The key *Sinorhizobium meliloti* succinoglycan biosynthesis gene *exoY* is expressed from two promoters

Hai-Ping Cheng *, Shi-Yi Yao

Biological Sciences Department, Lehman College, the City University of New York, 250 Bedford Park Boulevard West, Bronx, NY 10468, USA

Abstract

Bacterial exopolysaccharide, succinoglycan, plays an important role in eliciting infection thread formation, which is a key step in the establishment of *Sinorhizobium meliloti*–alfalfa (*Medicago sativa*) nitrogen fixing symbiosis. To understand the regulatory mechanisms that control production of succinoglycan, the expression of the key succinoglycan biosynthesis gene, *exoY*, was analyzed by constructing a set of nested deletions of the *exoY* promoter region. Two *exoY* promoters were identified based on the promoter activities and confirmed by direct detection of the transcripts. The expression from both promoters was induced in the *exoR95* and *exoS96* mutant backgrounds suggesting that both promoters are regulated by the ExoR protein and the ExoS/ChvI two-component signal transduction system. The identification of the *exoY* promoters provides additional avenue for further analysis of the role of succinoglycan in *S. meliloti*–alfalfa symbiosis.

© 2003 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Symbiosis; Gene expression; Nitrogen fixation; *Sinorhizobium meliloti*; Succinoglycan biosynthesis; *exoR*; *exoS*; Alfalfa; *Medicago sativa*

1. Introduction

Sinorhizobium meliloti establishes a nitrogen fixing symbiosis with its plant host alfalfa (*Medicago sativa*) through continuous interactions that elicit plant structure changes, which enable *S. meliloti* cells to enter and colonize alfalfa root nodules [1–4]. One of the early and critical steps of the symbiosis is the formation of infection threads inside the curled root hairs that are colonized by *S. meliloti* cells [5–8]. The infection threads are tube-like structures and they are filled with growing *S. meliloti* cells [7,8]. The infection threads elongate and extend into developing nodule primordium in the root cortex where they release bacterial cells into newly formed plant cells in the middle of root nodules [1,9,10].

The initiation of the formation of infection threads in the curled alfalfa root hairs requires succinoglycan as well as the nodulation factor [7,11]. Succinoglycan is a *S. meliloti* exopolysaccharide with different degrees of polymerization of a single repeating unit that consists of seven

glucoses and one galactose with succinyl, pyruvyl, and acetyl modifications [12,13]. Succinoglycan is produced in small amounts by free-living *S. meliloti* cells but it is produced in large amounts by *S. meliloti exoR95*::Tn5 and *exoS96*::Tn5 mutants [14,15]. The ExoR protein is an unknown regulatory protein [16]. The ExoS is the sensor of the ExoS/ChvI two-component regulatory system [17]. Although it is not clear how ExoR and ExoS/ChvI are related, they both regulate succinoglycan production by regulating the expression of succinoglycan biosynthesis genes, most of which cluster in a 19 kb region of the genome and are organized into several operons [14,18–20]. The ExoR protein and ExoS/ChvI system are also involved in regulating flagella biosynthesis (Cheng, H.-P., manuscript submitted).

While the expression of multiple *exo* genes, *exoA*, *exoF*, *exoP*, *exoQ*, and *exoT*, appear to be regulated [14,18], the expression of the *exoY* gene appears to be the primary target of regulation [21,22]. The *exoY* gene is the first gene of the *exoYFQ* operon and it encodes a galactosyl transferase that carries out the first step of succinoglycan biosynthesis [13,21]. The *exoYFQ* transcribes divergently from the *exoX* gene with a 769 bp intergenic region [22]. Transposon insertions in the intergenic region close to the *exoY* gene suppressed the succinoglycan overproduction

* Corresponding author. Tel.: +1 (718) 960-7190;
Fax: +1 (718) 960-8236.
E-mail address: haiping@lehman.cuny.edu (H.-P. Cheng).

caused by *exoR95* and *exoS96* mutations and brought the levels of succinoglycan production to that of the wild-type [22]. However, transposon insertions in areas further away from the *exoY* gene did not suppress the succinoglycan overproduction caused by *exoR95* and *exoS96* mutations [22]. This suggests that the region of the *exoY* promoter is targeted by the ExoR protein and ExoS/ChvI system in regulating succinoglycan production. In addition to ExoR and ExoS/ChvI, the production of succinoglycan appears to be regulated also by the ExoD [23], ExoX [22], MucR [24], SyrM [25], and SyrA [26] proteins.

