***Rhizobium leguminosarum* contains a group of genes that appear to code for methyl-accepting chemotaxis proteins**

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Methyl-accepting chemotaxis proteins (MCPs) play important roles in the chemotactic response of many bacteria. Oligonucleotide primers designed to amplify the conserved signalling domain of MCPs by PCR were used to identify potential MCP-encoding genes in *Rhizobium leguminosarum*. Using a PCR-derived probe created from these primers a genomic library of *R. leguminosarum* VF39SM was screened; at least five putative MCP-encoding genes (termed *mcpB* to *mcpF*) were identified and isolated from the library. One of these putative genes (*mcpC*) is located on one of the indigenous plasmids of VF39SM. Fifteen different cosmids showing homology to an *mcpD* probe were also isolated from a genomic library. The complete DNA sequences of *mcpB*, *mcpC* and *mcpD* were obtained. All three genes code for proteins with characteristics typical of MCPs. However, the protein encoded by *mcpB* has a relatively large periplasmic domain compared to that in other MCPs. Partial DNA sequences of *mcpE* and *mcpF* had strong similarity to sequences from the methylation domains of known MCPs. Mutants defective in *mcpB*, *mcpC*, *mcpD* or *mcpE* were created using insertional mutagenesis strategies. Mutation of *mcpB* resulted in impairment of chemotaxis to a wide range of carbon sources on swarm plates; phenotypes for the other three mutants have yet to be elucidated. The *mcpB*, *mcpC* and *mcpD* mutants were tested for loss of nodulation competitiveness. When co-inoculated with the wild-type, the *mcpB* and *mcpC* mutants formed fewer nodules than the wild-type, whereas the *mcpD* mutant was just as competitive as the wild-type. The results overall suggest that *R. leguminosarum* possesses *mcp*-like genes, and that at least some of these play a role in early steps in the plant-microbe interaction.

Keywords: rhizobia, chemotaxis, sensory proteins

INTRODUCTION

The study of bacterial chemotaxis has provided substantial information about the molecular mechanisms by which bacteria process and react to environmental information. Most of the knowledge of chemotaxis comes from research using enteric organisms such as *Escherichia coli* and *Salmonella typhimurium*. In these bacteria, many of the chemotaxis genes are contained

within a single operon (*cheA*, *cheY*, *cheW*, *cheR*, *cheB*) (for reviews see Stock *et al.*, 1991; Manson, 1992). Proteins CheA and CheY belong to a two-component regulatory system that modulates direction of flagellar rotation and thus determines the behaviour of the bacterium (reviewed by Matsumura *et al.*, 1990; Parkinson, 1993). Methyl-accepting chemotaxis proteins (MCPs) may also play a role in this signalling pathway by serving as environmental sensors. They are trans-membrane proteins with the carboxyl-terminus located in the cytoplasm and the amino-terminus exposed to the external environment (Boyd *et al.*, 1983; Hazelbauer *et al.*, 1990). The amino-terminal domain detects attractants and repellents, and the carboxyl-terminal domain relays this information to CheA, allowing the

Abbreviations: DIG, digoxigenin; MCP, methyl-accepting chemotaxis protein.

The GenBank/EMBL/DBJ accession numbers for the sequences reported in this paper are: *mcpB*, AF022807; *mcpC*, AF036168; *mcpD*, U81828.

bacterium to swim towards attractants and away from repellents (Hazelbauer *et al.*, 1990). Sensory adaptation occurs through methylation and demethylation of glutamate residues contained in the carboxyl-terminus of the protein (Hazelbauer, 1988). Generally, the binding of attractants leads to increased methylation of the MCP. Conservation of MCP amino acid sequences among bacteria occurs predominantly in the carboxyl-terminal region, where the MCP interacts with the signalling components CheW and CheA and where methylation occurs. A surge of interest in the chemotactic behaviour of other bacterial species has demonstrated that the chemotactic signalling pathway is highly conserved. Elements of the signalling pathway including *cheA*, *cheY*, *cheW*, *cheR*, *cheB* and MCP homologues have been found in a wide range of bacteria, such as *Bacillus subtilis* (Hanlon & Ordal, 1994; Zuberi *et al.*, 1990), *Caulobacter crescentus* (Alley *et al.*, 1992), *Myxococcus xanthus* (McCleary *et al.*, 1990; McCleary & Zusman, 1990), and the archaeon *Halobacterium halobium* (Alam *et al.*, 1989).

