Regulation of flagellar, motility and chemotaxis genes in *Rhizobium leguminosarum* by the VisN/R-Rem cascade

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In this paper, we describe the regulatory roles of VisN, VisR and Rem in the expression of flagellar, motility and chemotaxis genes in *Rhizobium leguminosarum* biovar *viciae* strains VF39SM and 3841. Individual mutations in the genes encoding these proteins resulted in a loss of motility and an absence of flagella, indicating that these regulatory genes are essential for flagellar synthesis and function. Transcriptional experiments involving *gusA*–gene fusions in wild-type and mutant backgrounds were performed to identify the genes under VisN/R and Rem regulation. Results showed that the chemotaxis and motility genes of *R. leguminosarum* could be separated into two groups: one group under VisN/R-Rem regulation and another group that is independent of this regulation. VisN and VisR regulate the expression of *rem*, while Rem positively regulates the expression of *flaA*, *flaB*, *flaC*, *flaD*, *motA*, *motB*, *che1* and *mcpD*. All of these genes except *mcpD* are located within the main motility and chemotaxis gene cluster of *R. leguminosarum*. Other chemotaxis and motility genes, which are found outside of the main motility gene cluster (*che2* operon, *flaH* for VF39SM, and *flaG*) or are plasmid-borne (*flaE* and *mcpC*), are not part of the VisN/R-Rem regulatory cascade. In addition, all genes exhibited the same regulation pattern in 3841 and in VF39SM, except *flaE* and *flaH*. *flaE* is not regulated by VisN/R-Rem in 3841 but it is repressed by Rem in VF39SM. *flaH* is under VisN/R-Rem regulation in 3841, but not in VF39SM. A kinetics experiment demonstrated that a subset of the flagellar genes is continuously expressed in all growth phases, indicating the importance of continuous motility for *R. leguminosarum* under free-living conditions. On the other hand, motility is repressed under symbiotic conditions. Nodulation experiments showed that the transcriptional activators VisN and Rem are dramatically downregulated in the nodules, suggesting that the symbiotic downregulation of motility-related genes could be mediated by repressing the expression of VisN/R and Rem.

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INTRODUCTION

Rhizobia are motile members of the α -proteobacteria that are known for establishing nitrogen-fixing symbioses inside the nodules of leguminous plants. These nodule bacteria exist either as free-living organisms in the soil or in association with their host plant. In the soil environment, there is a limited availability of utilizable carbon sources for rhizobia. Most of the organic matter in the soil is resistant to microbial degradation and can also form complexes with other compounds (Morita, 1988). There is a narrow region in the soil, the rhizosphere, that is rich in organic and

inorganic substances secreted by plants (Walker *et al.*, 2003). The ability of rhizobia to reach the rhizosphere and to colonize plant roots enables these soil bacteria to escape nutrient starvation in the soil (Thorne & Williams, 1997). The ability to sense nutrients and move towards them would therefore provide a competitive advantage for motile and chemotactic rhizobia over non-motile/non-chemotactic strains. The importance of motility and chemotaxis in the ecology of rhizobia is supported by a number of studies that have shown that non-motile or non-chemotactic strains are less competitive and less efficient in nodulation (Ames & Bergman, 1981; Soby & Bergman, 1983; Mellor *et al.*, 1987; Caetano-Anollés *et al.*, 1988; Bauer & Caetano-Anollés, 1990; Liu *et al.*, 1989; Malek, 1992; Yost *et al.*, 1998).

The motility system of rhizobia differs from the known enterobacterial system in terms of genetic organization, flagellar filament assembly, and mode of flagellar rotation.

Abbreviations: MU, Miller units; TEM, transmission electron microscopy. Two supplementary tables, showing primers used to amplify *visN*, *visR* and *rem*, and details of fragments used for gene–*gusA* fusions, and a supplementary figure, showing restriction maps of *visN*, *visR* and *rem*, are available with the online version of this paper.

The flagellar regulon, which consists of the flagella, motility, chemotaxis and regulatory genes, of *Salmonella enterica* and *Escherichia coli* is located in three and four separate clusters, respectively (Kutsukake *et al.*, 1990; Macnab, 1992). On the other hand, the majority of rhizobial genes involved in flagellar assembly and function are located in a single region in the chromosome of rhizobia (Sourjik *et al.*, 1998; Deakin *et al.*, 1999). In *Sinorhizobium meliloti*, 51 chemotaxis (*che*), flagellar (*fla*, *flg*, *flh*, *fli*), motility (*mot*) and regulatory (*visN*, *visR*, *rem*) genes are arranged in a single 56 kb region in the chromosome (Sourjik *et al.*, 1998; Eggenhofer *et al.*, 2006; Rotter *et al.*, 2006). The flagellar filament of enteric bacteria consists of one flagellin subunit, while the flagellar filaments of *S. meliloti* and *Agrobacterium tumefaciens* are assembled from four closely related flagellin subunits (Pleier & Schmitt, 1989, 1991; Sourjik *et al.*, 1998; Deakin *et al.*, 1999). Rhizobia also differ from enteric bacteria in terms of flagellar movement. The rhizobial flagellum rotates in one direction only, i.e. clockwise, as opposed to the bidirectional rotation of the enteric flagellum (Sourjik & Schmitt, 1996; Berg, 2003; Sourjik *et al.*, 1998). It has been suggested that the direction of movement is reversed by changes in flagellar rotational speed (Sourjik & Schmitt, 1996; Miller *et al.*, 2007). This type of flagellar movement is thought to be an adaptation of the rhizobia to the specific condition of swimming in viscous fluids that is prevalent in the soil (Scharf *et al.*, 2001).

