Regulation of exopolysaccharide synthesis in *Rhizobium* sp. strain TAL1145 involves an alternative sigma factor gene, *rpoH2*

Pakieli H. Kaufusi,¹ L. Scott Forsberg,² Panlada Tittabutr¹ and Dulal Borthakur¹

Correspondence
Dulal Borthakur
dulal@hawaii.edu

¹Department of Molecular Biosciences and Bioengineering, University of Hawaii at Manoa, 1955 East-West Road, Ag. Science 218, Honolulu, HI 96822, USA

²Complex Carbohydrate Research Center, University of Georgia, Athens, 315 Riverbend Road, GA 30602, USA

Exopolysaccharide (EPS) produced by *Rhizobium* sp. strain TAL1145 has been shown to be essential for effective nodulation on *Leucaena leucocephala* (leucaena). This paper reports the isolation and characterization of an alternative sigma factor gene, *rpoH2*, involved in the regulation of EPS synthesis in TAL1145. Disruption of this gene in TAL1145 resulted in a Calcofluor-dim mutant RUH102 that produced approximately 18% of the amount of EPS made by TAL1145. This mutation did not affect the normal growth of RUH102 in free-living state. RUH102 induced few nitrogen-fixing nodules, resulting in a significant reduction in total nitrogen content in leucaena. It was complemented for EPS production and nodulation by a 2.0 kb *Hind*III fragment of TAL1145. Sequence analysis of this fragment revealed the *rpoH2* ORF of 870 bp that encoded a protein of 32 kDa. Expression of the *rpoH2* ORF in *Escherichia coli* also revealed a 32 kDa protein. A PCR-constructed clone of 1263 bp, containing the *rpoH2* ORF and its upstream putative regulatory region, complemented RUH102 for EPS defects. Comparison of the RpoH2 sequence to proteins in the databases showed significant similarity to RpoH-like sigma factors of other Gram-negative bacteria. By constructing several *exo::Tn3Hogus* fusions and transferring them to the backgrounds of TAL1145 and RUH102, it was demonstrated that RpoH2 positively regulates the transcription of some *exo* genes.

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INTRODUCTION

The association of leguminous plants and soil bacteria of the genus *Rhizobium* is a complex process involving the coordinated exchange of signals (for review, see Niehaus & Becker, 1998). Exopolysaccharides (EPSs) have been shown to be involved in this association; the phenotypes of EPS-deficient mutants of various *Rhizobium* species range from pseudo-nodule formation to effective nodulation of the hosts (for review, see Frayssé *et al.*, 2003).

In the past few years, most of the genes relevant to EPS synthesis in *Sinorhizobium meliloti* have been sequenced and characterized. These EPS genes were associated with one of the two main EPS types, succinoglycan (EPS I) and galactoglucan (EPS II) (for review, see Becker *et al.*, 2002).

Abbreviations: EPS, exopolysaccharide; EPS I, succinoglycan; EPS II, galactoglucan; Gus, β -glucuronidase; MU, 7-hydroxy-4-methylcoumarin; MUG, 4-methylumbelliferyl β -D-glucuronide; TMS, trimethylsilyl.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AY536267.

The biosynthesis of EPS I is directed by the products of the *exo* genes, whereas EPS II is produced by the enzymes encoded by the *exp* genes. EPS I, the Calcofluor-binding EPS, plays a crucial role in effective nodule invasion in alfalfa (Gonzalez *et al.*, 1996).

Regulation of EPS I production in rhizobia is complex and may involve several regulatory proteins, including MucR, ExoR, ExoS, ExoX and SyrA. MucR is a zinc finger protein that positively regulates EPS I production in *S. meliloti*. Disruption of *mucR* results in a mutant strain that produces EPS II instead of EPS I but with no defects in nodule invasion (Zhan *et al.*, 1989; Keller *et al.*, 1995). ExoR is believed to act as a negative regulator of *exo* genes because *exoR* mutants produce colonies that are more mucoid than the wild-type, and are unable to colonize nodules (Doherty *et al.*, 1988; Reed *et al.*, 1991; Ozga *et al.*, 1994). ExoS is a negative regulator (Glazebrook *et al.*, 1990; Ozga *et al.*, 1994), and a part of a two-component regulatory system with ChvI, involved in the expression of *exo* genes (Cheng & Walker, 1998). ExoS mutants produce more EPS than the wild-type and invade nodules, but vary in their ability to

produce nitrogen-fixing nodules (Doherty *et al.*, 1988; Ozga *et al.*, 1994). ExoX, which is homologous to Psi (Borthakur & Johnston, 1987), also appears to be a negative regulator of EPS I production (Zhan & Leigh, 1990). Lastly, SyrA represents a new class of regulatory protein because a disruption in *syrA* abolishes EPS I synthesis (Barnett *et al.*, 1998).

Rhizobium sp. strain TAL1145 forms nitrogen-fixing nodules on the tree-legume *Leucaena leucocephala* (leucaena). TAL1145 produces large amounts of EPS in medium containing mannitol and the EPS-deficient Tn3Hogus-insertion mutants of TAL1145 are defective in nodulation of leucaena (Parveen *et al.*, 1997). We have also isolated one spontaneous EPS-deficient mutant, which was complemented by a DNA region of TAL1145, that is outside the *exo* gene cluster. Characterization of this mutant has led us to the identification of a gene encoding an alternative sigma factor involved in EPS synthesis in TAL1145. The aims of this study are to characterize this alternative sigma factor, and to establish its possible regulatory role in EPS synthesis in TAL1145.