To further characterize the regulation of the *exoY* gene expression and understand the role of succinoglycan in symbiosis, a set of nested deletions of the *exoY* promoter regions was constructed and fused to the *exoY-gfp* (green fluorescence protein) fusion. The analyses of fluorescence intensity and mRNA transcripts suggest that there are two different *exoY* promoters and none of them appear to be controlled directly by the ExoR protein or the ExoS/ChvI two-component regulatory system.

2. Materials and methods

2.1. Strains and growth media

S. meliloti Rm1021 (Str^r) was used as the wild-type strain [27], and Rm7095 (*exoR95*) and Rm7096 (*exoS96*) were used to determine the effect of the *exoR95*::Tn5 and *exoS96*::Tn5 mutations on the expression of the *exoY* gene. *Escherichia coli* DH5α was used for plasmid constructions and preparations [17], and *E. coli* MT616 (pRK600, Cm^r) was used as a helper in conjugation [27].

Luria–Bertani (LB) medium was used for the growth of *E. coli* strains, and LB supplemented with 2.5 mM MgSO$_4$ and 2.5 mM CaCl$_2$ (LB/MC) was used for all *S. meliloti* strains [27]. Z-MGS minimal medium [27] was used to prepare cells for *exoY* promoter activity analyses. Agar (1.5%) was added to make solid media. Antibiotics were used at the indicated concentrations: chloramphenicol, 10 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; neomycin, 200 μg ml^{-1}; and streptomycin 500 μg ml^{-1}.

2.2. Constructing a set of plasmids with nested deletions of the exoY promoter region

To determine the numbers of the *exoY* promoter, a set of nested deletions in the *exoY* promoter region was constructed and fused to an *exoY-gfp* translational fusion in two steps. A DNA fragment containing the entire *exoX-exoY* 769 bp intergenic region as the *exoY* promoter region and the first 282 bp of the *exoY* open reading frame was generated by PCR (polymerase chain reaction) using primers pexoxy588 and pexoy1610r (Table 1). The PCR cycles were performed as 1 min at 95 °C, 1 min at 56 °C, and 1–2 min at 72 °C for 35 cycles. PCR products were

Table 1
Oligo DNA primers used in the construction of nested deletion of the *exoY* promoter region fused with the *exoY-gfp* fusion

Name	Sequence of primers
pexoxy588	5′-GGCCGGAAGCTTGGCGCTAACCTACCTTCGGGTC-3′
pexoy1610r	5′-CTCTTCGAGGACCTCGTCGCC-3′
pGFP	5′-ATGGCTACGAAAGGAGAAGAACTC-3′
pGFPr	5′-CCGCTCGAGACCCGTCCTGTGGATATCCGG-3′
Primer XY2	5′-ATACCCAAGCTTGGGGCCACTATATTAGCGCCC-3′
Primer XY3	5′-ATACCCAAGCTTGGGTTGCAGTCGAGCATACATC-3′
Primer XY4	5′-ATACCCAAGCTTGGGTCCATTTCGCACAATTCAA-3′
Primer XY5	5′-ATACCCAAGCTTGGGCCGGGGCAGTTTGCCGC-3′
Primer XY6	5′-ATACCCAAGCTTGGGTCCCCTAAAATTGCCCGG-3′
Primer XY7	5′-ATACCCAAGCTTGGGTGGGGCGTGTGGCCGGC-3′
Primer XY8	5′-ATACCCAAGCTTGGGTGAGCGGGTAGCCTCAGC-3′
Primer XY9	5′-ATACCCAAGCTTGGGAAAAAAGTGAGGGAAAGTTG-3′
Primer XY10	5′-ATACCCAAGCTTGGGCGCTGTCCGTCAGTGCAG-3′
Primer XY11	5′-ATACCCAAGCTTGGGCGAAATAACTAGCCCGCG-3′
Primer XY12	5′-ATACCCAAGCTTGGGGAACTATCCTAACCCCTG-3′
Primer XY13	5′-ATACCCAAGCTTGGGTCGCGACTTTCGCCACC-3′
Primer XY14	5′-ATACCCAAGCTTGGGGCCATCATTCCGCCTTCA-3′
Primer XY15	5′-ATACCCAAGCTTGGGATGAGCCCGCGTCCCAC-3′