While it is advantageous for bacteria to be able to move towards favourable environments and away from detrimental conditions, motility is energetically costly. The bacterial cell expends 2% of its biosynthetic energy for the synthesis and function of the flagellum. Thus, flagellar gene expression is highly regulated by environmental conditions (Soutourina & Bertin, 2003). The regulation of genes involved in flagellar assembly and function is well studied in enteric bacteria (Silverman & Simon, 1974; Shi *et al.*, 1992; Pruss & Matsumura, 1997; Soutourina *et al.*, 2001; Berg, 2003). On the other hand, the regulation of motility-related genes is not well understood in rhizobia. To date, the only member of the rhizobia for which the regulation of the flagellar genes has been analysed is *S. meliloti* (Sourjik *et al.*, 2000; Rotter *et al.*, 2006). The motility-related genes of *S. meliloti* are tightly regulated in a hierarchical manner. Class IA includes the LuxR-type master regulatory proteins VisN and VisR, which may act as a heterodimer exerting control over the transcription of *rem* (class IB) (Rotter *et al.*, 2006; Sourjik *et al.*, 2000). Rem is an OmpR-like transcriptional activator that controls the expression of class II genes, which comprise flagellar assembly and motility genes (Rotter *et al.*, 2006). The class IIA genes *fliM* and *orf 38* have been suggested to regulate the expression of the class III genes, which include flagellin and chemotaxis genes. The class IIB genes *motA*, *motB* and *motC* do not exert transcriptional control over the class III genes (Sourjik *et al.*, 2000).

In this paper, we will describe the roles of VisN/R and Rem in the regulation of motility and chemotaxis genes in *Rhizobium leguminosarum* under free-living and symbiotic

conditions. We performed the studies using strains 3841 and VF39SM because the two strains exhibit substantial differences in pattern of flagellation and swarming motility (Tambalo *et al.*, 2010). Using transcriptional fusions to a large number of genes involved in chemotaxis and motility, we demonstrate that only a subset of these genes is regulated by the VisN/R cascade. We also looked at the kinetics of flagellin and motility gene expression to determine their expression patterns at different growth phases. The experiments revealed that the flagellin genes are continuously expressed, indicating that motility may be continuously needed in *R. leguminosarum* under free-living conditions, though it is downregulated during symbiosis.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *R. leguminosarum* strains were cultured in TY medium (Beringer, 1974), while *E. coli* strains were grown in LB medium (Sambrook *et al.*, 1989). Antibiotic concentrations used to culture *R. leguminosarum* were: 500 µg streptomycin ml⁻¹, 30 µg gentamicin ml⁻¹, 100 µg neomycin ml⁻¹, 100 µg spectinomycin ml⁻¹ and 5 µg tetracycline ml⁻¹. *E. coli* strains were grown in the following antibiotic concentrations: 100 µg ampicillin ml⁻¹, 50 µg kanamycin ml⁻¹, 15 µg gentamicin ml⁻¹ and 10 µg tetracycline ml⁻¹.

Motility tests. Strains were grown in TY broth for 24 h. Swimming motility was determined using a soft agar motility assay, which was done by inoculating the strains into a motility medium containing 0.3% agar, 0.01% yeast extract and 1 mM MgSO₄ (Ames *et al.*, 1980; Yost *et al.*, 1998). The swimming motility of each of the mutant strains was compared with that of the wild-type 3–4 days after inoculation. Motility was also confirmed by observation under a phase-contrast microscope.

Recombinant DNA techniques. DNA manipulations were done using standard methods (Sambrook *et al.*, 1989). Restriction endonucleases used in this study were purchased from Invitrogen and used according to the manufacturer's specifications. DNA fragments were isolated from the gel using a QIAquick Gel Extraction kit (Qiagen). Plasmids were isolated from *E. coli* strains using a GeneJET Plasmid Miniprep kit (Fermentas Life Sciences). Total DNA was isolated from *R. leguminosarum* strains using an AquaPure Genomic DNA Isolation kit (Bio-Rad Laboratories). Primers were designed by Sigma-Genosys (Sigma-Aldrich) and amplification was carried out using a MultiGene II thermal cycler (Labnet International). Southern blots were performed using a non-radioactive technique with reagents and protocols supplied by Roche Applied Science.

Cloning of *visNR* and *rem* genes. The regulatory genes *visN*, *visR* and *rem* were PCR-amplified from *R. leguminosarum* and then cloned individually into the vector pCR2.1-TOPO using the TOPO Cloning kit (Invitrogen). The primers used for amplifying these genes are shown in Supplementary Table S1.

Insertional mutagenesis. The three regulatory genes *visN*, *visR* and *rem* were excised from the TOPO vector and then ligated into the suicide vector pJQ200SK (Quandt & Hynes, 1993). Mutants were constructed by introducing either a spectinomycin-resistance or a kanamycin-resistance cassette (Prentki & Krisch, 1984; Fellay *et al.*, 1987) (Supplementary Fig. S1) within the regulatory gene. The mutated gene was introduced into the genome of *R. leguminosarum* by homologous recombination, as described by Quandt & Hynes

Table 1. Bacterial strains and plasmids used in this study

Abbreviations: Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Nm^r, neomycin resistance; Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance; Tc^r, tetracycline resistance.