METHODS

Strains, plasmids and media. Bacterial strains and plasmids are listed in Table 1. *Rhizobium* strains were grown in yeast extract mannitol (YEM) (Vincent, 1970) and tryptone yeast (TY) (Beringer,

1974) at 28 °C. TY was supplemented with 0.1% mannitol and 0.1% NH₄Cl, or 0.1% glucose and 0.1% NH₄Cl, or 0.1% glucose alone for the growth experiment. *Escherichia coli* strains were grown in LB, SOB and SOC media (Sambrook *et al.* 1989). Antibiotics were used in the following concentrations: for *Rhizobium* strains, streptomycin, 100 µg ml⁻¹; rifampicin, 20 µg ml⁻¹; tetracycline, 5 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; gentamicin, 5–7 µg ml⁻¹; for *E. coli*, kanamycin, 50 µg ml⁻¹; tetracycline, 10 µg ml⁻¹; gentamicin, 5 µg ml⁻¹; ampicillin, 100 µg ml⁻¹; chloramphenicol, 20 µg ml⁻¹.

DNA manipulations and subcloning. Genomic DNA was isolated from *Rhizobium* strains using standard procedure (Sambrook *et al.*, 1989). Plasmid DNA was isolated from overnight culture of *E. coli* or 2 day culture of *Rhizobium* by the alkaline lysis method (Sambrook *et al.* 1989). Restriction enzyme digests and ligations were performed according to the specifications of the supplier (New England Biolabs). ³²P-labelling of DNA probes and Southern blotting were performed according to the manufacturers' instructions.

Subclones were made from the CsCl gradient-purified pUHR17, which were digested with *Eco*RI and *Hind*III. Various fragments were gel purified and ligated into the *Eco*RI site in the vector pLAFR1 and into the *Hind*III site in the vector pRK404. These subclones were separately transferred by triparentals with the helper plasmid pRK2013 as described by Figurski & Helinski (1979) into EPS-defective mutant RUH7 for possible complementation. The 6 kb *Eco*RI fragment in plasmid pUHR101, and the smallest plasmids pUHR37/pUHR38, which contained 2.0 kb *Hind*III fragment in either orientation, complemented RUH7. This fragment was sequenced for both strands at the University of Hawaii Molecular Biology and Biotechnology Facilities (GenBank accession no. AY536267). Another 7.9 kb *Eco*RI fragment of plasmid pUHR223 (Parveen *et al.*, 1997) was gel purified

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
<i>Rhizobium</i>		
TAL1145	Wild-type, Sm ^r Rf ^r	George <i>et al.</i> (1994)
RUH7	Spontaneous EPS-defective mutant of TAL1145	This study
RUH102	TAL1145 <i>rpoH2</i> mutant, Kan ^r	This study
Plasmids		
pRK2013	ColE1-Tra[RK2] ⁺ (Kan ^r)	Figurski & Helinski (1979)
pRK404	Wide-host range plasmid vector, Tc ^r , IncP	Ditta <i>et al.</i> (1985)
pLAFR1	IncP1 <i>cos oriT</i> (Tc ^r), <i>mob</i> RK2 cosmid	Friedman <i>et al.</i> (1982)
pUHR13–17	Cosmid clones that complement the EPS defect of RUH7 and RUH102, Tc ^r	This study
pUHR101	pLAFR1::6 kb <i>Eco</i> RI fragment of pUHR17 containing <i>rpoH2</i>	This study
pUHR113	pUHR101 with a Kan cassette inserted in <i>Bam</i> HI site of <i>rpoH2</i>	This study
pUHR37	2.0 kb <i>Hind</i> III fragment containing <i>rpoH2</i> of pUHR17, cloned into pRK404	This study
pUHR38	2.0 kb <i>Hind</i> III fragment containing <i>rpoH2</i> of pUHR17 in the opposite orientation to pUHR37, cloned into pRK404	
pUHR241	1.4 kb <i>Eco</i> RV– <i>Hind</i> III fragment of pUHR37 in pRK404	This study
pUHR304	1263 bp fragment containing <i>rpoH2</i> of TAL1145, cloned into pRK404	This study
pUHR323	1323 bp fragment containing <i>rpoH2</i> of <i>S. meliloti</i> Rm1021, cloned into pRK404	This study
pUHR298	pET21a::rpoH21 (first ATG)	This study
pUHR299	pET21a::rpoH22 (second ATG)	This study
pUHR300	pET21a::rpoH23 (third ATG)	This study
pUHR301	pET21a::rpoH23 (fourth ATG)	This study
pUHR223	pLAFR3::23 kb TAL1145 fragment	Parveen <i>et al.</i> (1997)
pUHR290	pRK404::7.6 kb <i>Hind</i> III fragment of 23 kb region in pUHR223	This study

Phenotypes: Sm^r, Km^r, Tc^r, Rf^r, and Amp^r indicate resistance to streptomycin, kanamycin, tetracycline, rifampicin, and ampicillin, respectively.

and cloned into pRK404 to give plasmid pUHR290, which was found by sequencing to contain ORFs that are similar to *exo* genes in *S. meliloti* (P. H. Kaufusi & D. Borthakur, unpublished).

The *rpoH2* gene of *S. meliloti* Rm1021 was amplified by PCR as a 1323 bp fragment, using primers 5'-CGCTTAAGCACTCCTTGCG-TAA-3' and 5'-AAATGACCTCGGAAGCCTTAC-3'. The amplified fragment was first cloned into the TOPO cloning vector to verify the sequence. The cloned fragment was then removed as an *EcoRI* fragment and cloned into pRK404 to obtain plasmid pUHR323.