It has frequently been suggested that chemotaxis and motility must play an important role in the interaction of root-nodule-forming bacteria (collectively known as rhizobia) with their legume hosts (Currier & Strobel, 1977; Ames & Bergman, 1981; Gulash *et al.*, 1984; Caetano-Anollés *et al.*, 1988, 1992; Munoz Aguilar *et al.*, 1988; Bauer & Caetano-Anollés, 1990; Dharmatilake & Bauer, 1992; reviewed by Bauer, 1991, and Vande Broek & Vanderleyden, 1995). To confirm this hypothesis more information is needed about the chemotactic signalling pathways in rhizobia. Quite recently, Greck *et al.* (1995) have shown that *Sinorhizobium meliloti* contains a chemotaxis operon homologous to the *E. coli* chemotaxis operon. Genes identified using sequence homology to *E. coli* genes include *cheA*, *cheR*, *cheB*, two *cheY* genes and two ORFs. The products of the two ORFs show amino acid similarity to the signalling domain of MCPs, but their predicted amino acid sequence lacks the other functional domains, such as transmembrane regions and the two methylation domains, characteristic of MCPs. The roles of the two ORFs in chemotaxis remain unclear. Previously, conflicting reports of *in vivo* methylation studies questioned the existence of MCP-encoding genes in *Sinorhizobium* and *Rhizobium*. Robinson & Bauer (1993) observed no increase or decrease in protein methylation after *Sinorhizobium meliloti* cells were incubated with attractants such as L-amino acids or D-mannitol. However, Armitage *et al.* (1988) demonstrated an increase in protein methylation after *Rhizobium leguminosarum* cells were incubated with the chemoattractant L-serine. The latter experiment was in agreement with a study conducted by Morgan *et al.* (1993). Probing an *S. meliloti* Western blot with an antibody directed against an *E. coli* MCP (Trg) gave three hybridizing bands: a doublet between 65 and 70 kDa, and a band at 80 kDa. This provided further evidence that MCPs might exist in the rhizobia. When this research was initiated, the existence of MCP-

encoding genes in *Rhizobium* had not been clearly established. More recently, genes with homology to MCPs have been found on plasmids in a strain of *R. leguminosarum* (Brito *et al.*, 1996) and *Rhizobium* sp. NGR234 (Freiberg *et al.*, 1997); however, no role in chemotaxis has been shown. This paper documents research conducted to identify and isolate MCP-encoding genes from *R. leguminosarum* bv. *viciae* strain VF39SM.

METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. *R. leguminosarum* strains were grown on TY medium (Beringer, 1974) at 30 °C. *E. coli* strains were grown on LB medium (Sambrook *et al.*, 1989) at 37 °C. Swarm medium was composed of 0.01% yeast extract, 1 mM MgSO₄ and 0.3% (w/v) agar. The chemotactic response of *R. leguminosarum* to specific carbon sources was assayed using swarm plates containing Vincent's minimal medium (VMM; Vincent, 1970) with 0.15% agarose and the potential chemoattractant as the sole carbon source (1 mM final concentration). Carbon sources were purchased from Sigma-Aldrich. When necessary, *Rhizobium* strains were cultured in media containing antibiotics at the following concentrations: neomycin, 100 µg ml⁻¹; spectinomycin, 500 µg ml⁻¹; tetracycline, 5 µg ml⁻¹; streptomycin, 500 µg ml⁻¹; and gentamicin, 30 µg ml⁻¹. Antibiotic concentrations used when culturing *E. coli* strains were as follows: kanamycin, 50 µg ml⁻¹; tetracycline, 10 µg ml⁻¹; streptomycin, 500 µg ml⁻¹; gentamicin, 15 µg ml⁻¹; and spectinomycin 100 µg ml⁻¹.