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i> strains		
DH5 α	<i>EndA1, hsdR17, supE44, thi-1, recA1, gyrA96, relA1 (argF-lacZYA)</i> , U169, ϕ 80dlacZ Δ M15	Invitrogen
S17.1	Sp ^r . RP4 <i>tra</i> region, mobilizer strain	Simon <i>et al.</i> (1983)
<i>R. leguminosarum</i> strains		
3841	Biovar <i>viciae</i> , JB300 derivative, Sm ^r	Poole <i>et al.</i> (1994)
VF39SM	Biovar <i>viciae</i> , Sm ^r	Priefer (1989)
VF39SM <i>flaA</i> [−]	<i>flaA::gusA</i> fusion integrated into the genome of VF39SM, Sm ^r , Gm ^r , Nm ^r	This work
3841 <i>flaA</i> [−]	<i>flaA::gusA</i> fusion integrated into the genome of 3841, Sm ^r , Gm ^r , Nm ^r	This work
3841 <i>visN</i> [−]	Sp ^r inserted into <i>visN</i> of 3841, Sm ^r , Sp ^r	This work
3841 <i>visR</i> [−]	Km ^r inserted into <i>visR</i> of 3841, Sm ^r , Nm ^r	This work
3841 <i>rem</i> [−]	Km ^r inserted into <i>rem</i> of 3841, Sm ^r , Nm ^r	This work
VF39SM <i>visN</i> [−]	Sp ^r inserted into <i>visN</i> of VF39SM, Sm ^r , Sp ^r	This work
VF39SM <i>visR</i> [−]	Km ^r inserted into <i>visR</i> of VF39SM, Sm ^r , Nm ^r	This work
VF39SM <i>rem</i> [−]	Sp ^r inserted into <i>rem</i> of 3841, Sm ^r , Sp ^r	This work
Plasmids		
pFus1	pMP220 derivative with promoterless <i>gusA</i> , Tc ^r	Reeve <i>et al.</i> (1999)
pFAJ1701	RK2-derived cloning vector carrying <i>gusA</i> , Ap ^r Tc ^r	Dombrecht <i>et al.</i> (2001)
pFus1P	pFus1, par cassette inserted from pRR71-H as a <i>Hind</i> III fragment, Tc ^r	Yost <i>et al.</i> (2004)
pRK7813	Broad-host-range cloning vector, Tc ^r	Jones & Gutterson (1987)
pCRS530	Contains a promoterless <i>gusA</i> –Nm cassette, Nm ^r	Reeve <i>et al.</i> (1999)
pRR71-H	Plasmid carrying RK2 par locus as a <i>Hind</i> III fragment	Easter <i>et al.</i> (1997)
pFche1	<i>Eco</i> RI fragment containing the promoter region of the <i>che1</i> operon cloned into pFUS1	Miller <i>et al.</i> (2007)
pFche2	<i>Eco</i> RI fragment containing the promoter region of the <i>che2</i> operon cloned into pFUS1	Miller <i>et al.</i> (2007)
pFus1:: <i>flaA</i>	<i>flaA</i> promoter introduced into pFus1, Tc ^r	This work
pFAJ1701:: <i>flaB</i>	<i>flaB</i> promoter introduced into pFAJ1701, Ap ^r Tc ^r	This work
pFus1:: <i>flaC</i> (3841)	<i>flaC</i> promoter from 3841 introduced into pFus1, Tc ^r	This work
pFus1:: <i>flaC</i> (VF39SM)	<i>flaC</i> promoter from VF39SM introduced into pFus1, Tc ^r	This work
pFus1:: <i>flaD</i> (3841)	<i>flaD</i> promoter from 3841 introduced into pFus1, Tc ^r	This work
pFus1:: <i>flaD</i> (VF39SM)	<i>flaD</i> promoter from VF39SM introduced into pFus1, Tc ^r	This work
pFus1:: <i>flaE</i> (3841)	<i>flaE</i> promoter from 3841 introduced into pFus1, Tc ^r	This work
pFus1:: <i>flaE</i> (VF39SM)	<i>flaE</i> promoter from VF39SM introduced into pFus1, Tc ^r	This work
pFus1:: <i>flaH</i>	<i>flaH</i> promoter from 3841 introduced into pFus1, Tc ^r	This work
pFus1:: <i>flaG</i>	<i>flaG</i> promoter from 3841 introduced into pFus1, Tc ^r	This work
pFus1:: <i>motA</i>	<i>motA</i> promoter from 3841 introduced into pFus1, Tc ^r	This work
pFus1:: <i>motB</i>	<i>motB</i> promoter from 3841 introduced into pFus1, Tc ^r	This work
pFAJ1701:: <i>motB</i>	<i>motB</i> promoter introduced into pFAJ1701, Ap ^r Tc ^r	This work
pFus1:: <i>mcpC</i>	<i>mcpC</i> promoter introduced into pFus1, Tc ^r	Yost <i>et al.</i> (2004)
pFus1:: <i>mcpD</i>	<i>mcpD</i> promoter introduced into pFus1, Tc ^r	Yost <i>et al.</i> (2004)
pFus1:: <i>rem</i> (3841)	<i>rem</i> promoter from 3841 introduced into pFus1, Tc ^r	This work
pFus1:: <i>rem</i> (VF39SM)	<i>rem</i> promoter from VF39SM introduced into pFus1, Tc ^r	This work
pFus1:: <i>visN</i> (3841)	<i>visN</i> promoter from 3841 introduced into pFus1, Tc ^r	This work
pFAJ1701:: <i>visN</i> (3841)	<i>visN</i> promoter from 3841 introduced into pFAJ1701, Ap ^r Tc ^r	This work
pFus1:: <i>visN</i> (VF39SM)	<i>visN</i> promoter from VF39SM introduced into pFus1, Tc ^r	This work

(1993). Southern hybridization was done for each gene to confirm replacement of the wild-type gene with the mutated gene.

Transmission electron microscopy (TEM). Electron microscopy was performed by slightly modifying the procedure of Miller *et al.* (2007). The regulatory mutants were grown on TY plates at 30 °C for 48 h. A culture suspension was prepared using sterile double-distilled water. A Formvar-carbon-coated grid was placed on top of a cell suspension drop for 3 min and excess liquid was removed. Staining

was performed using 1% uranyl acetate for 30 s. Samples were observed using a Hitachi 7650 transmission electron microscope and images were taken using the AMT Image Capture Engine.

