-30 M30 mocE::Tn5 Sm^R Km^R Ino^+ Mos^+ Moc^-</td>
<td>133/318</td>
<td>(23)</td>
</tr>
<tr>
<td>MOCF</td>
<td>S. meliloti L5-30 M32 mocF::Tn5 Sm^R Km^R Ino^+ Mos^+ Moc^-</td>
<td>972/1227</td>
<td>(23)</td>
</tr>
</tbody>
</table>
Ten, 15 and 20 weeks post-inoculation the nodules were harvested and surface-sterilized with 70% ethanol. Rhizobia were re-isolated by homogenizing the nodules in sterile H₂O and serial dilutions were prepared. The wild type versus the mutant strain output ratio was determined by selective plating on TY Sm and TY Sm Km. The values represent the average of one typical experiment ± SEM with 7 plants each.

Table 4.2: Primer used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>idhA Forward</td>
<td>5' GCC GAC GGA TAC TCA TGC CGA TCT C 3'</td>
</tr>
<tr>
<td>mocA Forward</td>
<td>5' CCT TAA TTT GGC GGG CAA TGG GAT 3'</td>
</tr>
<tr>
<td>mocB Forward</td>
<td>5' CAA GTT CGG AAC CCT GCT AC 3'</td>
</tr>
<tr>
<td>mocC Forward</td>
<td>5' GCG GGA TGT GGA TGC AGA ATT TGA 3'</td>
</tr>
<tr>
<td>mocD Forward</td>
<td>5' CGA GAT TAC AGT CTG CTT GGT CG 3'</td>
</tr>
<tr>
<td>mocF Forward</td>
<td>5' GAG CCG TAT TGT GAT CGT TGG CGC G 3'</td>
</tr>
<tr>
<td>mocR Forward</td>
<td>5' CAT CTC AAG GCT GAC AC A CG 3'</td>
</tr>
<tr>
<td>ORF293 Forward</td>
<td>5' GTT GAG AAA AGC GAC AGC G 3'</td>
</tr>
<tr>
<td>ORF334 Forward</td>
<td>5' CGC AAT CGC CGT TGA GAA GGT TAT 3'</td>
</tr>
<tr>
<td>Tn5Ext</td>
<td>5' GAA AGT TAC CAT GTT AGG AGG TC 3'</td>
</tr>
</tbody>
</table>

Results

Determination of transposon insertion sites by PCR and DNA sequencing

The exact locations of the Tn5 transposons in the mutant strains used in this study were determined via PCR and DNA sequencing. We constructed primers (Table 4.2) based on the published DNA sequence of the rhizopine catabolism genes (Genbank Accession Nos. X78503 and AF076471). The purified PCR products were
subjected to DNA sequencing, and for each mutant strain, the location inside the targeted open reading frame was confirmed (Fig. 4.2).

Figure 4.2: The structure of the moc operon in *S. meliloti* L5-30. The horizontal arrows indicate the location of the open reading frames in the moc region. The locations of the Tn5 insertions are marked with vertical arrows, and labeled with the strain designations. The scyllo-inosamine catabolic phenotype is indicated by + (catabolism) and (no catabolism).

**Rhizopine catabolism assay**

To test the chemically synthesized scyllo-inosamine for its biological efficacy, we used the *S. meliloti* 1021 (Moc−) and L5-30 (Moc+) wild-type strains as well as transposon-induced L5-30 moc mutant strains in growth studies. When grown in minimal medium with 0.2% scyllo-inosamine as sole C- and N-source, the Moc+ wild-type strain L5-30 grew to an optical density of 1.4 (OD600), whereas the Moc− wild-type strain 1021 was not able to grow as expected (Fig. 4.3A). The mocA, mocB, mocC, and two mocR mutants were also not able to use the chemically synthesized scyllo-inosamine as sole C- and N-source (Fig. 4.3A). The mutants with insertions in ORF334 and ORF293 grew to around half of the optical density as the wild-type strain. The mutant strains with insertions in mocD, mocE, and mocF grew to almost the same final optical density as the wild type. The *S. meliloti* L5-30 idhA mutant
Figure 4.3: Growth of *S. meliloti* wild-type and mutant strains in minimal medium with (A) 0.2% *scyillo*-inosamine, and (B) proline as sole carbon and nitrogen source as well as (C) 0.2% *myo*-inositol as carbon and 0.1% KNO3 as nitrogen source. The growth was determined spectrophotometrically at 600 nm (OD600). Bars represent the average values from duplicate cultures of one typical experiment. Error bars denote SEM.
strain could not use scyllo-inosamine as sole C- and N-source. Thus, our results
confirm that the mocABCR genes and the idhA genes are essential for the degradation
of scyllo-inosamine. As controls, we found that all strains grew with 0.2% proline as
sole C- and N-source (Fig. 4.3B), and all strains grew with 0.2% myo-inositol as sole
C-source, but the idhA mutant could use myo-inositol (Fig. 4.3C).