Tn3Hogus mutagenesis. Tn3Hogus mutagenesis of the recombinant plasmid, pUHR290, was performed by using a Tn3Hogus-containing strain HB101 as described by Stachel *et al.* (1985). This 6.62 kb transposon, constructed by Brian Staskawicz (University of California, Berkeley, USA), has a kanamycin-resistance marker and a promoterless β -glucuronidase (*gus*) gene near the left inverted repeat (B. Staskawicz, personal communication). The derivatives of pUHR290 carrying Tn3Hogus insertions were transferred to TAL1145 with selection for cotransfer of the plasmid and transposon antibiotic resistance markers. The transconjugants showing Gus activity on YEM plates containing 4-methylumbelliferyl β -D-glucuronide (MUG) (Sigma) were selected for fluorometric assay.

Genetic manipulation. The 2.0 kb *HindIII* fragment in plasmid pUHR37/38 is a part of the 6 kb *EcoRI* fragment in plasmid pUHR101. A kanamycin-resistance cassette was inserted into a single *BamHI* site in the 6 kb *EcoRI* fragment to obtain plasmid pUR113. This plasmid was transferred by triparental matings with the helper plasmid pRK2013 as described above. This plasmid was recombined into TAL1145 genomic DNA by homogenization (Ruvkun & Ausubel, 1981) using a P1-group incompatible plasmid, pPH1JL, to obtain the EPS-defective mutant RUH102.

Exopolysaccharide analyses. The EPS mutants were selected on YEM agar plates containing 0.02% Calcofluor White M2R (Cellufluor; Polysciences) with a laboratory-bench long-wave UV lamp as described by Glazebrook *et al.* (1989). RUH102 and TAL1145 were grown in 1500 ml YEM liquid medium at 28 °C for about 3 days to an OD₆₀₀ of approximately 1.0 for EPS production. Cells were precipitated by centrifuging at 15 000 r.p.m. (Sorvall RC-5B Plus) for 30 min. The supernatant was filtered through a pad of Celite 545 (Fisher Scientific, Diatomaceous Earth) on two layers of glass fibre filter paper (Whatman GF/C). The EPS was harvested by sequential precipitations with cetyltrimethylammonium bromide and acetone, and dialysed extensively (Robertsen *et al.*, 1981). For structural studies, the purified EPS from each bacterial strain was subjected to size-exclusion chromatography on Superose-12 HR 10/30, and on Superdex-75 HR 10/30 (both from Amersham Biosciences), equilibrated in 50 mM ammonium formate. Fractions were monitored by refractive index, and for carbohydrate content using a colorimetric procedure (York *et al.*, 1985). The glycosyl composition of these EPS samples was determined by preparing the trimethylsilyl (TMS)-methyl glycosides, with GC-MS analysis using a 30 m DB-5 capillary column as described by York *et al.* (1985). Proton NMR spectra of the EPS samples were recorded in D₂O at 25 °C using a Varian 300 MHz spectrometer. In separate experiments, the size distributions of the soluble EPSs produced by RUH102 and TAL1145 were compared by fractionation of the crude culture medium directly on a Bio-Gel A-5M column, as described previously (Parveen *et al.*, 1996).

Plant nodulation tests. Strain TAL1145 and its derivatives were tested for their symbiotic phenotypes with *L. leucocephala* variety K8 seedlings in modified Leonard jar assemblies containing nitrogen-free nutrient solution described by Borthakur & Gao (1996). One- to two-week-old seedlings were inoculated with approximately 10⁶ cells in sterile plant nutrient solution from a 2 day culture. Each

strain was tested on at least 12 plants. Seedlings were observed for healthy growth after 3–4 weeks. After 10 weeks, the roots were harvested for nodule counts, and the above-ground parts were oven-dried at 70 °C for 3 days for dry matter content and total nitrogen determination. For nodule assay, nodules were surface sterilized with 1% sodium hypochlorite, washed with sterile water, and crushed in YEM liquid medium before being transferred onto YEM agar plates. Nitrogen-fixing nodules were those that strains could be recovered from; otherwise they were considered pseudonodules.

β -Glucuronidase (Gus) activity assay. A fluorometric assay for Gus activity using MUG substrate was performed according to the method of Jefferson *et al.* (1987). The enzymic by-product is a fluorescent compound, 7-hydroxy-4-methylcoumarin (MU). Fluorometric measurements were made using a fluorescence spectrophotometer (Hitachi F2500), which was pre-calibrated with 50, 100, 500, 1000 and 3300 nM MU, corresponding to 140, 312, 1590, 3145 and 9400 fluorescence units, respectively. The wavelength of the spectrophotometer was set to 365 nm for excitation and 455 nm for emission. The fluorescence readings of the samples were automatically converted to nM MU by the spectrophotometer. The Gus activity was measured as the amount of MU produced per h per ml of cells (OD₆₀₀ of 1.0).