Construction of gene fusions and β -glucuronidase (*gusA*) reporter gene assays. To construct gene fusions, the promoters of each of the genes were cloned upstream of a promoterless *gusA* gene in the vector pFus1 (Reeve *et al.*, 1999), as described by Yost *et al.* (2004). The regulatory genes *visN* and *visR* are cotranscribed in

one operon in *S. meliloti* (Sourjik *et al.*, 2000), and their proximity to each other in *R. leguminosarum* (Young *et al.*, 2006) suggested that this was also the case for this species. For this reason, the *gusA* fusion was constructed using the upstream region from *visN*. For stabilized fusions, the promoter region of the gene was cloned into the par-stabilized *gusA* fusion vector pFAJ1701 (Dombrecht *et al.*, 2001) or pFusIpar (Yost *et al.*, 2004). Genome-integrated fusions were constructed by inserting a promoterless *gusA* gene within the corresponding flagellar gene (downstream of the promoter region) in a pJQ200SK gene construct. The gene fusion was integrated into the genome by homologous recombination. Correct integration was confirmed by Southern hybridization. The details of the generation of gene fragments used for constructing *gusA*-gene fusions are shown in Supplementary Table S2.

For the *gusA* assays involving the fusion strains in the wild-type and mutant backgrounds, the strains were grown in TY broth for 48 h at 30 °C. The kinetics study was performed by growing the strains in TY broth at 30 °C with shaking (75 r.p.m.) and then samples were taken periodically for the *gusA* assay. β -Glucuronidase activity was measured as described by Jefferson *et al.* (1986) and modified by Yost *et al.* (2004). All data given are the means of at least triplicate experiments.

Nodulation conditions and *gusA* reporter gene assay for bacteroids. Plant experiments and *gusA*-gene expression assays for bacteroids were conducted as described by Yost *et al.* (2004). The nodules of plants inoculated with the *R. leguminosarum gusA* fusion strains were crushed and the supernatant containing the bacteroids was used for *gusA* assay. All data given in the tables and figures are means of triplicates, and nearly all experiments were repeated independently several times with similar results. The nodulation experiments were repeated at least twice, with similar trends.

RESULTS AND DISCUSSION

Motility genes of *R. leguminosarum* 3841

Analysis of the genome of *R. leguminosarum* 3841 (Young *et al.*, 2006) showed that this strain has a large flagellar gene cluster (Fig. 1) similar to that of *S. meliloti*. This gene cluster in *R. leguminosarum* contains the majority of the genes involved in flagellar assembly and function, including four flagellin genes, the regulatory genes *visN*, *visR* and *rem*, and the motility genes *motABCfliK*. Although *R. leguminosarum* and *S. meliloti* are closely related, they exhibit significant differences, such as the number of flagellin subunits and chemoreceptor genes. In addition to the four *fla* genes of *R. leguminosarum* (*flaA* to *flaD*) shown in Fig. 1, we have cloned three *fla* genes, *flaE* (pRL110518), *flaH* (RL3268) and *flaG* (RL 4729). Both *flaH* and *flaG* are chromosomally located in loci separate from the main flagellar regulon shown in Fig. 1. The other flagellin gene, *flaE*, is located on plasmid pRL11JI. The flagellin genes of *R. leguminosarum* encode proteins of around 300 amino acids, which are considerably shorter than the flagellins (321–401 amino acids) of *S. meliloti* (Capela *et al.*, 2001). Five of the flagellin genes (*flaABCEG*) encode highly similar proteins, while *flaD* and *flaH* are quite divergent. There are also two complete *che* operons in *R. leguminosarum* (Miller *et al.*, 2007) and 26 chemoreceptor genes, as opposed to nine in *S. meliloti*. We have completed

Fig. 1. Chromosomal region of *R. leguminosarum* 3841 containing the major chemotaxis, flagellin, motility and regulatory genes. Genes used in this study are in bold type. Adapted from RhizoDB (<http://xbase.bham.ac.uk/rhizodb/>).

sequence analyses and functional studies of the seven *fla* genes, which we describe in a separate paper (D. D. Tambalo and others, unpublished results).

The roles of VisN, VisR and Rem in the regulation of the flagellar and motility genes of *R. leguminosarum*

Very few studies have been done on the regulation of flagellar and chemotaxis genes in rhizobia, under both free-living and symbiotic conditions. Three regulatory genes (*visN*, *visR* and *rem*) have been identified in *S. meliloti*, and these genes have been shown to function as global transcriptional activators of flagellar, motility and chemotaxis genes (Sourjik *et al.*, 2000; Rotter *et al.*, 2006). *visN* and *visR*, the LuxR-type genes, encode proteins that control the expression of *rem*. The transcriptional activator Rem, which is expressed only during the exponential phase, acts as an intermediate between VisN/R and the motility and flagellar genes (Rotter *et al.*, 2006). The roles of these regulators in the expression of flagellar and chemotaxis genes in *R. leguminosarum* were determined by cloning the

orthologous genes from the sequenced strain 3841 and our laboratory strain VF39SM. We used both strains because these strains exhibit differences in the pattern of flagellation (Tambalo *et al.*, 2010) and also in swarming motility (Tambalo *et al.*, 2010). Strain VF39SM is peritrichously flagellated, while 3841 has one to two subpolar flagella.

BLAST searches showed that VisN (RL0696), VisR (RL0697) and Rem (RL0727) have 77, 71 and 92 % similarity, respectively, to the corresponding proteins in *S. meliloti*. We constructed strains with mutations in the genes encoding the three transcriptional activators VisN, VisR and Rem for both 3841 and VF39SM. The VF39SM mutants were non-motile on swimming motility plates (Fig. 2) and appeared non-flagellated under TEM (Fig. 3), which indicated that these regulatory genes are necessary for flagellar synthesis. Complementation of the *visN* and *visR* mutants with cosmid pIJ9861 (Miller *et al.*, 2007) resulted in restoration of motility.