**Competition assay for nodule occupancy**

To investigate if scyllo-inosamine catabolism plays an important role during
host-bacteria interactions, a co-challenge experiment for nodule occupancy was
performed with the *S. meliloti* L5-30 wild type and each of the mutants that failed to
use scyllo-inosamine as the sole C-source, namely the idhA, mocA, mocB, mocC,
mocR1 and mocR2 mutants. The wild type and individual mutant strains were
inoculated onto axenic alfalfa plants in a 1:1 ratio and single inoculants served as
control. All strains nodulated the host successfully when inoculated individually.
Nodules were harvested 10, 15 and 20 weeks post inoculation and the mutant versus
wild-type output ratio was determined. The mocC, mocR1 and mocR2 mutants were
outcompeted by the wild type after 10 weeks and only represented a small number of
the rhizobia reisolated from the nodules (15–25 %) (Fig. 4.4A). The total number of
mocC and mocR1 mutants decreased further over time (2–8%). The idhA, mocA and
mocB mutants were initially not impaired in their ability to compete with the wild
type (Fig. 4.4A). Nevertheless, the total number of idhA and mocA mutants decreased
over time
Figure 4.4: Competition assay for nodule occupancy elucidating the role of rhizopine catabolism. The rhizopine strain L5-30 and its corresponding \textit{idhA} (LIDHA) and \textit{moc} mutants \textit{moca} (MOCA), \textit{mocb} (MOCA), \textit{mocc} (MOCC), \textit{mocr1} (MOCR1), \textit{mocr2} (MOCR2) were inoculated on alfalfa plants in a 1:1 ratio. The wild type versus the mutant output ratio of reisolated rhizobia was determined after 10 (A), 15 (B) and 20 (C) weeks. Bars represent the average of 7 plants and error bars denote the SEM.
and represented only 30 and 37% of the reisolated rhizobia after 20 weeks, while the *mocB* mutant persisted inside the nodules (Fig. 4.4B and C).

**Discussion**

*scyllo*-inosamine catabolism requires functional *mocABCR* genes

We have shown that the same mutant strains that were reported earlier as being unable to degrade the rhizopine purified from nodules (22), namely the *mocA*, *mocB*, *mocC*, and *mocR* mutants were also not able to use the chemically synthesized *scyllo*-inosamine as sole C- and N-source (Fig. 4.3A). This confirms that the proposed structure of *scyllo*-inosamine is probably correct and its designation as rhizopine is warranted. The functional *mocD*, *mocE*, and *mocF* genes were initially not found to be required for the catabolism of the crude rhizopine (22); and the corresponding mutant strains were able to use *scyllo*-inosamine as sole C- and N-source. Nevertheless, later rhizopine catabolism studies in *R. leguminosarum* bv. *viciae* and *S. meliloti* L5-30 conducted by Bahar et al. showed that the *mocD*, *mocE*, and *mocF* genes were required for the growth with 3-O-MSI in crude extracts (1, 2). The authors suggested that the *mocD*, *mocE*, and *mocF* gene products catalyze the demethylation of 3-O-MSI resulting in SIA, and that this had not been found in earlier studies because SIA was the major rhizopine in the crude extract (1).

ORF334 and ORF293 were not required for the growth with the crude rhizopine as sole C-source (22). In this study we show that the mutants in both open reading
frames were impaired in their ability to grow with the chemically synthesized SIA. This is an interesting finding and requires further investigation.

