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An AraC-like transcriptional activator is required for induction of genes needed for α-galactoside utilization in *Sinorhizobium meliloti*

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Abstract

The nodulating bacterium *Sinorhizobium meliloti* can utilize α-galactosides like melibiose and raffinose as sole sources of carbon and energy. We show that this utilization requires an AraC-like transcriptional activator, AgpT. When *agpT* was inactivated, *Rhizobium meliloti* could not utilize α-galactosides or induce genes required for transport and catabolism of these sugars. The *agpT* gene was not essential for the establishment of an effective nitrogen-fixing symbiosis.

Keywords: AraC; AgpT; Melibiose; Raffinose; *Sinorhizobium meliloti*

1. Introduction

Bacteria belonging to the genera *Sinorhizobium*, *Rhizobium* and *Bradyrhizobium* are able to induce a developmental pathway which leads to the formation of root nodules on host plants of the Leguminosae family [1–6]. The inducing bacteria enter the plant root as the nodule develops, invade nodule cells, and then differentiate into nitrogen-fixing bacteroids. The genes and genetic circuits which are uniquely required for free-living *Rhizobia* to establish productive symbioses have been targets of recent research, and some of these pathways are now well understood. Of growing interest are those bacterial activities which are not uniquely involved in this differentiation, but which are, nevertheless, important for symbiosis. Some of the important bacterial processes under investigation include: growth in soil and in the rhizosphere [7–10], competition for access to infection sites on roots of host plants [11], control of central metabolic pathways [12–17], horizontal gene transfer [8,18,19] and intercellular communication between bacteria [20,21]. An understanding of these types of processes is essential for a full understanding of the differentiation which occurs during the symbiosis between *Rhizobia* and their plant hosts.

We have recently described two neighboring genes in *Sinorhizobium meliloti* required for the utilization and uptake of α-galactosides such as melibiose, raffinose and stachyose. The upstream-most gene, *melA*, encodes an α-galactosidase, and the adjacent gene, *apgA*, encodes the periplasmic binding protein component of a binding protein-dependent α-galactoside permease [22]. Both genes are induced by galactose and α-galactosides. Interestingly, the regulation of these genes is tied to the regulation of genes important for symbiosis through the *syrA* gene. *syrA* encodes a small membrane protein which is upregulated by the NodD3-SyrM regulatory circuit [22]. SyrA also upregulates exopolysaccharide synthesis in *Rhizobium meliloti*, probably by post transcriptional mechanisms [23]. It has been shown that the *agpA* gene is downregulated by SyrA, and that *syrA* is highly expressed in bacteroids suggesting that the SyrA protein may downregulate the *agp* genes during bacteroid differentiation. The *agp* and *melA* genes are not essential for symbiosis, but they do play a supporting role because they are induced when *S. meliloti* grows in the presence of alfalfa seed wash, and when it grows on the surface of alfalfa seedlings (unpublished data).

In this paper, we describe *agpT*, which encodes an AraC-like transcriptional activator. We show that a *S. meliloti* strain carrying an *agpT* mutation is unable to grow on α-galactosides, and is unable to induce *agpA*, a gene required for α-galactoside transport.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains and their relevant genotypes are listed in Table 1. *S. meliloti* was cultured at 30°C and growth was followed by measuring the OD_{415} of 100 μl of cell culture in a 96-well microtiter dish using a Bio-Rad 550 plate reader.

2.2. Growth media

Bacteria were grown on Luria Broth (LB), M9 minimal medium with various carbon sources or basal medium. Basal medium is M9 minimal medium containing Difco yeast extract at a concentration of 65 μg ml^{-1}.

2.3. DNA sequencing

The *agpT* gene was sequenced during our characterization of genes required for α-galactoside utilization in *S. meliloti* [22]. Briefly, DNA from the regions flanking an *agpA*::Tn*phoA* insertion site was amplified by inverse PCR with primers which hybridized to the left end and center of Tn*phoA* (5′-GCAGAGCGGCAGTCTGATCACCCGTTA and 5′-AAGGTCGCGCGCATTCCCGATGAA, respectively). The PCR fragments were used to screen a *S. meliloti* lambda library constructed in the vector Lambda Fix II. DNA from positive lambda clones was subcloned and sequenced using standard methods. The gene sequence of *agpT* was deposited with GenBank, and the accession number is AF235048.