We constructed transcriptional fusions for *visN*, *rem*, all flagellin genes, *motA* (RL0703) and *motB* (RL0723), and also used pre-existing fusions to chemotaxis genes (*mcpC*, *mcpD*, *che1* and *che2*) (Yost *et al.*, 2004; Miller *et al.*, 2007) to determine whether VisN/R and Rem control their transcription. The *gusA*-gene fusions were introduced into the strains carrying mutations in each of the regulatory genes and in wild-type backgrounds. The ratio of the promoter activity in the mutant to that in the wild-type background was obtained (Table 2). If the mutated gene did not exert transcriptional control over the promoter being tested, the ratio would be close to 1, whereas the ratio would be less than 0.50 and close to 0 if the wild-type gene were needed for transcription. The *gusA* assays showed that for both VF39SM and 3841, the *visN/visR* promoter (*visN*

and *visR* are in an operon) was negatively autoregulated, as indicated by higher promoter activities in the *visN* and *visR* mutant backgrounds (Table 2). We also confirmed that for both strains the expression of *visN* was not heavily influenced by Rem, as the values in *rem* mutant backgrounds, although significantly different statistically, were not radically lower than in the wild-type backgrounds. The expression of the 3841 and VF39SM *rem* promoters was lower in *visN* and *visR* mutant backgrounds, suggesting that *rem* is under the regulation of VisN/R, as is the case in *S. meliloti*. The expression levels of the four flagellin genes (*flaA*, *flaB*, *flaC* and *flaD*) in both VF39SM and 3841 were reduced in the regulatory mutant backgrounds compared with gene expression in the wild-type backgrounds. For VF39SM, *flaE* and *flaH* did not exhibit a significant decrease in gene expression when the respective fusions were introduced into *visN*, *visR* and *rem* mutant backgrounds. *flaG* expression, while lower at a statistically significant level in the regulatory mutants, did not exhibit the same magnitude of dependence on VisN/R and Rem as that of other flagellin genes. For 3841, *flaH* was regulated by VisN/R and Rem, while *flaE* and *flaG* were not. Additionally, for both 3841 and VF39SM, *motA* and *motB* exhibited a reduction in gene expression in mutant backgrounds. The promoter activities of the *mcpC* and *che2* operon for both strains did not exhibit a significant decrease in gene expression in the mutant backgrounds, while those of the *mcpD* and *che1* operon were reduced.

These results obtained from the transcriptional studies revealed a hierarchy in the regulation of motility-related genes in *R. leguminosarum* (Fig. 4). The transcriptional activator VisN is on top of the hierarchy and exhibits autoregulation. VisN and VisR, which are believed to function as a heterodimer (Sourjik *et al.*, 2000), appear to regulate the expression of *rem*. The flagellar genes of *R.*

Fig. 2. Swimming motility plates for wild-type and regulatory mutants. (a) 3841 wild-type, (b) 3841 *visN* mutant, (c) 3841 *visR* mutant, (d) 3841 *rem* mutant, (e) VF39SM wild-type, (f) VF39SM *visN* mutant, (g) VF39SM *visR* mutant, (h) VF39SM *rem* mutant.

Fig. 3. TEM images of (a) *R. leguminosarum* VF39SM wild-type, (b) VF39SM *visN* mutant, (c) VF39SM *visR* mutant and (d) VF39SM *rem* mutant. Bars, 500 nm.

leguminosarum are grouped into two categories: genes that are regulated by the VisN/R-Rem regulatory cascade and genes that are independent of this central motility regulation system. The genes that are positively controlled by VisN/R-Rem include *flaA*, *flaB*, *flaC*, *flaD*, *motA*, *motB*, *mcpD* and *che1*, while *che2*, *flaE*, *flaH* (for VF39SM), *flaG* and *mcpC* did not show clear evidence of transcriptional activation by these three regulatory genes. The four flagellin genes *flaA*, *flaB*, *flaC* and *flaD*, and *motA*, *motB* and *che1*, are located in the main flagellar motility region of strain 3841. The second *che* operon, *flaH* and *flaG* are located outside the main flagellar gene cluster, while both *flaE* and *mcpC* are plasmid-borne. Hoang *et al.* (2008) have also reported a subset of flagellar genes (*flaF*, *flbT* and *flaC*) that are not regulated by Rem in *S. meliloti* strain Rm8530. However, unlike the flagellar genes of *R. leguminosarum*, which exhibit complete independence from VisN/R-Rem regulation, the flagellar genes of *S. meliloti* that are not controlled by Rem are still under the regulation of VisN. It is therefore possible that in *R. leguminosarum*, there is a second regulatory system that controls the expression of the genes that are not under the VisN/R-Rem regulatory cascade. The regulation pattern described above is the same for VF39SM and 3841, except for *flaH*, which is regulated by VisN/R and Rem in 3841 but not in VF39SM, and *flaE*, which is not under the described regulation in 3841 but repressed by Rem in VF39SM. Pairwise alignment of the promoter regions of the flagellar genes of VF39SM to the corresponding promoter regions of 3841 revealed that these regions have high homology (98–100% identity),

supporting the same regulation patterns observed in the transcription experiments.