Recombinant DNA techniques. All restriction endonucleases and modifying enzymes were purchased from Gibco-BRL, and used according to the manufacturer's specifications. Southern blots were hybridized with non-radioactive DIG-labelled DNA probes using reagents and protocols obtained from Boehringer Mannheim. Hybridizations were performed overnight at 67 °C followed by washes of 2 × SSC, 0.1% SDS at room temperature, and 0.1 × SSC, 0.1% SDS at 67 °C. All washes were done in duplicate. Hybridization signals were detected via chemiluminescence using CSPD substrate and a protocol supplied by Boehringer Mannheim.

Visualization of *Rhizobium* plasmids. When necessary the plasmids of VF39SM were visualized on agarose gels using a modified Eckhardt technique (Eckhardt, 1978) described by Hynes *et al.* (1985), as modified by Hynes & McGregor (1990).

DNA sequencing and analysis. DNA sequencing was performed using an Applied Biosystems automated sequencer operated by University Core DNA Services (University of Calgary). *ExoIII* deletions were used to obtain the complete DNA sequences of *mcpB*, *mcpC* and *mcpD*. Enzymes and protocols for the *ExoIII* deletions were obtained from Pharmacia. Partial DNA sequences of *mcpE* and *mcpF* were obtained from subclones in pBSISK(+) using T7 and T3 primers. Sequence homology searches were performed using the BLAST sequence alignment program (Altschul *et al.*, 1990).

Identification of MCP-encoding genes in *R. leguminosarum* VF39SM. Primers designed from the DNA sequence of *dcrA*, an MCP-encoding gene from *Desulfovibrio vulgaris* (Dolla *et al.*, 1992), were used to amplify VF39SM genomic DNA by

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)*	Source or reference
<i>E. coli</i>		
DH5 α	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(argFlacZYA) U169 ϕ 80dlacZ ΔM15</i>	Gibco-BRL
S17-1	Sp ^r ; RP4 <i>tra</i> region, mobilizer strain	Simon <i>et al.</i> (1983)
<i>R. leguminosarum</i>		
VF39SM	biovar <i>viciae</i> , St ^r	Priefer (1989)
LRS39201	VF39SM cured of pRleVF39b	Hynes & McGregor (1990)
LRS39301	VF39SM cured of pRleVF39c	Hynes & McGregor (1990)
LRS39401	VF39SM cured of pRleVF39d	Hynes & McGregor (1990)
LRS39501	VF39SM cured of pRleVF39e	Hynes & McGregor (1990)
LRS39601	VF39SM cured of pRleVF39f	Hynes & McGregor (1990)
VF-MCPB ⁻	VF39SM, <i>mcpB</i> :: Ω Tc; Tc ^r	This work
VF-MCPC ⁻	VF39SM, <i>mcpC</i> :: Ω Sp; Sp ^r	This work
VF-MCPD ⁻	VF39SM, <i>mcpD</i> :: Ω Nm; Nm ^r	This work
VF-MCPE ⁻	VF39SM, <i>mcpE</i> :: Ω Sp; Sp ^r	This work
VF-MCPC ⁻ /D ⁻	VF39SM, <i>mcpC</i> :: Ω Sp, <i>mcpD</i> :: Ω Nm; Sp ^r Nm ^r	This work
Plasmids		
pJQ200mp18	Suicide vector with <i>sacB</i> system; Gm ^r	Quandt & Hynes (1993)
pJQ200SK	Suicide vector with <i>sacB</i> system; Gm ^r	Quandt & Hynes (1993)
pRK7813	Broad-host-range cloning vector; Tc ^r	Jones & Guttererson (1987)
pBSIISK(+)	Cloning vector; Ap ^r	Stratagene
MCPB.B	8 kb VF39SM <i>Bam</i> HI fragment cloned in pBSIISK(+); contains a fragment of <i>mcpB</i>	This work
VGL-747	VF39SM cosmid clone in pRK7813; cosmid contains the entire <i>mcpB</i> gene	This work
MCPB.E	1.8 kb VF39SM <i>Eco</i> RI fragment cloned in pBSIISK(+)	This work
MCPC.B	6.7 kb VF39SM <i>Bam</i> HI fragment cloned in pBSIISK(+)	This work
MCPC.E	3 kb VF39SM <i>Eco</i> RI fragment cloned in pBSIISK(+)	This work
MCPD.B	7 kb VF39SM <i>Bam</i> HI fragment cloned in pBSIISK(+)	This work
MCPD.P	2.5 kb VF39SM <i>Pst</i> I fragment cloned in pBSIISK(+)	This work
MCPE.B	5 kb VF39SM <i>Bam</i> HI fragment cloned in pBSIISK(+)	This work
MCPE.C	1.7 kb VF39SM <i>Cla</i> I fragment cloned in pBSIISK(+)	This work
MCPF.B	6.7 kb VF39SM <i>Bam</i> HI fragment cloned in pBSIISK(+)	This work
MCPF.PL	1.2 kb VF39SM <i>Pst</i> I fragment cloned in pBSIISK(+)	This work
MCPB Ω Tc	Ω Tc cassette cloned in internal <i>Not</i> I site of MCPB.E; contained in pJQ200mp18	This work
MCPC Ω Sp	Ω Sp cassette cloned in internal <i>Xho</i> I site of MCPC.E; contained in pJQ200SK	This work
MCPD Ω Nm	Ω Nm cassette cloned in internal <i>Hind</i> III site of MCPD.P; contained in pJQ200SK	This work
MCPE Ω Sp	Ω Sp cassette cloned in internal <i>Sma</i> I site of MCPE.C; contained in pJQ200SK	This work