purified according to the protocol of QIAquick PCR purification kit (Qiagen Inc., Valencia, CA, USA). This DNA fragment was digested with restriction enzyme *Hin*dIII at 37 °C for 2 h to generate a *Hin*dIII compatible end at the unique *Hin*dIII site at the promoter side of the DNA fragment introduced using the PCR primer pexoxy588 (Table 1). A DNA fragment carrying the *gfp* open reading frame and its transcriptional terminator with an *Xho*I restriction enzyme compatible end following the transcription terminator was similarly generated using PCR primers pGFP and pGFPr (Table 1), and *Xho*I digestion at the unique *Xho*I site introduced through PCR primer pGFPr (Table 1). The DNA fragment containing *exoY* promoter and the first half of the *exoY* gene was ligated with the DNA fragment containing the *gfp* gene at the blunt end of each of the fragments using T4 DNA ligase (New England Biolabs, Beverly, MA, USA) at 4 °C overnight. The ligated DNA fragment with *exoY* promoter region and *exoY-gfp* fusion was directionally cloned in between the *Hin*dIII and *Xho*I sites on pMB393 [28] to generate plasmid pHC77. The *exoY-gfp* fusion can not be expressed by any promoters on the vector itself. To generate the nested deletion of the *exoY* promoter region, a set of oligo DNA primers pexoxy588, and pXY2-15 that anneal to regions of the *exoY* promoter that are 50 bp apart were individually paired with the primer pGFPr to generate a set of DNA fragments. The set of DNA fragments were similarly digested with *Hin*dIII and *Xho*I restriction enzymes, cloned in between the *Hin*dIII and *Xho*I sites on pMB393, and transformed into *E. coli* DH5α to generate a set of plasmids, pHC201–pHC215, containing a set of nested deletions of the *exoY* promoter in front of the *exoY-gfp* fusion (Table 2). The plasmid pHC201 is the same as the pHC77 and it was reproduced as a control

Table 2
Plasmids used in the study

Plasmid	exoY promoter region	Sources
pMB393	none	[28]
pHC201 (pHC77)	−769 to −1	this work
pHC202	−701 to −1	this work
pHC203	−651 to −1	this work
pHC204	−591 to −1	this work
pHC205	−551 to −1	this work
pHC206	−504 to −1	this work
pHC207	−454 to −1	this work
pHC208	−404 to −1	this work
pHC209	−355 to −1	this work
pHC210	−304 to −1	this work
pHC211	−254 to −1	this work
pHC212	−207 to −1	this work
pHC213	−154 to −1	this work
pHC214	−104 to −1	this work
pHC215	−54 to −1	this work

construction. The plasmids pMB393 and pHC201–pHC215 were extracted from same numbers *E. coli* DH5α cells carrying the plasmids Wizard^R *Plus* SV Minipreps DNA purification system kit (Promega Corporation, Madison, WI, USA) following manufacturer's instruction. The plasmids were digested with *Hin*dIII and *Xho*I restriction enzymes and resolved on agarose gel using electrophoresis to confirm the nested deletion in the *exoY* promoter region (data not shown). The similarity of the intensity of the DNA bands on agarose gel suggests that copy numbers of the plasmids were not significantly affected by the difference in the regions of the *exoY* promoter on the plasmids.

2.3. Measuring exoY promoter activities

The level of average GFP fluorescence intensity per cell ('specific GFP expression' for short) was determined and used to represent the activities of the *exoY* promoters since the copy numbers of the plasmids with different parts of the *exoY* promoter region appear to be similar. To determine the specific GFP expression, *S. meliloti* cells were collected from LB/MC liquid cultures, washed, resuspended to OD_{600} 0.1 in Z-MGS media, and incubated with shaking at 30 °C for two more days. Z-MGS cultures were diluted 1:10, and transferred to wells in a transparent 96-well plate and wells in a black 96-well plate in equal amounts. The cultures in transparent 96-well plates were used to determine the cell density using an absorbance microplate reader (Spectra Max 340PC, Molecular Device, Sunnyvale, CA, USA) and the cultures in black 96-well plates were used to determine the intensity of GFP fluorescence using a fluorescence microplate reader (Spectra Max Germini XS, Molecular Device, Sunnyvale, CA, USA). Both cell density and GFP fluorescence intensity of individual culture were collected and analyzed using a computer program (SpectroSoft, Molecular Device, Sun-

nyvale, CA, USA). The GFP fluorescence intensity of a culture was normalized to its cell density to generate the average specific GFP expression. The specific GFP expression was used to represent the *exoY* promoter activities.