The pUHR290::Tn3Hogus insertions that showed positive Gus activity were transferred into the wild-type TAL1145 and *rpoH2* mutant, RUH102. To demonstrate the role of RpoH2 in the regulation of *exo* genes, equal amounts of the transconjugants were grown in 50 ml YEM liquid medium to an OD₆₀₀ of 1.0. Cells were incubated at 28 °C for approximately 2 days, and 1.0 ml of the cell culture (OD₆₀₀ of 1.0) was transferred into a 1.5 ml microfuge tube. Each transconjugant was tested in triplicate. Cells were precipitated from the culture by centrifuging, and the pellet was washed with 0.5 M NaCl. The cells were lysed by suspending them in 1.0 ml Gus extraction buffer containing 50 mM Na₂HPO₄ (pH 7.0), 10 mM β -mercaptoethanol, 10 mM EDTA, 0.1% sodium lauroyl sarcosine and 0.1% Triton X-100. Lysozyme was added to a concentration of 1 mg ml⁻¹ and incubated at 37 °C for 1 h. The cell debris was precipitated from the cell lysate by centrifuging. A 100 ml sample of the supernatant was added to 400 μ l of assay buffer containing 1 mM MUG in Gus extraction buffer and incubated at 37 °C for 1 h; the enzymic reaction was then stopped by adding 9 vols of a buffer containing 0.2 M Na₂CO₃. One millilitre of each sample was measured in the fluorescence spectrophotometer.

RESULTS

Isolation of a 6.0 kb fragment of TAL1145 that complements EPS-defective mutants

A spontaneous EPS-defective mutant, RUH7, was isolated from *Rhizobium* sp. strain TAL1145 based on its altered colony morphology. The colonies of RUH7 are small, opaque and nonmucoid, compared to the large, semi-translucent and mucoid colonies of TAL1145 on YEM agar. When grown on YEM agar containing the fluorescent dye Calcofluor white, RUH7 shows only a dim UV-fluorescence in contrast to the bright fluorescence of TAL1145, indicating that the gene mutated in RUH7 is involved in succinoglycan (EPS I) production. To identify the gene that complements this mutant, RUH7 was mated en masse with a cosmid clone library of TAL1145 and 12 EPS⁺ transconjugants were isolated. These EPS⁺ derivatives made large, semi-translucent and mucoid colonies like

the wild-type strain TAL1145 on YEM. From these trans-conjugants, five overlapping cosmid clones, pUHR13–pUHR17, were isolated that complemented RUH7.

Cosmid pUHR17, containing a 23 kb DNA insert, was used for further analysis. To identify a shorter DNA fragment containing the gene or genes that complement RUH7, various *EcoRI* and *HindIII* fragments of pUHR17 were subcloned into the wide-host-range plasmid vectors pLAFR1 and pRK404, respectively. These subclones were separately transferred into RUH7 for possible complementation. In this way, a plasmid, pUHR101, containing a 6 kb *EcoRI* fragment, was identified which complemented this mutant for EPS synthesis. Similarly, a 2.0 kb *HindIII* fragment of pUHR17 was identified which, when cloned in either orientation in the recombinant plasmids pUHR37 and pUHR38, complemented RUH7 for EPS synthesis. Restriction analyses and Southern hybridization show that the 2 kb *HindIII* fragment is a part of the 6 kb *EcoRI* fragment. There is a single *BamHI* site common to both fragments. A kanamycin-resistance cassette was inserted into this *BamHI* site in plasmid pUHR101 to obtain plasmid pUHR113 (Fig. 1). Introduction of pUHR113 into RUH7 did not complement for the EPS-defective phenotype, indicating that the gene for EPS synthesis in the 6.0 kb fragment was disrupted.

The gene disrupted in plasmid pUHR113 was marker-exchanged into the equivalent position in the genome of strain TAL1145 to obtain mutant RUH102. The colonies of RUH102 were small, opaque, nonmucoid and dim UV-fluorescent, similar to those of RUH7, in YEM containing

Calcofluor. The EPS defect of RUH102 was complemented by the 6.0 kb *EcoRI* fragment in pUHR101 and the 2 kb *HindIII* fragment in plasmids pUHR37 and pUHR38 (Fig. 1). Since RUH102 is a mutant with a precise kanamycin-resistance marker insertion, this mutant was used instead of RUH7 for further analyses.

Exopolysaccharide production by mutant RUH102

Since RUH102 shows an attenuated Calcofluor response and abnormal colony morphology relative to TAL1145, we are interested in determining the amount and the nature of EPS produced by this mutant. Liquid YEM cultures were inoculated with 10^6 cells of RUH102 and TAL1145. The acidic EPS was isolated from the culture filtrate after 4 days of growth, using sequential precipitation and dialysis as described by Robertsen *et al.* (1981). The purified EPS fraction was then analysed by size-exclusion chromatography to estimate the molecular size, and by GC-MS and ^1H NMR to determine glycosyl and non-carbohydrate components.

As shown in Table 2, RUH102 produced only 18 % of the amount of total EPS synthesized by TAL1145, (as determined by weighing the dried product) indicating that total EPS synthesis is diminished in the mutant compared to the wild-type. GC-MS analyses of the glycosyl derivatives prepared from the two samples indicated that both EPS preparations consisted only of glucose and galactose, in ratios of 2:1 (TAL) and 2.6:1 (RUH). This suggested that the two EPS preparations (TAL and RUH) each consisted of a mixture of both EPS I and EPS II type polysaccharides. Previous studies have shown that purified EPS I has a glucose:galactose ratio of 7:1, and that EPS II has a glucose:galactose ratio of 1:1 (Reinhold *et al.*, 1994; Her *et al.*, 1990). NMR analyses also revealed the presence of proton resonances characteristic of O-acetyl (2.01 p.p.m.), pyruvate acetal (1.35 p.p.m.) and succinyl (2.52 p.p.m. and 2.37 p.p.m.) substituents, in both the TAL and RUH preparations. These noncarbohydrate substituents are typical of EPS type I polysaccharides (Reinhold *et al.*, 1994), while EPS II is devoid of succinate (Her *et al.*, 1990). The predominant anomeric proton signals were attributed to β -linked glucose residues (4.42 p.p.m.), in close agreement with that reported for EPS I, which contains only β -linked residues (Reinhold *et al.*, 1994). Anomeric signals of weaker intensity were also detected at 5.20 p.p.m., and were attributed to α -linked residues, probably arising from EPS type II polysaccharides, which are known to contain both α - and β -linked residues in a 1:1 ratio (Her *et al.*, 1990). Together, these findings suggest that both the TAL and RUH preparations consisted of a mixture of EPS type I and type II polysaccharides, and that the overall level of both polysaccharides was reduced in the RUH mutant relative to TAL.