* Antibiotics: Ap, ampicillin; Gm, gentamicin; Nm, neomycin; Sp, spectinomycin; St, streptomycin; Tc, tetracycline.

PCR. The primer sequences used were 5'-CATGGTCTTC-TCGGCAGCTTGCG-3' and 5'-GAATCGCCGACCAGAC-CAACC-3', and PCR cycling conditions were 1 min at 94 °C, 30 s at 60 °C and 1 min at 72 °C for 30 cycles. Using the protocol outlined by Boehringer Mannheim, the amplified product was PCR labelled with DIG. The DIG-labelled product was gel-purified using band interception as described by Sambrook *et al.* (1989) and was subsequently used as a probe to identify MCP-encoding genes from a size selected partial genomic library of VF39SM.

The partial genomic library was constructed by digesting VF39SM genomic DNA with *Bam*HI to completion. The digest was electrophoresed in 0.8% agarose gel and the smear of fragments ranging in size from 5 to 12 kb was isolated using Prep-a-Gene (Bio-Rad). The fragments were ligated to *Bam*HI-digested pBSIISK(+). The ligation products were used to transform *E. coli* DH5 α .

A putative MCP-encoding gene was isolated from this first round of screening, and named *mcpB*. Its identity was verified by subcloning and sequencing the region that hybridized to the PCR probe. The subcloned DNA was labelled by the random primer method (Boehringer Mannheim) and used to reprobe the partial genomic library. This second round of screening discovered four previously undetected putative MCP-encoding genes, termed *mcpC* through *mcpF*. Each fragment was subcloned and sequenced to identify regions of homology to MCP-encoding genes. New primers C1 (5'-AGGCGGAC-CGAGCAGCAGGC-3') and C2 (5'-CTTGATTTCTTTG-CCGC-3') were designed from the DNA sequence of *mcpD* and were used to create a new PCR-amplified probe from VF39SM genomic DNA. PCR cycling parameters were as described above. Subsequently, this probe was used to probe a complete genomic library of VF39SM.

Preparation of a VF39SM genomic library. Total genomic DNA from VF39SM was partially digested with *Sau*3AI to give a maximum number of fragments in the 30–40 kb size range. The fragments were ligated to the dephosphorylated cosmid pRK7813. The ligation mixture was packaged *in vitro* using the Boehringer Mannheim packaging kit according to the manufacturer's recommendation. Titration of the genomic library yielded approximately 2500 c.f.u. Cosmid DNA was isolated as alkaline lysis minipreps from 1000 independent tetracycline-resistant clones and verified on 0.8% agarose gels. Cosmid DNA from each individually purified clone was then dot-blotted on positively charged membranes. Two membranes contained 400 clones each and a third membrane contained the remaining 200 clones in the library. The membranes were dried for 30 min at 120 °C.