2.4. Detecting transcripts from both exoY promoters

Three special oligo DNA primers were designed to detect the transcripts from the *exoY* promoters on the plasmid carrying the set of nested deletions of the *exoY* promoter using RT-PCR (reverse transcription-PCR) (Fig. 2A). Primer 1 (5′-TTTGTATAGTTCATCCATGCC-3′) anneals to the end of the *gfp* open reading frame on the promoter fusion plasmids and it was used to synthesize the first strand of cDNA based on the RNA transcripts in RT reactions (Fig. 2A). Because the *gfp* gene is only carried on the plasmid, using a primer that anneals only to the *gfp* gene, makes it possible to measure the impact of the nested deletions on the expression of the *exoY* gene from the plasmids in different genetic backgrounds. Primer 2 (5′-ATGGCT AGCAAAGGAGAAGA-3′) anneals to a region just downstream of the P$_{exoYdown}$ promoter and primer 3 (5′-TGAGCGGGTAGCCTCAGC-3′) anneals to just downstream of the P$_{exoYup}$ promoter. Total RNA was extracted from the wild-type strain Rm1021 carrying plasmids pHC201 (both promoters), pHC207 (both promoters), pHC208 (P$_{exoYdown}$ promoter), pHC214 (P$_{exoYdown}$ promoter), and pHC215 (no promoter) using RiboPure™-Bacteria kit (Ambion Inc. Austin, TX, USA) following manufacturer's instructions. Briefly, the first strand cDNA synthesis was carried out by mixing MMLV-RT with 2.0 μg total RNA in a final volume of 20 μl. After RT reaction, 2 μl of reaction mixture was used as a template for PCR. Both the minus-RT and the minus-template were used as negative controls for the PCRs to ensure the total RNA used free of DNA. The total RNAs were mixed with primer 1 for RT to generate the cDNA based on the transcript(s). Primers 1 and 2 were added together to the RT reactions for the subsequent PCRs. The cDNA copy of the short transcripts from the P$_{exoYdown}$ promoter can be amplified by primers 1 and 2 in a PCR. The cDNA copy of the long transcript from the P$_{exoYup}$ promoter can be amplified by primers 1 and 3 in a PCR. The RT-PCR products were purified and resolved on agarose gel (Fig. 2B).

3. Results and discussion

3.1. Locating exoY promoters and determining their activities in free-living cells

To identify the location of the putative *exoY* promoters, the set of plasmids (pHC201–pHC215) carrying nested deletions of the *exoY* promoter region (Table 2) were introduced into the wild-type strain Rm1021 cells by con-
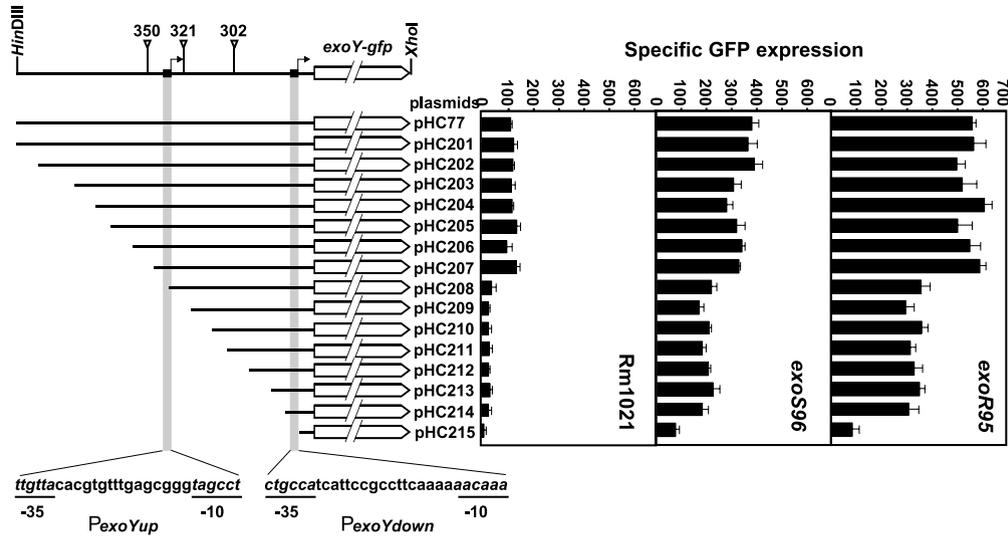
Fig. 1. Schematic representation of nested deletions in the *exoY* promoter region and the effects of the deletions on the expression of the *exoY* gene in the wild-type, *exoR95*, and *exoS96* mutant backgrounds. A line graph is used to show the *exoY* and *exoX* genes, and the positions of three transposon insertions. The positions of the proposed *exoY* promoters are marked by short solid bars and arrows. The solid lines are used to represent the regions of the *exoY* promoter carried on each of the plasmids. Two gray vertical bars represent the relative position of the proposed *exoY* promoters. The sequences of the proposed *exoY* promoters are shown at the bottom of the bars. The promoter activities of the set of plasmids carrying nested deletions in wild-type Rm1021, *exoR95*, and *exoS96* mutant backgrounds are shown in three different bar graphs.