The two EPS preparations (TAL and RUH) were analysed by size-exclusion chromatography on two different

Fig. 1. Physical map of the 6.0 kb *EcoRI* region of *Rhizobium* sp. strain TAL1145 containing *rpoH2* (open arrow). Plasmid pUHR323 contains *rpoH2* of *S. meliloti* Rm1021 in a 1323 bp fragment that complements RUH102 for EPS synthesis. Restriction sites: E, *EcoRI*; H, *HindIII*; B, *BamHI*; Ev, *EcoRV*. Δ , Kanamycin cassette insertion; +, complementation; -, no complementation.

Table 2. Relevant characteristics of *rpoH2* mutant RUH102

The amount of EPS was obtained by weighing the total mass of the extracted EPS from 1500 ml liquid culture (see Methods). For nodule counts and dry matter content, each value represents the mean $\pm$ SEM of 15 plants. The values for percentage total nitrogen represent the mean $\pm$ SEM of three ground samples, each of which consisted of five plants. Cfw, Calcofluor white; DMC, dry matter content; ND, not determined; (+), halo; (–), haloless.

Strain	Cfw	Amount of EPS (%)	Halo	Nodules per plant	DMC (mg per plant)	% Total N (mg per g)
No rhizobia	–	–	–	0	171 $\pm$ 13	0.69 $\pm$ 0.04
TAL1145	Bright	100	+	8 $\pm$ 1	351 $\pm$ 26	2.37 $\pm$ 0.08
RUH102	Dim	18	–	3 $\pm$ 1	155 $\pm$ 44	1.33 $\pm$ 0.15
RUH102:: <i>rpoH2</i> (pUHR37/38)	Bright	ND	+	9 $\pm$ 2	288 $\pm$ 32	2.47 $\pm$ 0.09

chromatography resins as described in Methods. Both preparations migrated at the void volume on Superose-12, indicating high molecular masses at or exceeding 300 kDa. These molecular mass estimates are in accordance with previously reported estimates for the high-molecular-mass forms of EPS I and EPS II type polysaccharides (Wang *et al.*, 1999; Gonzalez *et al.*, 1996). Both the TAL and RUH samples were also excluded from Superdex-75, and low-molecular-mass carbohydrates, such as oligosaccharide subunits and EPS-derived fragments, were not observed. Polysaccharides of less than 30 kDa would be expected to be partially retained by the Superdex-75 column. The absence of any low-molecular-mass forms of EPS from these preparations probably reflects the fact that such components are produced at only very low levels, and that losses may have occurred during dialysis and work-up of the high-molecular-mass EPS forms. Together, these results indicated that the overall size, glycosyl composition, and non-carbohydrate substituents of the two high-molecular-mass EPS preparations were similar. Therefore, the small opaque colonies of RUH102 are most likely due to a reduced amount of total EPS production for RUH, compared to the parent strain.

On YEM agar containing Calcofluor, the morphologies of the RUH102 colonies were similar to those of class III EPS-defective mutants of TAL1145 described previously (Parveen *et al.*, 1997). These morphological features included partially mucoid colonies with reduced amount of EPS and dim blue fluorescence under UV. To determine if the size distribution of the soluble EPS produced by RUH102 is similar to that of the class III mutants, we analysed the supernatant of RUH102 grown in YEM broth on a column of Bio-Gel A-5M. The supernatant (culture medium) was concentrated using a rotary evaporator, then filtered before lyophilizing and resuspending in a sodium phosphate buffer. When the resuspended solution from RUH102 was fractionated using a Bio-Gel A-5M column, two distinct peaks, representing high- and lower-molecular-mass components, were obtained. Both components were estimated to be well in excess of 300 kDa, based on the fractionation range of Bio-Gel A-5M. The peak area for the high-molecular-mass EPS fraction was approximately half

that of the peak area for the lower-molecular-mass fraction. Under similar growth conditions TAL1145 yielded a larger peak area for the high-molecular-mass EPS and a smaller peak for the lower-molecular-mass EPS (data not shown). Thus, RUH102 showed a similarity to class III mutants in terms of EPS profile (Parveen *et al.*, 1997). The identities of the polysaccharide components in the Bio-Gel A-5M fractions were not investigated further in the present study.

The mutation in RUH102 does not affect its growth

The mutant RUH102 does not have auxotrophic defects. To determine if this mutation affects its growth in normal conditions, RUH102 and TAL1145 were grown in TY supplemented with three different combinations of glucose, mannitol and NH₄Cl. Cultures were inoculated with less than 10⁶ cells per ml and the growth of the bacteria was measured as the OD₆₀₀ of the cultures twice a day for 4 days. Mutant RUH102 grew as well as did TAL1145 in TY, TY-0.1 % mannitol-0.1 % NH₄Cl, TY-0.1 % glucose-0.1 % NH₄Cl, and TY-0.1 % glucose (data not shown). Thus, the mutation in RUH102 does not affect its normal growth in standard conditions.