2.4. Construction of an insertion mutation in agpT

In order to determine the function of AgpT, we constructed a mutant strain, RB6, which contained an integrated suicide plasmid (pRB1) in the *agpT* gene of wild-type *S. meliloti* strain Rm1021. The suicide plasmid, pRB1, was directed to *agpT* by a 678-bp internal fragment of *agpT*. PCR and Southern analysis were used to confirm that the suicide plasmid had integrated, and disrupted, *agpT* as anticipated (unpublished results).

The suicide plasmid vector used to construct plasmid pRB1 was pMB439. Plasmid pMB439 was constructed by replacing the ampicillin resistance gene in pBlueScript SK(−) with the *aadA* gene from Tn21. AadA conferred resistance to spectinomycin and allowed for selection of the integrated plasmid in *S. meliloti*. An origin of transfer, *oriT*, was also present which allowed conjugal transfer of the plasmid (M. Barnett, Stanford University, personal communication).

2.5. Alkaline phosphatase assays

0.1–1.0-ml samples were taken from 96-h-old cultures, pelleted, resuspended in 0.5 ml of 1 M Tris–HCl pH 8.0, and permeabilized with a drop of chloroform. 180 μl of this extract was added to 20 μl of a 4% solution of *ortho*-nitro-phenyl-phosphate (ONPP) in a 96-well microtiter dish. Controls contained 180 μl of sample extract and 20 μl of water. Hydrolysis of ONPP was monitored at 415 nm with a Bio-Rad 550 plate reader. The OD_{415} of the samples was taken every 2–10 min and each reading was corrected by subtracting the OD_{415} of the respective control at each time point. The alkaline phosphatase specific activity for each sample was estimated by taking the slope of the resultant curve (change in OD_{415} per min) and dividing it by the amount of cell material present in the control sample (OD_{415}) at the first time point, and multiplying the result by 1000.

3. Results and discussion

3.1. Sequence of the agpT gene

Sequencing of the region upstream of the *S. meliloti melA* gene revealed a divergent open reading frame of 939 bp encoding a putative protein of 313 amino acids. Data base searches revealed that the protein product of

Table 1
Strains and plasmids

Strains	Relevant characteristics	Source
S. meliloti		
Rm1021	Str	[15]
Rm8002	Str, Pho$^-$	[27]
RB6	*agpT*::pRB1. Plasmid integrated into *agpT*	This study
RB9	*agpT*::pRB1, *agpA*::Tn*phoA* Pho$^-$. Derived from SG1001	This study
SG1001	*agpA*::Tn*phoA*. Derived from Rm8002	[22]
E. coli		
XL1 Blue	Used for cloning and sequencing	Stratagene
Plasmids		
pMB439	Suicide plasmid made by cloning the Sp^r gene from Tn21 and the *oriT* origin of transfer into pBlueScript SK(−)	M. Barnett
pRB1	Contains a internal 678-bp *Sac*II fragment of the *agpT* open reading frame cloned into pMB439.	This study
pBlueScript KS(+)	Cloning vector	Stratagene