We also noted that the transcript of the *flaE* gene from VF39SM increased to about threefold in a *rem* mutant background. This indicates that, contrary to the activator role of Rem in the expression of the other flagellin genes (*flaABCD*), Rem appears to repress the expression of *flaE*, though this effect may not be direct. Rotter *et al.* (2006) have shown through sequence alignment that Rem is a member of the OmpR family of transcriptional regulators. This family of regulators is characterized by a regulatory domain at the N terminus and a DNA-binding domain with a 'winged helix–turn–helix' pattern at the C terminus (Martínez-Hackert & Stock, 1997; Rotter *et al.*, 2006). The possible dual roles of Rem as an activator and a repressor of flagellin genes in *R. leguminosarum* have a precedent in *E. coli*, where it has been reported that OmpR can act as an activator and a repressor of a gene encoding a major outer-membrane protein (OmpF) (Rampersaud *et al.*, 1994). At low levels of phosphorylated OmpR, OmpR binds to the activator site of *ompF* to activate its expression. On the other hand, elevated levels of OmpR-phosphate would cause OmpR to bind to the repressor site, leading to *ompF* repression. A more recent study has also described opposite roles of OmpR in regulating the genes encoding outer-membrane channels in *Dickeya dadantii* (formerly *Erwinia chrysanthemi*; Condemine & Ghazi, 2007). While OmpR activates *kdgN*, which encodes an outer-membrane protein, it also acts as a repressor of *kdgM*, which encodes another

Table 2. Expression of *gusA* fusions to regulatory, flagellar, motility and chemotaxis genes in wild-type and mutant (*visN*, *visR*, *rem*) backgrounds

All values for wild-type strains are expressed as MU and are the means of at least three independent cultures ($\pm$ SD for the wild-type backgrounds). Expression levels in the *visN*, *visR* and *rem* mutants are shown as the ratio of mutant expression to that of the wild-type. Fusions were made using pFus1 unless otherwise indicated. The promoter regions were obtained from both VF39SM and 3841 unless otherwise indicated. ND, Not determined.

Fusion	Promoter activity (MU), VF39SM wild-type	Relative promoter activity			Promoter activity (MU), 3841 wild-type	Relative promoter activity		
		VF39 <i>visN</i>	VF39 <i>visR</i>	VF39 <i>rem</i>		3841 <i>visN</i>	3841 <i>visR</i>	3841 <i>rem</i>
Regulatory genes								
<i>visN</i>	3203 $\pm$ 68	1.32*	1.82*	1.02	2152 $\pm$ 88	1.77*	1.55*	0.70*
<i>rem</i>	7637 $\pm$ 834	0.12*	0.07*	0.96	5712 $\pm$ 80	0.14*	0.10*	0.60*
Flagellin genes								
<i>flaA</i>	6516 $\pm$ 200	0.39*	0.11*	0.33*	2376 $\pm$ 35	0.20*	0.38*	0.14*
<i>flaB</i> [†]	195 $\pm$ 7	0.22*	0.15*	0.14*	154 $\pm$ 11	0.22*	0.23*	0.17*
<i>flaC</i>	5207 $\pm$ 138	0.16*	0.11*	0.17*	3826 $\pm$ 494	0.31*	0.26*	0.22*
<i>flaD</i>	2522 $\pm$ 28	0.12*	0.07*	0.10*	2928 $\pm$ 115	0.11*	0.09*	0.05*
<i>flaE</i>	109 $\pm$ 5	1.78*	0.68*	9.45*	68 $\pm$ 6	0.76	0.97	0.56
<i>flaH</i>	112 $\pm$ 3	1.40	1.57*	1.44	542 $\pm$ 79	0.21*	0.21*	0.16*
<i>flaG</i>	348 $\pm$ 14	0.75*	0.68*	0.70*	79 $\pm$ 1	1.32*	1.00	0.86
Motility genes								
<i>motA</i> (3841)	1857 $\pm$ 382	0.01*	0.04*	0.01*	3591 $\pm$ 29	0.01*	0.02*	0.02*
<i>motB</i> (3841)	195 $\pm$ 92	0.39	0.42	0.37*	928 $\pm$ 19	0.08*	0.38*	0.08*
<i>motB</i> [†] (3841)	123 $\pm$ 1	0.20*	0.10*	0.15*	219 $\pm$ 2	0.12*	0.08*	0.07*
Chemotaxis genes								
<i>mcpC</i> (VF39SM)	266 $\pm$ 17	0.97	ND	1.20	242 $\pm$ 6	0.60*	0.70*	0.66*
<i>mcpD</i> (VF39SM)	843 $\pm$ 92	0.12*	ND	0.16*	421 $\pm$ 10	0.25*	0.18*	0.26*
<i>che1</i> operon (3841)	2735 $\pm$ 471	ND	0.06*	0.04*	1839 $\pm$ 231	ND	0.09*	0.25*
<i>che2</i> operon (3841)	1030 $\pm$ 28	0.88	ND	0.80	1021 $\pm$ 839	0.81	0.82	ND

*Difference between the wild-type and mutant is statistically significant at $P < 0.001$ (Student's *t* test).

[†]Fusion in pFAJ1701.

outer-membrane protein. Although the studies described above have demonstrated that OmpR can function as both an activator and a repressor for outer-membrane proteins, this is believed to be the first report of a dual function for an OmpR-type protein, specifically Rem, in the regulation of flagellin genes. It is curious that *flaE* expression does not increase in a *visN* or *visR* mutant, since Rem abundance should be low in such a mutant. However, this could be explained if VisN/R were required, either directly or indirectly, for expression of the *flaE* gene itself.

Kinetics of flagellin and motility gene expression

We looked at the expression patterns of flagellar genes, which represent the different classes in the regulatory scheme established in *S. meliloti* (Sourjik *et al.*, 2000; Rotter *et al.*, 2006). The regulatory genes *visN* and *rem* represent class I genes, whereas *motB* and *flaA* represent class II and class III, respectively. *R. leguminosarum* strains carrying transcriptional fusions for *flaA* (pFus1::*flaA*) and *motB*

(pFAJ1701::*motB*) were constructed. The strains were grown in TY broth and their expression was measured at different growth phases using a *gusA* assay. The expression of *flaA* increased during the exponential phase and peaked at an enzyme activity of 6112 ± 955 Miller units (MU; Miller, 1972) (Fig. 5a). Promoter activity was still maintained at relatively high values (66–88 %) during the stationary phase. The same trend was observed for the expression of *motB* (Fig. 5b). The activity of the *motB* promoter increased during the exponential phase and the highest gene expression was observed after 48 h of incubation. The activity was also maintained at relatively high rates during the stationary phase before it dropped to around 60 % at 288 h.