Insertional mutagenesis of VF39SM *mcp* genes. *mcp* genes were mutated by inserting antibiotic-resistance cassettes into the ORFs of the cloned *mcp* genes. The antibiotic-resistance cassettes used were those developed by Prentki & Krisch (1984) and Fellay *et al.* (1987). The gene replacement in VF39SM was performed using a suicide vector and a positive selection strategy (Quandt & Hynes, 1993) for double homologous recombinational events. Southern blotting (data not shown) was used to verify the replacement of the wild-type gene with the mutant gene.

Nodulation competition experiments. Peas (*Pisum sativum* cv. Trapper) were surface-sterilized by washing in 50% bleach for 5 min, then with 70% ethanol, followed by three rinses in sterile distilled water. The seeds were germinated on water agar plates for 3 d and then transferred to modified magenta

jars which were modelled to resemble Leonard jars (Vincent, 1970). The peas were grown in a vermiculite substrate.

After transfer to the magenta jars the germinating pea seeds were inoculated with the appropriate VF39SM strains. For competition experiments, the wild-type was co-inoculated with the *mcp* mutant in a 1:1 ratio. The ratios were confirmed by performing viable plate counts on the inoculum. The inoculated peas were then grown for 5 weeks, after which the nodules were harvested, surface-sterilized and crushed. The macerate was plated on the appropriate selective media to determine whether the wild-type or the *mcp* mutant strain had formed the nodule. For each competition experiment 100 nodules were crushed and plated.

RESULTS

Cloning of putative *mcp* genes

PCR reactions using VF39SM genomic DNA and the *dcra* primers (Dolla *et al.*, 1992) amplified a single fragment approximately 350 bp in size. This fragment was used to probe a genomic blot of VF39SM digested with a variety of restriction endonucleases. Digestion of VF39SM genomic DNA with *Bam*HI gave at least seven hybridizing bands ranging in size from 5 to 12 kb (data not shown). Consequently, to enrich for fragments of this size a size-fractionated library of VF39SM was created. Four hundred colonies containing inserts, based on blue/white selection, were included in the library. Five putative *mcp* clones were subsequently isolated from this library. Fig. 1 provides restriction maps of *mcpB* through *mcpF*. Fifteen additional clones showing homology to an *mcpD* probe (all unique based on the size of the hybridizing fragment) were identified from a cosmid library of VF39SM. No further characterization

Fig. 1. Restriction maps of the original *Bam*HI fragments cloned from the size-fractionated genomic library of VF39SM (see text for details). B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; N, *Not*I; P, *Pst*I; S, *Sac*I; Sa, *Sal*I; Sm, *Sma*I; X, *Xho*I. The locations and omega cassettes used for insertional mutagenesis of *mcpB*, *mcpC*, *mcpD* and *mcpE* are indicated by the triangles above each restriction map.

McpC

Identities = 153/383 (39%), Positives = 205/383 (53%)

McpC: IRSIASAMRRLSDGDLSDIPYAGRADEVGEMAGAVEIFRQNALNVVRLEKNPLNPAARA
 I IA+A +LS GD D+ R DE+G + A+++FR N +++ +L + AA
 McpA: 222 INDIAAATDKLSKGDNSIDLEKMTRGDELGGIVTALKVFRDNQVHLEQLRADQEKSAALT 281

McpC: MQRAPHSSAPNARREQLRFATTTTIGBGLRRLASGDIFQLSEQFAAEYEALREDFNASL
 +A A ++ + L EGL +LASGD++F+++ F +Y L++DFNA++
 McpA: 282 ADERRSKEAAAAAAQEAASLVVSNLAEGLEKIASGDLTFRVTTADFPDGRKLKDDFNAAAM 341