Symbiotic properties of mutant RUH102

To determine the symbiotic properties of RUH102, leucaena seedlings were inoculated with 10⁶ cells of RUH102 or TAL1145 by pipetting to the root rhizosphere. The plants inoculated with RUH102 showed chlorosis and stunting symptoms after 4 weeks, whereas the plants inoculated with TAL1145 were green and healthy. RUH102 induced fewer nitrogen-fixing nodules; these nodules were dispersed and their number was 30 % of the total number induced by TAL1145. The dry matter and total nitrogen content of the plants nodulated by RUH102 were 44 % and 56 %, respectively, of those of the plants nodulated by TAL1145 (Table 2). The control plants, which were not inoculated with rhizobia, carried no nodules and produced about the same dry matter but contained 49 % less nitrogen compared to the plants inoculated with RUH102 (Table 2). Bacteria isolated from the nodules retained their original phenotypes. When RUH102, reisolated from nodules, was used to

inoculate leucaena, the plants exhibited similar phenotypes as the original RUH102. These results suggest that the number of nitrogen-fixing nodules induced by RUH102 is not enough to support normal plant growth.

The locus disrupted in RUH102 is a sigma factor gene, *rpoH2*

To identify the gene disrupted in RUH102, the 2.0 kb *Hind*III fragment of plasmid pUHR38 was sequenced. Analysis of the sequence revealed one ORF of 870 bp as a possible gene disrupted in the mutant RUH102. A single *Bam*HI site at nucleotide 302 from the first ATG of this ORF is the site where the kanamycin-resistance cassette was inserted to create mutant RUH102 (Fig. 1). A 1.4 kb

*Eco*RV–*Hind*III fragment, cloned in plasmid pUHR241, complemented RUH102 for EPS synthesis and nodulation. To further demonstrate that the identified ORF is the gene disrupted in RUH102, a PCR constructed clone of 1263 bp, containing the ORF and its upstream putative regulatory region, was introduced to RUH102 for possible complementation for EPS synthesis and nodulation. This fragment is flanked with artificial *Eco*RI sites at both ends and cloned to pRK404 to give plasmid pUHR304. Introduction of pUHR304 into RUH102 complemented for EPS synthesis (Fig. 1) and nodulation.

The identified ORF encodes a putative protein of 32 585 Da with a pI of 7.99. Comparison of the deduced amino acid sequence to proteins catalogued in the databases revealed

Fig. 2. Phylogenetic tree representing 30 RpoH sigma factor proteins in other bacteria catalogued in the NCBI databases including RpoH-like RpoH2 in *Rhizobium* sp. strain TAL1145. This tree was constructed using the CLUSTAL program available in version 9.1 of the Genetics Computer Group (GCG) package (Oxford Molecular Group). The bacteria and NCBI accession numbers used were: *Escherichia coli*, NP_417918; *Shigella flexneri*, AAN44938; *Salmonella enterica*, NP_458353; *Salmonella typhimurium*, NP_462469; *Yersinia pestis*, NP_407258; *Haemophilus influenzae*, NP_438438; *Pasteurella multocida*, NP_246523; *Colwellia maris*, BAC76410; *Pseudomonas putida*, AAN70673; *Pseudomonas syringae*, AAO53974; *Pseudomonas aeruginosa*, NP_249067; *Coxiella burnetii*, AAF26609; *Xanthomonas axonopodis*, NP_644130; *Xanthomonas campestris*, NC_003902; *Xylella fastidiosa*, NP_780223; *Ralstonia metallidurans*, CAC42416; *Ralstonia solanacearum*, NP_518495; *Bordetella pertussis*, CAE44005; *Chromobacterium violaceum*, AAQ61866; *Neisseria meningitidis*, NP_283704; *Agrobacterium tumefaciens*, BAA09439; *Sinorhizobium meliloti*, *rpoH1*: BAB13517; *Bradyrhizobium japonicum*, *rpoH2*: BAC52602; *Caulobacter crescentus*, NP_421892; *Bradyrhizobium japonicum*, *rpoH1*: NP_771871; *Rickettsia conorii*, NP_360044; *Rickettsia prowazekii*, NP_220687; *Sinorhizobium meliloti*, *rpoH2*: NP_387362; *Rhizobium* sp. TAL1145, AY536267; *Mesorhizobium loti*, *rpoH*-like gene C: NP_104874. (1), RpoH1; (2), RpoH2; (C), RpoH C.

significant similarity to RpoH-like sigma factors (Fig. 2). It has 75 % identity with sigma factor RpoH2 in *S. meliloti*, 65 % identity with RpoH-like sigma factor C in *Mesorhizobium loti* and up to 66 % identity with alternative sigma factors from many bacteria including *Agrobacterium tumefaciens*. This gene has been designated *rpoH2* due to high similarity of the deduced amino acid sequence with RpoH2 proteins of other rhizobia. Alignment of the RpoH2 sequence with sigma factors of other rhizobia displayed many identical residues (data not shown). RpoH2 has two highly conserved regions, located at amino acid residues 46–116 and 225–276, which are common to regions 2 and 4, respectively, of the sigma-70 family. Region I of RpoH2 at the amino terminus with 45 residues and region 3 with 109 residues do not show significant similarity with regions 1 and 3 of other RpoH sigma factors.