The strains used in the above assay carried the *gusA* gene fusions in plasmids pFus1 and pFAJ1701. In these strains the genes are present in more than one copy; thus, the values for the promoter activities could have been higher than the actual expression values. To circumvent this

Fig. 4. Proposed model for the regulation of motility-related genes in *R. leguminosarum*. Genes are indicated as horizontal arrows and the corresponding gene products as ellipsoids. Translation to gene products is indicated by open vertical arrows. Black vertical arrows and bars indicate positive and negative regulation, respectively. Negative autoregulation of *visN* is shown as dotted arrows. All genes exhibited the same regulation pattern in 3841 and in VF39SM, except *flaE* and *flaH*. *flaE* is not regulated by VisN/R-Rem in 3841 but is repressed by Rem in VF39SM. *flaH* is under VisN/R-Rem regulation in 3841 but not in VF39SM.

problem, we repeated the assay for the *flaA* gene using strain A3SG/3841, which contains a genome-integrated *flaA-gusA* fusion. The highest promoter activity observed using the genome-integrated fusion was 935 ± 11 MU, which was almost seven times lower than the highest value (6112 ± 955 MU) obtained using the *flaA-gusA* fusion in pFus1. Although the values differ in plasmid-borne and genome-integrated fusions, the same trend in gene expression was observed. Fig. 5(c) shows the kinetics of *flaA* gene expression until 201 h of incubation. The promoter activity started to increase at the onset of the exponential phase and reached its peak 33 h after incubation (late-exponential phase). During the stationary phase, gene expression of the chromosomally integrated *flaA-gusA* fusion in 3841 ranged from 61 to 88 % of the highest value.

The transcriptional results described above indicate that the flagellin and motility genes are expressed throughout growth. During the lag and early exponential phases, flagellin and motility gene expression was minimal. At these stages of growth, the medium still contains a high concentration of nutrients and low concentrations of toxic metabolites, and therefore is still favourable for growth and reproduction (Amsler *et al.*, 1993). The flagellin gene *flaA* and the motility gene *motB* were continuously expressed

during the stationary phase of the cultures. Wei & Bauer (1998) have suggested the presence of compounds in stationary phase cultures that act as active inducers of motility. Thus, when filtrates from stationary phase cultures are added to *S. meliloti* cells under starvation conditions, cell motility is restored. They also indicated that the active substances in the culture filtrates are not necessarily metabolizable and do not need to be present at a certain concentration to prevent downregulation of motility under starvation conditions.

Since we had shown that *flaA* and *motB* are controlled by the regulatory genes *visN*, *visR* and *rem*, we determined whether these regulatory genes exhibit the same expression patterns as those of *flaA* and *motB*. The activities of both *rem* and *visN* promoters started to increase at the onset of the exponential phase and peaked at the onset of the stationary phase (Fig. 5d, e). The activity started to decrease during the stationary phase and then dropped to 33 % of the highest promoter activity for both regulatory genes. VisN acts as a transcriptional activator of *rem*; hence, we observed a drop in the transcription of *rem* when the amount of *visN* transcript decreased during the stationary phase. The expression patterns of *visN* and *rem* differ from what has been described above for *flaA* and *motB*. The values obtained for *visN* and *rem* dropped

Fig. 5. Kinetics of flagellar gene expression in wild-type strains. Gene expression was measured as β -glucuronidase activity and expressed in MU. Values are means of three independent replicates; error bars, SD. (a) *flaA-gusA* fusion in VF39SM. The *flaA-gusA* gene fusion was constructed using pFus1 as vector. (b) *motB-gusA* fusion in VF39SM. The *motB-gusA* gene fusion was constructed using pFAJ1701. (c) The *flaA* promoter-*gusA* gene fusion integrated into the genome of strain 3841. (d) *visN-gusA* fusion in 3841. The *visN-gusA* gene fusion was constructed using pFus1. (e) *rem-gusA* fusion in 3841. The *rem-gusA* gene fusion was constructed using pFus1.

during the stationary phase, while *flaA* and *motB* exhibited relatively high expression at this phase. Although the transcription values for *visN* and *rem* were lower in the stationary phase compared with expression during the exponential phase, we speculate that the amount of *visN* and *rem* transcripts available might still be enough to drive the expression of the flagellin and motility genes.

To confirm the motility of the cells at the different growth phases, the cells were observed under a phase-contrast microscope. At the stationary phase, 30–40 % of the cells exhibited swimming motility, which is approximately the same number as during exponential phase. TEM also revealed that the cells at the stationary phase were flagellated (Fig. 6). Most of the cells observed had two flagella per cell, which is less than the number (four to seven flagella per cell) observed for plate cultures. The broth culture was grown with shaking, thus some of the flagella could have been sheared off from the cells; the same lower number of flagella in broth culture was observed during exponential phase. There were also polysaccharides produced by the cells and salts in the TY medium that may have reacted with the stain used; thus, some flagella may not have been observed by TEM.

Similar growth-independent gene expression has been reported for *mcp* fusions in *R. leguminosarum* (Yost *et al.*, 2004). Motility alone is not sufficient for adaptation. The cells should be able to detect unfavourable changes to be able to respond; thus, at least some of the *mcp* genes of *R. leguminosarum* are also continuously expressed. Continuous cell motility has also been reported in *E. coli*. An increase in motility and flagellar expression has been observed in *E. coli* from the late-exponential phase to the early stationary phase (Amsler *et al.*, 1993). *Rhodobacter*

capsulatus, which is also a member of the α -proteobacteria, exhibits the same gene expression pattern as *R. leguminosarum*. The transcripts for *flgE*, which encodes the major hook protein, and for *motB* are higher in the late-exponential and stationary phases. The flagellin gene is also expressed in both the exponential and stationary phases. It has been suggested that the flagellin gene of *Rhodobacter capsulatus* is continuously transcribed because the flagellin protein is required in high amounts. In this way, a continuous pool of the protein is available for flagellum synthesis when the other flagellar proteins are transcribed (Lang & Beatty, 2002).