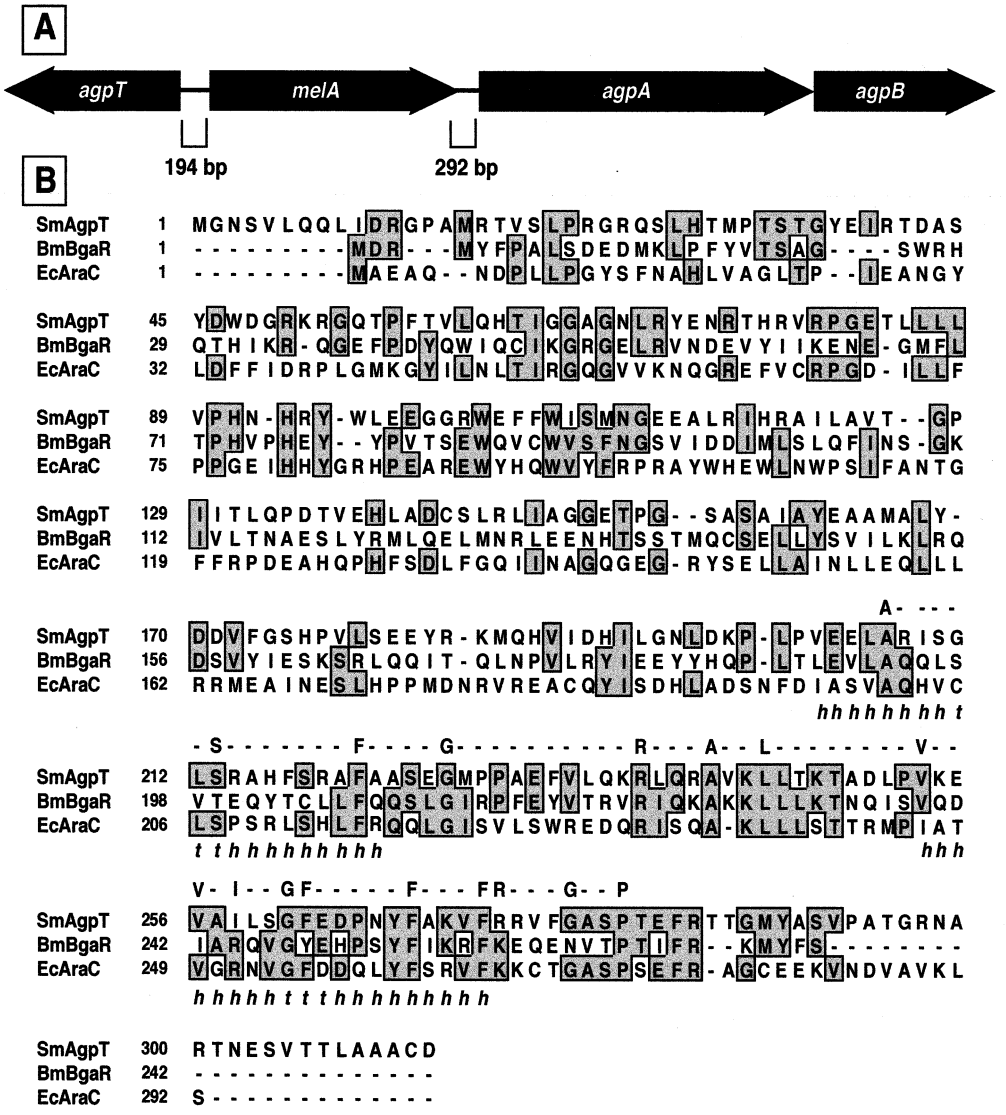

Fig. 1. (A) Map of the *agpT* and the nearby *melA*, *agpA* and *agpB* genes. (B) AgpT is similar to AraC-like transcriptional activators. The deduced amino acid sequence of AgpT was compared to that of the *B. megaterium* BgaR and *E. coli* AraC proteins. The lineups were done using Clustal W. Identical residues are boxed. Indicated are the residues where AgpT sequence matches the AraC/Xyls DNA-binding consensus: A— ——S— L— F— — G— ————————R— A— L— ————(I/V)–(I/V)–(I/V)–G(F/Y)— ——F— F(R/K)— G–P [25]. Also shown are the two putative helix-turn-helix motifs. Helix forming residues are indicated with an '*h*', and turn forming residues with a '*t*' [28].

this open reading frame, AgpT, was most similar to transcriptional regulators of the AraC/XylS family (Fig. 1). AgpT was most similar (22% identical) to the AraC-like activator, BgaR, from *Bacillus megaterium* which is an activator required for transcription of genes needed for utilization of β-galactosides [24], and it was 19% identical to AraC from *Escherichia coli*.