McpC: RQLGATIGAVLQTVYSIDNGTGEIASAAQDLKRTQQAASLEETAAALEITSNVTMAT
 L T+ + + + G EIA A+ DLS+RTEQAASLEETAAAL E+T+ V
 McpA: 342 GSIQETMKVIAASTDGLSTGADEIAHASDDLSRTEQAASLEETAAALDELATVRRTA 401

McpC: KRTDEARNVAKEADISAQRSAAVVSQAEGAMRRIEDSSQISNIIGAIDEIATQTNLLAL
 +A +V A S VV QA AM IE SS QIS IIG IDEIATQTNLLAL
 McpA: 402 AGARQASDVVSTTRGEATHSGQVHVQAVSAMGEIEKSSGQISQIIGVIDEIAFQTNLLAL 461

McpC: NAGVEAARAGEAGKGFVAVQAEVRELAQRAAQAQAEIKGFIQKSSADVENGKLVLETGT
 NAGVEAARAGEAG GFVAVQAEVR LAQR+A+AAKEIK I S+ V GV LV +TG
 McpA: 462 NAGVEAARAGEAGRFVAVQAEVRLAQRSAEAAKEIKALISSSTQVSVQVSLVQTGE 521

ΩSp

McpC: SLKSIGEVVYHINQLMDATATSAREQSTGLAEINTAVNQMDQATQQAAMVQSTAAVAS
 +L+ I V I+ L+ IA SA EQ+TGL E+NTAVNQMDQ TQQAAMVQSTAA S
 McpA: 522 ALQRIVTKVGEIDALVTEIAASAAEQATGLNEVNTAVNQMDQVTTQQAAMVQSTAAHS 581

McpC: LSSEAGRLRDVLVNFQDLGDKSA
 L E L L+ +FQ+ S+
 McpA: 582 LKGETAELVRLMARFQVSGSGSS 604

McpE

Identities = 24/58 (41%), Positives = 37/58 (63%)

ΩSp

McpE: GIGAGSNEIRSSGDQVSERTEQESVSVEETEALEETITTTVRDADKRTTEETQVVALT
 G+ G+EI + D +S RTEQ++ S+EEET A L+E+T TVR + + VV+ T
 McpA: 357 GLSTGADEIAHASDDLSRTEQAASLEETAAALDELATVRRTAAGARQASDVVSTT 414

McpF

Identities = 36/71 (50%), Positives = 49/71 (69%)

McpF: ADLGQKYAVLRDNFNDALSHLEAMVKVSAKGTDTGTSKEIRIRASNELSQRTERRQAASL
 AD Y L+D+FN A+ L+ M ++A + T +EI AS++LS+RTE+QAASL
 McpA: 324 ADFFPGDYRKLKDDFNAAAGSLQETMKVIAASTDGLSTGADEIAHASDDLSRTEQAASL 383

McpF: EETSAAALDEL
 EET+AAALDEL
 McpA: 384 EETAAALDEL 394

Fig. 2. Predicted amino acid sequences of McpC, McpE and McpF derived from complete or partial DNA sequencing of *mcpC*, *mcpE* and *mcpF* were submitted to a BLASTX search (Altschul et al., 1990). The *C. crescentus* McpA sequence is shown below; the numbers flanking the McpA sequence represent the amino acid residue numbers of McpA. Plus signs between the sequences indicate similar residues. The methylation regions are represented by bold characters. The bold asterisks indicate sites of potential methylation in McpA (Alley et al., 1992). The McpC sequence contains both the methylation domains and the highly conserved signalling region of McpA, while the sequences from McpE and McpF contain the first methylation domain. The locations of the omega antibiotic-resistance cassettes used in insertional mutagenesis in relation to the amino acid sequence of each putative MCP are also indicated.

of any of these clones is available to verify that they code for MCPs.