Galbraith et al. showed that the \textit{idhA}-encoded functional \textit{myo}-inositol dehydrogenase is required for the catabolism of the crude rhizopine \cite{7} and were the first to show that rhizopine and inositol catabolism are linked. Here we confirm the requirement of the \textit{myo}-inositol dehydrogenase for SIA catabolism, since the \textit{idhA} mutant could not grow with the chemically synthesized SIA as sole C- and N-source.

\textbf{The moc genes are required for successful competition during alfalfa nodulation}

It was reported earlier that a functional \textit{moc} locus of \textit{S. meliloti} L5-30 is required for competitive host nodulation \cite{9, 12, 24}. Our data from the co-challenge experiment confirm these findings. The mutants with transposon insertions in the \textit{mocA}, \textit{mocC} and \textit{mocR} genes were outcompeted by the wild type during the cause of the experiment \cite{fig. 4.4}. In addition, we found that an \textit{idhA} mutant of L5-30 was also strongly impaired in the ability to compete with the wild type. It is interesting to note that the mutants affected in the genes encoding the two dehydrogenases, IdhA and MocA could initially persist in the nodules, but were outcompeted by the wild type over time. The \textit{mocB} mutant did not show any reduced ability to compete during the process of nodule occupancy, although the strain was not able to grow with rhizopine as the sole C- and N-source. This finding is enigmatic and requires further investigation.
The results of the co-challenge experiment indicate that the ability to catabolize rhizopine seems to be more important for maintaining than for establishing the symbiotic relationship. This is different from our findings from the *S. meliloti* 2011 inositol catabolism mutants. The *idhA*, *iolYRCDEB* and *mmsA* mutants of the non-rhizopine strains were already outcompeted by the wild type after 10 weeks (Fig. 4.5, Fig. 2.7 Chapter II) showing that the rhizobial inositol catabolism is required also for early stages of the symbiotic relationship.

This study does not only confirm *scyлlo*-inosamine as a rhizopine but also strongly supports the rhizopine concept with respect to the role of the rhizopines as nutritional mediators in symbiotic nitrogen fixation. The rhizopines are thought to be proprietary carbon sources sent out into the rhizosphere by the nitrogen-fixing-bacteroids to provide their kin with a competitive advantage. A study by MacSpadden-Gardener and de Brujin (1998) revealed that other rhizosphere isolates, belonging to the genera *Arthrobacter*, *Pseudomonas*, *Aeromonas* and *Alcaligenes* also use 3-*O*-MSI a sole C-source (16). Interestingly, two free-living *S. meliloti* spp., lacking the symbiotic plasmids, and therefore are not able to engage in symbiotic relationships with plants, were also among the isolates (16). These rhizopine degraders, including *Sinorhizobium*, are known to be dinitrifiers (3). A legume plant will only allow the rhizobia to nodulate under nitrogen-limiting conditions (28).
Figure 4.5: Competition assay for nodule occupancy elucidating the role of inositol catabolism. The non-rhizopine strain 2011 and its corresponding idhA (WIDHA) and iolY (W63-1 and W63-2), iolR (WIOLR), iolC (WIOLC), iolD (WIOLD) and iolE (WIOLE) mutants were inoculated on alfalfa plants in a 1:1 ratio. The wild type versus the mutant output ratio of reisolated rhizobia was determined after 10 (A), 15 (B) and 20 (C) weeks. Bars represent the average of 7 plants and error bars denote the SEM.
The presence of denitrifying organisms in the rhizosphere may decrease the concentration of nitrogen available to plants, creating an additional advantage for the rhizobia, and better their chances to be accepted by a potential host. In addition, the microbial community of the rhizosphere impacts symbiotic nitrogen fixation, e.g. it has been shown for *S. meliloti* that the presence of phosphate mobilizing *Pseudomonas* and *Bacillus* spp. increases nodulation efficiency (10). *Pseudomonas* and *Bacillus* spp. are also known to promote plant growth and to protect plant roots from potential pathogens, and are therefore used as biocontrol agents (11, 19, 21). Hence the benefit gained from the production of the rhizopines by the rhizobia might be greater if the rhizopines would not be a proprietary carbon source for just one species. Instead the rhizopine could promote a shift in rhizosphere community towards microorganisms that create conditions under which symbiotic nitrogen fixation becomes more favorable. Therefore it can be argued that the rhizopine strains among the rhizobia might be capable of creating biased rhizospheres that promote plant health and growth.
References


CHAPTER 5

CONCLUSION

This work analyzes the hypothesis that not only the inositol-derived rhizopines but also naturally occurring inositol isomers, such as myo-, scyllo-, and D-chiro-inositol function as nutritional mediators in the Sinorhizobium meliloti - alfalfa nitrogen-fixing symbiosis.