jugation. The levels of average specific GFP expression were determined for each Rm1021 carrying different plasmids in three independent experiments to determine the levels of the *exoY* gene expression (Fig. 1).

The level of the specific GFP expression by Rm1021 carrying the plasmid pHC215 with the first 54 bp of the promoter, was the same as Rm1021 carrying the original vector pMB393 (data not shown). This suggests that the *exoY-gfp* gene fusion was not expressed in Rm1021 (pHC215) cells, which also suggests that the first 54 bp of the *exoY* promoter region does not contain active *exoY* promoter. The level of specific GFP expression of the Rm1021 (pHC214) was consistently higher than that of Rm1021 (pHC215), which suggests that the first 104 bp of the *exoY* promoter region contains a complete *exoY* promoter that expressed the *exoY-gfp* fusion. The levels of the specific GFP expression were about the same for the Rm1021 (pHC214) and Rm1021 (pHC213), which suggests that there is no additional sequence required for the *exoY* promoter on the pHC214 plasmid. Further analysis of the −1 to −153 region of the *exoY* promoter in the pHC214 using a promoter prediction program (www.fruit-fly.org/seq_tools/promoter.html), as well as direct comparison of the sequences suggest that the downstream *exoY* promoter, P$_{exoYdown}$, is most likely located in the −78 to −104 region of the *exoY* promoter region (Fig. 1).

The levels of the specific GFP expression by Rm1021 cells carrying the plasmids pHC208–pHC214 were about the same, suggesting there was no additional complete *exoY* promoter in the −105 to −404 *exoY* promoter region. The level of the specific GFP expression by Rm1021 (pHC207) was consistently higher than that of Rm1021 (pHC208), suggesting there is another *exoY* promoter in

the −356 to −454 region of the *exoY* promoter region. Further analysis suggested that this upstream *exoY* promoter, P$_{exoYup}$, is most likely located in the −395 to −425 region (Fig. 1). The plasmid pHC208 carries only the −10 region of the P$_{exoYup}$ promoter so it was not expressing *exoY* gene from the P$_{exoYup}$ promoter.

The levels of the specific GFP expression by Rm1021 cells carrying plasmids pHC207–pHC201 were similar, which suggests that there is no additional *exoY* promoter in the −426 to −769 region.

All together, these results suggest that the *exoY* gene can be expressed from two different promoters: an upstream P$_{exoYup}$ promoter and a downstream P$_{exoYdown}$ promoter. The proposed locations of the *exoY* promoters provide a perfect explanation of the previous transposon insertion analysis of the *exoY* promoter region [22]. The insertion of Tn5 transposon in the Ω302 and Ω321 mutants (Fig. 1) blocks the *exoY* expression from the P$_{exoYup}$ promoter but not the P$_{exoYdown}$ promoter in both *exoR95* and *exoS96* mutant backgrounds so that the colonies appear dim but not dark. This is because the expression of the *exoY* gene from the P$_{exoYdown}$ promoter was sufficient to support succinoglycan biosynthesis. The Tn5 insertion in the Ω351 mutant was upstream of both *exoY* promoters, so it can not suppress the overexpression of the *exoY* gene in neither *exoR395* nor *exoS396* mutant backgrounds. The *exoR395*Ω351 and *exoS395*Ω351 double mutants continue to overproduce succinoglycan.