Proteins are included in the AraC/XylS family based on the similarities of their DNA-binding domains. These domains are generally about 100 amino acids long, contain two helix-turn-helix motifs, and are usually located at the C-terminus of the protein [25]. Depending on the protein, amino acids outside of the DNA-binding domain may be involved in dimerization, ligand-binding or environmental sensing [25,26]. Sequence data show that the C-terminal portion of the AgpT protein closely matches the AraC/XylS family consensus (Fig. 1).

3.2. *agpT* mutants cannot utilize α-galactosides

In order to determine the function of AgpT, we constructed a mutant strain, RB6, which contained an integrated suicide plasmid (pRB1) in the *agpT* gene of wild-type *S. meliloti* strain Rm1021. The suicide plasmid was directed to *agpT* by a 678-bp internal fragment of *agpT*. PCR and Southern analysis were used to confirm that the suicide plasmid had integrated into, and disrupted, *agpT* as anticipated (unpublished results). The resulting strain, RB6, grew normally in complex media such as LB. However, strain RB6 grew poorly or not at all in defined M9

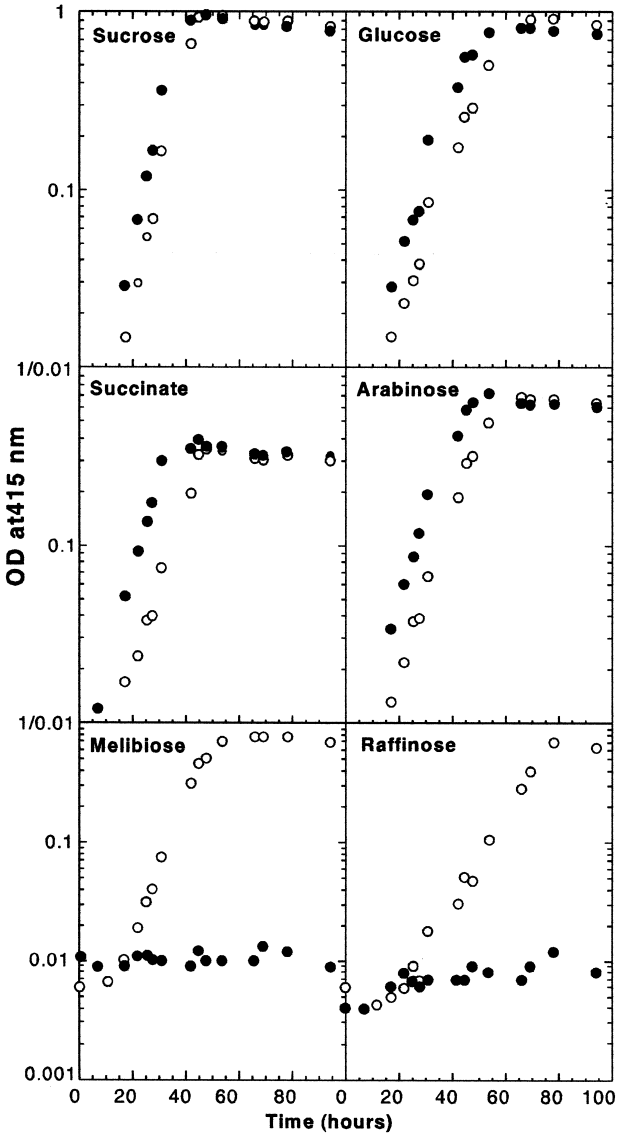

Fig. 2. *agpT* is required for growth of *S. meliloti* on α-galactosides. Strains Rm1021 (wild-type ○) and RB6 (*agpT* ●) were grown in basal medium containing the indicated carbons sources at 0.2%. Optical density at 415 nm was monitored by reading 100-μl samples of culture in a 96-well microtiter dish.

media, when α-galactosides such as melibiose or raffinose were used as the sole carbon source. In contrast, the growth of RB6 in M9 medium was normal on all other carbon sources tested, including galactose, organic acids, monosaccharides and disaccharides (Fig. 2 and unpublished results). Thus, it seemed likely that strain RB6 failed to grow on α-galactosides because *agpT* encoded a transcriptional activator required for the induction of *melA*, *agpA* and possibly other genes required for the transport and catabolism of these sugars.