*S. meliloti* catabolizes myo-, D-chiro and scyllo-inositol as sole carbon sources. The initial oxidation of myo- and D-chiro-inositol is catalyzed by the cytosolic myo-inositol dehydrogenase (IdhA). An additional dehydrogenase of the IdhA/MocA/GFO family encoded by the *iolY* gene was found to be essential for growth with scyllo-inositol, and is predicted to be located in the periplasm. The further degradation of all three inositol isomers requires the *iolC, iolD, iolE, iolB* and *iolA* genes.

The inositol catabolism (*iol*) genes are dispersed at three different loci: the pSymB located *idhA* gene, the chromosomal *iolA* gene and the *iolYRCDEB* gene cluster. The *idhA* and *iolA* genes are monocistronically transcribed while *iolYRCDEB* comprise an operon. The *iol* genes are weakly constitutively expressed in the absence of inositol and their transcription is repressed by the gene product of *iolR*. The *iolR*
gene encodes a repressor of the RpiR family. IolR recognizes a conserved binding motif (5'-GGAA\_5,1TTCC-3') in the promoter regions of the *idhA* and *iolY* genes. Two additional IolR-binding motifs are located within the *iolYRCDEB* operon, upstream of *iolR* and *iolC*. Nevertheless, no promoter structures could be identified in the regulatory regions of *iolR* and *iolC*, indicating that the *iolYRCDEB* operon contains two internal operators. IolR-mediated repression is antagonized by the late pathway intermediate 2-keto-5-deoxy-gluconic acid 6-phosphate and not by a specific inositol isomer.

The *iolA* gene is not part of the IolR regulon and is constitutively expressed in *S. meliloti*. Nevertheless *iolA* expression increases to yet a higher level in the presence of inositol. The *iolA* gene product, the methyl malonate semialdehyde dehydrogenase (MmsA), was found to be involved in valine catabolism. Therefore, it seems to play a more general metabolic role in *S. meliloti*. These findings allow the conclusion that MmsA is not part of the core inositol catabolic pathway in *S. meliloti*, limiting it to the gene products of the *idhA* and *iolYCDEB* genes. Hence we annotated *iolA* as *mmsA*.

The inositol derivative *scylo*-inosamine (SIA) has been proposed to be a rhizopine. We used chemically synthesized SIA in this study to confirm its suggested structure. We were able to show that the same genes essential for the degradation of
the crude rhizopine isolated from root nodules, namely the \textit{idhA} and \textit{mocABCR} genes, were also required for the utilization of the chemically synthesized SIA.

\textit{S. meliloti} mutants with insertions in the individual \textit{idhA, iolY, iolC, iolD, iolE, iolB, mmsA} genes and in the regulatory gene \textit{iolR} could no longer compete effectively with the wild-type in a co-challenge experiment for host plant infection. In addition, the transposon-induced \textit{mocA, mocC, and mocR} mutants were also outcompeted by the wild type. The fact that not only the ability to use inositol compounds as nutrient sources but also proper inositol pathway regulation are required to compete successfully for nodule occupancy indicates that inositol carbohydrates do not only function as an important nutrient source that sustains the rhizobia in the rhizosphere but also have a signaling role in plant bacteria interactions.

The genomic organization of the \textit{iol} genes is widely distributed and highly conserved in the \textit{Rhizobiaceae}, a family of important pathogenic and symbiotic bacteria. This work with the model system of \textit{S. meliloti} - alfalfa symbiosis contributes a better understanding of the beneficial relationship between rhizobia and legumes in general. In addition, information derived from this study can be relevant to understanding inositol and rhizopine metabolism in other \textit{Rhizobiaceae} and their roles in symbiosis and virulence.
APPENDIX

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Petra R Kohler, Silvia Rossbach

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Metabolism of inositol and rhizopine in Sinorhizobium meliloti: key pathways for competitive host plant nodule formation

Petra R Kohler

Department of Biology and Chemistry

Michigan State University

East Lansing, MI 48824-1310

United States

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