3.2. Detecting transcription products of the two exoY promoters

Analyses of the nested deletions of the *exoY* promoter
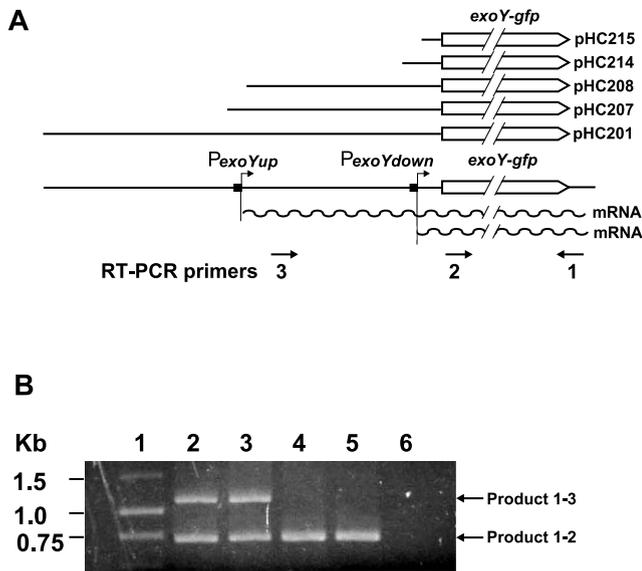
A

B

Fig. 2. Schematic representations showing the positions of the RT-PCR primers in the *exoY* promoter region in A and a picture showing the RT-PCR products in B. A: Line graphs showing the *exoY* promoter region, the positions of proposed *exoY* promoters, the predicted transcripts, the areas the RT-PCR primer anneals to, and the *exoY* promoter area in the plasmids used in the study. B: A picture of agarose gel showing DNA size standard (lane 1), RT-PCR products from cells carry plasmid pHC201 (lane 2), pHC207 (lane 3), pHC208 (lane 4), pHC214 (lane 5), and pHC215 (lane 6).

region suggested there were two *exoY* promoters, which should produce two different transcripts, one long transcript and one short transcript. RT-PCR and three specially designed primers (Fig. 2A) were used to detect the possible transcripts from the *exoY* promoter as described in details in Section 2. This will allow the simultaneous detection of both long and short *exoY-gfp* transcripts.

As shown in Fig. 2B, no DNA fragment was detected on the agarose gel when total RNA from Rm1021 (pHC215) was used as the template for RT-PCR. This suggests that there was no transcript produced and that the first 54 bp of the *exoY* promoter region does not include an active *exoY* promoter. The fact that no nonspecific DNA fragments were detected also confirmed the specificity of the primers 1, 2, and 3. A 0.7 kb DNA fragment was detected when total RNA from Rm1021 (pHC214) was used as the template. The size of this fragment matches the distance between the sites that primers 1 and 2 anneal to. This suggests that the short transcript of the *exoY-gfp* gene was produced by Rm1021 (pHC214). This is consistent with the suggestion that the −78 to −104 region contains the $P_{exoYdown}$ promoter. The 0.7 kb DNA fragment was detected again when total RNA from Rm1021 (pHC208) was used as template. This suggests the −1 to −404 region of the *exoY* promoter contains only the $P_{exoYdown}$ promoter. An additional 1.1 kb DNA fragment was detected when total RNA from either Rm1021 (pHC201) or Rm1021 (pHC207) was used as the template. The size of the 1.1 kb fragment matches the distance be-

tween the sites that primers 1 and 3 anneal to. This suggests it was amplified from the long transcript expressed from the P_{exoYup} promoter. These results suggest that the *exoY* promoter region (−1 to −454) on the plasmid 207 contains another active *exoY* promoter, which is consistent with the proposed location of the P_{exoYup} promoter.

3.3. Both exoY promoters are induced by the exoR95 and exoS96 mutations

The expression of the *exoY* gene is upregulated in *exoR95* and *exoS96* mutant backgrounds [22]. This could be the result of the induction of one or both of the $P_{exoYdown}$ and P_{exoYup} promoters. It is also possible that there are additional *exoY* promoters that are only active in the *exoR95* and *exoS96* mutant backgrounds. To examine all these possibilities, the entire set of the plasmids carrying the nested deletions of the *exoY* promoter was introduced into the *S. meliloti* strains Rm7095 (*exoR95* mutant) and Rm7096 (*exoS96* mutant) cells by conjugation. The specific GFP expression was determined for each of strains carrying one of the plasmids (Fig. 1). In the *exoR95* mutant background, the specific GFP expression was 80 for Rm7095 (pHC215), 330 for Rm7095 carrying plasmids pHC208–pHC214, and 550 for Rm7095 carrying plasmids pHC77, and pHC201–pHC207. In the *exoS96* mutant background, the specific GFP expression was 70 for Rm7096 (pHC215), 200 for Rm7096 carrying plasmids pHC208–pHC214, and 340 for Rm7096 carrying plasmids pHC77, and pHC201–pHC207. The *exoY* gene was expressed at two levels in both *exoR95* and *exoS96* mutant background as measured by specific GFP expression, which is similar to that in the wild-type background. The fact that the *exoY* gene was expressed in two levels by the same plasmids suggests that the *exoY* gene was expressed from the same two promoters in the *exoR95* and *exoS96* mutant backgrounds as in the wild-type background. The two levels of the *exoY* gene expression in the *exoR95* and *exoS96* mutant backgrounds also suggest that there is no additional *exoY* promoter that is regulated directly by either ExoR protein or ExoS/ChvI two-component regulatory system.