3.3. AgpT is required to induce genes needed for α-galactoside utilization

We tested whether *agpT* was required for induction of genes required for α-galactoside utilization by comparing alkaline phosphatase activity arising from an *agpA*::Tn*phoA* reporter in strain SG1001 (*agpT*$^+$, *agpA*::Tn*phoA*) to that arising from the same reporter in strain RB9 (*agpT*::pRB1, *agpA*::Tn*phoA*). As a control, strain Rm8002, which is the *pho*$^-$ parent of strains SG1001 and RB6, was also assayed for alkaline phosphatase activity. The strains were grown for 96 h in a basal salts medium containing one of a variety of carbon sources, and then the specific activity of alkaline phosphatase was measured. Basal medium is M9 minimal medium containing yeast extract at a concentration of 65 μg ml^{-1}. Yeast extract in the basal medium was necessary because it allowed cells to grow to a measurable density, even if they were unable to catabolize particular sugars.

Results from these experiments showed that *agpT* is required for induction of *agpA*::Tn*phoA* by α-galactosides (Table 2). The *agpT*$^+$ *agp*A::Tn*phoA* reporter strain SG1001 showed high levels of alkaline phosphatase activity when cells were grown on galactose or the α-galactosides melibiose or raffinose. When glucose or succinate were provided as carbon sources, the AgpA::PhoA activity was lower than that seen in basal medium due to the catabolite repression mediated by these carbon sources. When the *agpT*::pRB1 mutation was introduced into strain SG1001, the resulting strain, RB9, was unable to induce the *agpA*::Tn*phoA* reporter in response to galactose or α-galactosides.

3.4. Phenotypes due to the agpT mutation are not due to polar effects on downstream genes

Sequencing revealed that there is a gene downstream of the *agpT* gene that encodes a LacI-type transcriptional repressor. This gene is transcribed in the same direction as *agpT*. It was possible that the effects of the insertional mutation in *agpT* were really due to polar effects on the transcription of this gene. To rule this out, we created an insertional mutation in the gene, and tested the resulting strain for growth on α-galactosides. The mutation had no effect on the utilization of these sugars. This indicated that

Table 2
AgpA::PhoA activity of strains grown in various carbon sources

Medium	AP specific activity[a]		
	Rm8002 (*pho*$^-$)	SG1001 (*pho*$^-$ *agpA*::Tn*phoA*)	RB9 (*pho*$^-$ *agpA*::Tn*phoA agpT*::pRB1)
Basal	18 ± 5	34 ± 6	99 ± 15
Basal+glucose	2 ± 1	6 ± 4	23 ± 13
Basal+succinate	2 ± 0	10 ± 8	34 ± 2
Basal+lactose	3 ± 1	82 ± 58	62 ± 9
Basal+galactose	2 ± 1	400 ± 47	22 ± 9
Basal+melibiose	2 ± 1	3662 ± 135	88 ± 9
Basal+raffinose	2 ± 1	1452 ± 135	105 ± 7

[a]Average and standard deviation of three or four independent experiments.

the phenotypes seen in strains RB6 (*agpT*::pRB1) and RB9 (*agpT*::pRB1, *agpA*::Tn*phoA*) were due to the *agpT* mutation and not due to polar effects on downstream genes.

3.5. The agpT mutation has no effect on the symbiotic phenotype of R. meliloti

Strain RB6 was used to inoculate alfalfa in order to ascertain whether the *agpT* gene was required for effective symbiosis. The mutant strain was able to form nodules, and the nodules were able to provide enough fixed nitrogen to allow vigorous growth of the plants on nitrogen free medium. Thus *agpT*, like *agpA*, and *agpB* are not essential for nitrogen fixation.

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