The continuous flagellar gene expression described above is different from that reported in *S. meliloti*, where cell motility is confined within a window during the exponential phase (Sourjik *et al.*, 2000; Rotter *et al.*, 2006). Although both *visN* and *visR* are constitutively expressed throughout the entire growth of *S. meliloti*, other flagellar and motility genes (including *rem*) are not expressed in stationary or low-energy phases. Transcriptional analysis of *rem* in *S. meliloti* shows that its promoter activity increases rapidly at the start of the exponential phase and then abruptly drops by 75 % before it decreases to a final activity below 10 % (Rotter *et al.*, 2006). Hoang *et al.* (2008) have also reported that the motility of *S. meliloti* is under the regulation of the ExpR/Sin quorum-sensing system. The expression of the motility genes is suppressed at high population densities (at late-exponential phase) by quorum sensing. On the other hand, expression of flagellin and motility genes in *R. leguminosarum* is still relatively high even at high population densities. This difference in the temporal expression of flagellar genes between *S. meliloti* and *R. leguminosarum* suggests that although these nodule

Fig. 6. TEM image of VF39SM at the stationary phase. Arrows show flagella. Bar, 500 nm.

bacteria are related, they employ different strategies when confronted with less favourable growth conditions such as those associated with the stationary phase. When the nutrients in the environment become scarce, motile bacteria need to choose between searching for new nutrient supplies and turning off motility to conserve energy. Chemotaxis and motility are energetically costly, requiring approximately 50 gene products (Wei & Bauer, 1998) and approximately 2% of the cell's energy (Soutourina & Bertin, 2003) for flagellar synthesis and function; hence the repression of motility in *S. meliloti*. On the other hand, continued chemotaxis and motility under free-living conditions, as in the case of *R. leguminosarum*, may result in finding more nutrients and a more favourable environment.

Downregulation of regulatory genes under symbiotic conditions

Since *R. leguminosarum* can live either freely or in symbiosis with its host plant, we also looked at the expression of motility genes during symbiosis. Our nodulation experiments showed that all of the motility-related genes studied were significantly repressed inside the nodules (Table 3). The promoter activities of *flaA*, *flaB*, *flaC* and *motB* were reduced 19-, eight-, 43- and eight-fold, respectively. The activity of the *visN* promoter exhibited as much as a 40-fold reduction for VF39SM. The reduction in the activity observed for 3841 was slightly lower, i.e. a 17-fold reduction. This could be attributed to the difference in the copy numbers of the *gusA* fusion vectors used for strains 3841 (pFAJ1701) and VF39SM (pFus1). We obtained a higher promoter activity under free-living conditions using pFus1 (data not shown). For *rem*, the expression was substantially repressed in the bacteroid, exhibiting as much as 40- to 63-fold reductions in 3841 and VF39SM, respectively. The downregulation of *visN* and *rem* reported in this paper suggests that the symbiotic downregulation of motility-related genes such as the *mcp* genes (Yost *et al.*, 2004) as well as the *fla* and *mot* genes is

mediated by repressing the expression of the genes that encode the global activators VisN/R and Rem.

Conclusions

The results from this study demonstrate a hierarchy in the regulation of motility-related genes in *R. leguminosarum*. The transcriptional activators VisN and VisR regulate the expression of *rem*. Rem, on the other hand, positively controls the expression of genes located within the main flagellar regulon, which includes *flaA*, *flaB*, *flaC*, *flaD*, *motB* and *che1*. Other genes, which are located outside of the main flagellar gene cluster in the chromosome (*che2*, *flaH* for VF39SM, and *flaG*) or are plasmid-borne (*mcpC*), are not part of this regulatory cascade, and could be regulated by a secondary regulatory system. In addition, all genes exhibited the same regulation pattern in 3841 and in VF39SM, except *flaE* and *flaH*. *flaE* is not regulated by VisN/R-Rem in 3841 but its expression is repressed, though not necessarily directly, by Rem in VF39SM. *flaH* is under VisN/R-Rem regulation in 3841 but not in VF39SM. The kinetics experiment revealed that the flagellar genes (*flaA*, *motB*, *visN* and *rem*) are expressed at all growth phases, indicating that *R. leguminosarum* is continuously motile under free-living conditions. In contrast, the regulatory genes *visN* and *rem* are significantly downregulated when motility is not needed inside the nodules of the host plant.

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Table 3. Downregulation of flagellar and motility gene fusions in bacteroids

All values are expressed as MU and are the mean $\pm$ SD of three independent replicates.

Fusion	β -Glucuronidase activity (MU)	
	Free-living	Bacteroids
<i>flaA</i> – <i>gusA</i> fusion (3841); genome-integrated fusion	935 $\pm$ 11	50 $\pm$ 3
pFAJ1701:: <i>flaB</i> (3841)	155 $\pm$ 11	19 $\pm$ 2
pFus1:: <i>flaC</i> (3841)	3826 $\pm$ 494	89 $\pm$ 31
pFAJ1701:: <i>motB</i> (3841)	217 $\pm$ 5	28 $\pm$ 7
pFus1:: <i>visN</i> promoter (VF39SM)	3203 $\pm$ 68	109 $\pm$ 55
pFAJ1701:: <i>visN</i> promoter (3841)	852 $\pm$ 33	50 $\pm$ 5
pFus1:: <i>rem</i> promoter (VF39SM)	7637 $\pm$ 834	169 $\pm$ 94
pFus1:: <i>rem</i> promoter (3841)	6962 $\pm$ 133	276 $\pm$ 110

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