Further analysis of the levels of the *exoY* gene expression in different background also suggests that the expression is the highest in the *exoR95* mutant background and second highest in the *exoS96* mutant background, which is consistent with previous analyses based on *lacZ* and *phoA* fusions [14,15]. What is also interesting is that the expressions from P_{exoYup} and $P_{exoYdown}$ promoters were both upregulated in both *exoR95* and *exoS96* mutant backgrounds. The upregulation of transcription is often the results of the specific interactions between transcriptional regulators and specific DNA sequence elements. Similar DNA sequences were often found around the promoters that are similarly regulated, but no obviously similar DNA sequences or elements were found around P_{exoYup} and

$P_{exoY down}$ promoters. This raises the possibility that other unknown protein factors are directly involved in regulating *exoY* gene expression, and that neither ExoR protein nor ExoS/ChvI two-component regulatory system directly interact with the *exoY* promoters. The signals received by either ExoR protein or ExoS/ChvI system are transmitted downstream to other proteins in regulating succinoglycan biosynthesis. This model is consistent with our recent findings that both ExoR protein and the ExoS/ChvI system are involved in regulating flagella biosynthesis in addition to succinoglycan biosynthesis (Cheng, H.-P., manuscript submitted).

The finding of two inducible *exoY* promoters is consistent with our preliminary results that the expression of the *exoY* gene was upregulated in cells inside infection threads (Cheng, H.-P., unpublished results). The identification of two *exoY* promoters and the construction of the nested promoter deletion fused to *exoY-gfp* fusion will facilitate further analyses of the regulation of the succinoglycan biosynthesis gene expression during symbiosis and it will also provide better understanding of the role of succinoglycan and other bacterial exopolysaccharides in microbe–plant interactions.

Acknowledgements

References

[1] van Rhijn, P. and Vanderleyden, J. (1995) The *Rhizobium*–plant symbiosis. Microbiol. Rev. 59, 124–142.

[2] Brewin, N.J. (1993) The *Rhizobium*–legume symbiosis: plant morphogenesis in a nodule. Semin. Cell Biol. 4, 149–156.

[3] Broughton, W.J. and Perret, X. (1999) Genealogy of legume–*Rhizobium* symbioses. Curr. Opin. Plant Biol. 2, 305–311.

[4] Long, S.R. (1989) *Rhizobium*–legume nodulation: life together in the underground. Cell 56, 203–214.

[5] Kijne, J.W. (1992) In: Biological Nitrogen Fixation (Stacey, G., Burris, R.H. and Evans, H.J., Eds.), pp. 349–398. Chapman&Hall, New York.

[6] Downie, J.A. and Walker, S.A. (1999) Plant responses to nodulation factors. Curr. Opin. Plant Biol. 2, 483–489.

[7] Cheng, H.P. and Walker, G.C. (1998) Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. J. Bacteriol. 180, 5183–5191.

[8] Gage, D.J. and Margolin, W. (2000) Hanging by a thread: invasion of legume plants by rhizobia. Curr. Opin. Microbiol. 3, 613–617.

[9] van Brussel, A.A.N., Bakhuizen, R., van Spronsen, P.C., Spaink, H.P., Tak, T., Lugtenberg, B.J.J. and Kijne, J.W. (1992) Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of *Rhizobium*. Science 257, 70–72.

[10] Timmers, A.C., Auriac, M.C. and Truchet, G. (1999) Refined analysis of early symbiotic steps of the *Rhizobium*–Medicago interaction in relationship with microtubular cytoskeleton rearrangements. Development 126, 3617–3628.