Expression of the *rpoH2* gene in *Escherichia coli*

To determine if the *rpoH2* ORF produces a 32 kDa protein as predicted from the deduced amino acid sequence, we expressed the ORF in *E. coli*. A close examination of the *rpoH2* ORF revealed four possible ATG translation start sites in the same frame, at positions 1, 7, 16 and 34. We constructed four clones by PCR amplification of the ORFs between these possible start sites and the stop codon. Two restriction sites, *NdeI* and *EcoRI*, were introduced into the beginning and the end of the constructs, respectively, to allow their directional cloning into the expression vector pET-14b. The sequence of the cloned fragments was verified by sequencing. The resulting plasmids, pUHR298, pUHR299, pUHR300 and pUHR301, contained the gene constructs corresponding to the four ATG start codons above (Table 1). All four constructs were driven by a T7 promoter, and expressed equally in *E. coli* L21 (DES)pLysS, and the protein products migrated on an SDS-polyacrylamide gel at the expected size of about 32 kDa (data not shown). These results indicate that *rpoH2* does produce a 32 kDa protein in TAL1145.

RpoH2 is a transcriptional regulator of the *exo* genes

Cosmid pUHR223 containing a 24 kb region from strain TAL1145 complemented several EPS-defective mutants of TAL1145 (Parveen *et al.*, 1997). A 7.6 kb *HindIII* fragment of pUHR223, subcloned in plasmid pUHR290, complemented some of these EPS-defective mutants. Sequence analysis of this 7.6 kb region revealed six putative ORFs similar to *exo* genes, *exoHKLAMO*, in *S. meliloti* (our unpublished results). This 7.6 kb fragment includes a 414 bp upstream region of *exoH* that may contain the promoter of the *exo* gene cluster. To investigate if RpoH2 regulates the transcription of these *exo* genes in strain TAL1145, we constructed transcriptional *exo-gus* fusions by inserting the promoterless *gus* gene of Tn3Hogus into the *exo* gene cluster in pUHR290. The *gus* gene would be expressed if inserted into a target gene in the correct

orientation. We selected only those insertions that showed Gus activities in the background of TAL1145. Restriction analyses of these derivatives show that the Tn3Hogus inserted in the same orientation as the disrupted genes. Most of the insertions were mapped on the *exoH* gene, although some insertions were also located on the downstream region of *exoH*.

These fusions were introduced into the *rpoH2* mutant RUH102 and the wild-type strain TAL1145 backgrounds to report the role of RpoH2 in the transcriptional activation of these *exo* genes. The Gus activities for a particular *exo-gus* fusion in both backgrounds would be the same if RpoH2 did not transcriptionally regulate that *exo* gene. The Gus activities were analysed by fluorometric assay, and the data are summarized in Table 3. The *exo-gus* fusions showed 2–441-fold higher Gus activities in TAL1145 background when compared to the *rpoH2* mutant background. These results demonstrate that RpoH2 regulates the transcription of some *exo* genes in TAL1145.

DISCUSSION

This is believed to be the first report of an alternative sigma factor involved in the regulation of EPS synthesis in rhizobia. The alternative sigma factor in TAL1145 is an orthologue of the heat-shock sigma factor RpoH in several Gram-negative bacteria, and has homologues in *S. meliloti*,

Table 3. Gus activity of the *exo::gus* fusions in the backgrounds of strain TAL1145 and the *rpoH2* mutant strain RUH102

The β -glucuronidase (Gus) activity determined by fluorometric assay as described in Methods was normalized to OD₆₀₀. Each value represents the mean GUS activity $\pm$ SEM of triplicates. MU, fluorescent compound 7-hydroxy-4-methylcoumarin.

pUHR290::Tn3Hogus insertion	Gus activity (nM MU (ml cells) ⁻¹ h ⁻¹)	
	TAL1145	RUH102
<i>exo290-13</i>	30 727 $\pm$ 786	1 697 $\pm$ 127
<i>exoH-57</i>	19 823 $\pm$ 836	337 $\pm$ 46
<i>exo290-58</i>	5 620 $\pm$ 248	2 340 $\pm$ 121
<i>exo290-70</i>	19 907 $\pm$ 1 352	210 $\pm$ 165
<i>exo290-72</i>	53 757 $\pm$ 2 829	390 $\pm$ 137
<i>exo290-73</i>	121 543 $\pm$ 5 922	457 $\pm$ 32
<i>exo290-78</i>	89 183 $\pm$ 4 209	1 400 $\pm$ 76
<i>exo290-80</i>	24 620 $\pm$ 1 434	1 987 $\pm$ 101
<i>exo290-90</i>	12 613 $\pm$ 672	267 $\pm$ 25
<i>exo290-93</i>	9 130 $\pm$ 382	100 $\pm$ 17
<i>exoH-97</i>	34 257 $\pm$ 2 041	80 $\pm$ 17
<i>exo290-98</i>	29 363 $\pm$ 1 266	160 $\pm$ 27
<i>exo290-104</i>	19 807 $\pm$ 1 005	133 $\pm$ 15
Control (no plasmid)	80 $\pm$ 66	130 $\pm$ 135

M. loti and *Agrobacterium tumefaciens*. There are two homologues of RpoH in *S. meliloti* and *M. loti*. The RpoH2 in *S. meliloti* of size 288 aa displays 75% identity with RpoH2 of TAL1145 while the RpoH1 of 301 aa shows only 45% identity. The two homologues in *M. loti* are 287 and 303 aa and have 65% and 47% identities with RpoH2 of TAL1145, respectively. Multiple alignment analysis shows that *S. meliloti* RpoH2 of size 288 aa, and *M. loti* RpoH C of size 287 aa, and TAL1145 RpoH2 of size 289 aa are in the same group (Fig. 2).

In *S. meliloti*, involvement of RpoH2 in EPS synthesis has not been established. Even the *rpoH1* and *rpoH2* double mutant of *S. meliloti* has not been shown to be EPS defective (Ono *et al.*, 2001). However, the *rpoH2* mutant in TAL1145 has an obvious EPS-defective phenotype, which enabled us to isolate and characterize this gene. To determine if the *rpoH2* genes of *S. meliloti* could complement the EPS defect of RUH102, we amplified by PCR the *rpoH2* gene of *S. meliloti* Rm1021 as a 1323 bp fragment including 431 bp upstream and 25 bp downstream of the *rpoH2* ORF, and cloned it into the broad-host-range plasmid vector pRK404 (Fig. 1). The resulting plasmid, pUHR323, complemented RUH102 for EPS synthesis, suggesting that RpoH2 of TAL1145 and *S. meliloti* may have similar regulatory functions.

In some Gram-negative bacteria, alternative sigma factors are involved in the synthesis of the EPS alginate. Controlling alginate production by these sigma factors is common to *Pseudomonas* species and *Azotobacter vinelandii* as a possible mechanism for their adaptation to different ecological sites (DeVries *et al.*, 1994; Keith & Bender, 1999; Martinez-Salazar *et al.*, 1996). Mucoid-to-nonmucoid conversions in the alginate-producing *Pseudomonas aeruginosa* often result from mutations in *algT*, which encodes an alternative sigma factor required for the transcription of *algD* and *algR1* involved in alginate synthesis (DeVries *et al.*, 1994). The *algT* gene has also been isolated from the phytopathogen *Pseudomonas syringae* (Keith & Bender, 1999). In *A. vinelandii*, an alternative sigma factor gene, *algU*, has 93% identity to the *algT* gene in *P. aeruginosa*. Alginate production by *A. vinelandii* is strongly correlated with AlgU activity, and disruption of *algU* resulted in nonmucoid mutant (Martinez-Salazar *et al.*, 1996). AlgT or AlgU is functionally equivalent to the extreme heat shock sigma factor σ^E in *E. coli* and other Gram-negative bacteria. However, AlgT and AlgU showed very low similarity to the RpoH2 in TAL1145, suggesting that RpoH2 is different from these sigma factors.

The alternative RpoH-like sigma factor, RpoH2, found in this study represents a new class of positive regulator for EPS synthesis in strain TAL1145. Expression of the full-length *rpoH2*-coding region in *E. coli* revealed a protein of 32 kDa, similar to the predicted molecular mass. We have expressed the *rpoH2* ORF from the four putative start sites, at positions 1, 3, 6 and 12, in *E. coli*, which indicates that *rpoH2* can be translated from any of these sites. The ATG at

position 3 of *rpoH2* corresponds to the translation start sites of RpoH2 of *S. meliloti* and RpoH C of *M. loti* (Oke *et al.*, 2001; Kaneko *et al.*, 2000). However, a close examination of *rpoH2* for the presence of Shine-Dalgarno sequence reveals that the first ATG site with an AGGA sequence 9 nt upstream is the most likely translation start site. We have also shown that some of the *exo* genes for succinoglycan synthesis in TAL1145 require RpoH2 for transcription. This RpoH2 subunit contains conserved regions common to regions 2 and 4 of the sigma-70 family. Region 2 is involved in both the -10 promoter recognition and the primary core RNA polymerase binding determinant (Malhotra *et al.*, 1996; Campbell *et al.*, 2002). Region 4 is involved in binding to the -35 promoter element via a helix-turn-helix motif (Campbell *et al.*, 2002). Regions 2 and 4 of RpoH2 are found at amino acid residues 46-116 and 225-276, respectively.

The *rpoH2* mutant RUH102 synthesized EPS in reduced amounts, but with the same molecular mass as TAL1145, as verified by size-exclusion chromatography. Additionally, RUH102 was Calcofluor-dim, indicating the production of traces of succinoglycan. These characteristics are quite similar to the phenotypes of mutants in the previously identified positive regulators, *mucR* and *exoD*, of the *exo* genes in *S. meliloti* (Keller *et al.*, 1995; Reed *et al.*, 1991). The colony morphology and the EPS profile of RUH102 are similar to those of the EPS-defective class III mutants of TAL1145, described previously (Parveen *et al.*, 1997). However, the symbiotic phenotype of RUH102 is somewhat different from those of the class III mutants. RUH102 formed mostly small ineffective nodules on leucaena, which were similar to those induced by the class III mutants, and from which rhizobia could not be isolated. But unlike the class III mutants, RUH102 formed a few large nodules and rhizobia were isolated from these nodules. The isolated rhizobia exhibited the same phenotypic characteristics as RUH102 on YEM medium. When these rhizobia were reinoculated on leucaena seedlings, they again formed mostly small ineffective nodules and a few large effective nodules. We analysed the symbiotic properties of RUH102 on leucaena three times independently, and each time we observed the formation of a few nitrogen-fixing nodules, resulting in about 55% nitrogen content in leucaena compared to plants inoculated with TAL1145. Keller *et al.* (1995) reported a similar observation that a *mucR* mutant of *S. meliloti* induced the formation of few nitrogen-fixing nodules on alfalfa. We propose that partial nodule induction by mutant RUH102 was due to leakiness in the expression of the *exo* genes regulated by RpoH2, and that this also accounts for the traces of succinoglycan it produced. The results of this study suggest that RpoH2 plays an important role in the regulation of EPS biosynthesis in TAL1145. The discovery of this alternative sigma factor in strain TAL1145 will expedite the understanding of the mechanism of EPS biosynthesis in rhizobia, because the sigma factor protein family is well studied in other bacteria.

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