[11] Walker, S.A. and Downie, J.A. (2000) Entry of *Rhizobium leguminosarum* bv. *viciae* into root hairs requires minimal nod factor specificity, but subsequent infection thread growth requires *nodO* or *nodE*. Mol. Plant-Microbe Interact. 13, 754–762.

[12] Reinhold, B.B., Chan, S.Y., Reuber, T.L., Marra, A., Walker, G.C. and Reinhold, V.N. (1994) Detailed structural characterization of succinoglycan, the major symbiotically important exopolysaccharide of *Rhizobium meliloti* strain Rm1021. J. Bacteriol. 176, 1997–2002.

[13] Reuber, T.L. and Walker, G.C. (1993) Biosynthesis of succinoglycan, a symbiotically important exopolysaccharide of *Rhizobium meliloti*. Cell 74, 269–280.

[14] Reuber, T.L., Long, S. and Walker, G.C. (1991) Regulation of *Rhizobium meliloti exo* genes in free-living cells and in planta examined using Tn*phoA* fusions. J. Bacteriol. 173, 426–434.

[15] Doherty, D., Leigh, J.A., Glazebrook, J. and Walker, G.C. (1988) *Rhizobium meliloti* mutants that overproduce the *Rhizobium meliloti* acidic calcofluor-binding exopolysaccharide. J. Bacteriol. 170, 4249–4256.

[16] Reed, J.W., Glazebrook, J. and Walker, G.C. (1991) The *exoR* gene of *Rhizobium meliloti* affects RNA levels of other *exo* genes but lacks homology to known transcriptional regulators. J. Bacteriol. 173, 3789–3794.

[17] Cheng, H.P. and Walker, G.C. (1998) Succinoglycan production by *Rhizobium meliloti* is regulated through the ExoS-ChvI two-component regulatory system. J. Bacteriol. 180, 20–26.

[18] Becker, A., Kleickmann, A., Keller, M., Arnold, W. and Puhler, A. (1993) Identification and analysis of the *Rhizobium meliloti exoA-MONP* genes involved in exopolysaccharide biosynthesis and mapping of promoters located on the *exoHKLAMONP* fragment. Mol. Gen. Genet. 241, 367–379.

[19] Glucksmann, M.A., Reuber, T.L. and Walker, G.C. (1993) Genes needed for the modification, polymerization, export, and processing of succinoglycan by *Rhizobium meliloti*: a model for succinoglycan biosynthesis. J. Bacteriol. 175, 7045–7055.

[20] Glucksmann, M.A., Reuber, T.L. and Walker, G.C. (1993) Family of glycosyl transferases needed for the synthesis of succinoglycan by *Rhizobium meliloti*. J. Bacteriol. 175, 7033–7044.

[21] Müller, P., Weng, M.W.M., Quandt, J., Arnold, W. and Pühler, A. (1993) Genetic analysis of *Rhizobium meliloti exoYFQ* operon: ExoY is homologous to sugar transferases and ExoQ represents a transmembrane protein. Mol. Plant-Microbe Interact. 6, 55–65.

[22] Reed, J.W., Capage, M. and Walker, G.C. (1991) *Rhizobium meliloti exoG* and *exoJ* mutations affect the *exoX-exoY* system for modulation of exopolysaccharide production. J. Bacteriol. 173, 3776–3788.

[23] Reed, J.W. and Walker, G.C. (1991) The *exoD* gene of *Rhizobium meliloti* encodes a novel function needed for alfalfa nodule invasion. J. Bacteriol. 173, 664–677.

[24] Bertram-Drogatz, P.A., Quester, I., Becker, A. and Puhler, A. (1998) The *Sinorhizobium meliloti* MucR protein, which is essential for the production of high-molecular-weight succinoglycan exopolysaccharide, binds to short DNA regions upstream of exoH and exoY. Mol. Gen. Genet. 257, 433–441.

[25] Swanson, J.A., Mulligan, J.T. and Long, S.R. (1993) Regulation of *syrM* and *nodD3* in *Rhizobium meliloti*. Genetics 134, 435–444.

[26] Barnett, M.J., Swanson, J.A. and Long, S.R. (1998) Multiple genetic controls on *Rhizobium meliloti syrA*, a regulator of exopolysaccharide abundance. Genetics 148, 19–32.

[27] Leigh, J.A., Signer, E.R. and Walker, G.C. (1985) Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc. Natl. Acad. Sci. USA 82, 6231–6235.

[28] Gage, D.J., Bobo, T. and Long, S.R. (1996) Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). J. Bacteriol. 178, 7159–7166.