Sequencing of the *mcp* clones

The *Bam*HI fragments originally cloned from the VF39SM partial genomic library, labelled MCPB.B through MCPF.B, were subcloned to isolate smaller restriction fragments that hybridized to the *dcrA* primer derived PCR probe. The subclones were then sequenced from vector T7 or T3 primers, and deduced amino acid sequences were analysed by BLASTX for homology with protein databases. High similarities were reported to sequences of known MCPs. The highest alignment scores occurred with MCP sequences from *R. leguminosarum* (Brito et al., 1996), *Rhodobacter capsulatus* (Michotey et al., 1996) and *Caulobacter crescentus* (Alley et al., 1992). Sequence alignments of McpC, McpE and McpF with McpA of *C. crescentus* are shown in Fig. 2. The nucleotide sequence of *mcpB* has been deposited in GenBank (accession number AF022807). DNA sequence data were obtained using the clone MCPB.B and from a genomic cosmid clone carrying the entire *mcpB* gene. The predicted McpB protein is 716 aa in length with a molecular mass of

76.7 kDa. Based on a Kyte–Doolittle plot, two transmembrane domains are present. These encompass amino acid residues 18–37 and 298–317. The area flanked by the transmembrane domains is, therefore, 261 aa in size and is presumably located in the periplasm. The complete DNA sequence of *mcpC* (GenBank accession number AF036168) indicates that it encodes a protein containing two predicted transmembrane regions in the amino-terminal domain, and has all the characteristics common to known MCPs: a periplasmic amino-terminus, putative methylation domains, and a conserved signalling domain.

The *mcpD* gene sequence has been deposited in GenBank (accession number U81828). Two potential translational start sites exist for the *mcpD* ORF. The first is the ATG codon at nt 40, while the second is an ATG start codon at nt 61. Neither is preceded by a strong ribosome-binding site. The gene is, however, expressed in VF39SM as indicated by a promoterless *lacZ* fusion to *mcpD* (data not shown). The predicted protein based on the first start codon has 624 amino acids and a molecular mass of 66.3 kDa. A Kyte–Doolittle plot predicts two transmembrane regions, the first from residues 1 to 26 or 8 to 26, depending on the transcriptional initiation site. The second trans-

McpB	1	MLHFVNKFGIRAOITSGLVPLILLMSLLTVSAISGMNGLASIFSSYRAT
McpD	1	MF-QF-LK-TMPLTAK--LAAIIVAVNL-----CG----ISAFATY---
R.I. McpA	1	MK-HISIV-GKFFIIMAVFGVMALGLTF-----YQSRQMLKVNDYS---
C.c. McpA	1	MK-RIRLV-DLPLIIKIGFAP-AFALL-----MLAVMAGG----
Tsr	1	MLKRIKIVTSL-LLVLAVGLLQLTSGGLFFNALKNDKENFTVLQTTIRQQ
McpB	51	AGQSLAISDYSDQLNEIQMSAEAFRSTPTQAVVDRFRAGVKAFFDADDPRF
McpD	33	--TWMYETR--ALIDGAK-----ANWSKDAE-----QCASLAAGGVKW
R.I. McpA	40	--QELLDKDASAAALRLT-----QSNRSLEIAR-----ASISDMVMTR
C.c. McpA	33	--AILVQKSQSAALKQVVE-----RDMRQNLEIQRISKRISNINGELFV
Tsr	50	QST-TNGSWVALLQTRNTLNRAIRYMMQNNIGSGSTVAELMESASISL
McpB	101	AGNKDLQSVLAAIRODIATYGKAFEEIVALQARRDALISKVTEFGPWTSI
McpD	67	GKANAAAREAYSLEYRDPISLDLVQFAAFNA-----EPAXVDTWTRD
R.I. McpA	75	SKEARARAEEFGL--NDAR-----ENFV-----RFMDLAIAAVP
C.c. McpA	76	MTHKAGNIDVDK--NDARMAAVLVETDAV-----KKDLLALKSKL
Tsr	99	KQAEKNWADYEALPRDPR-----
McpB	151	ALNDVVRSAWRQNDVPLLQMTAATLEALNRSLYFSERFVHSDDDFAAYDTA
McpD	107	GISGLPT-----PXDLATRLSAK
R.I. McpA	106	EQSELPK-----LKADGFSVTDTC
C.c. McpA	114	PAEEQPK-----I-AELIKSLEEC
Tsr	117	-----QSTAAA-----AEIKRNYDIY
McpB	201	QAALAEAVTLNEAAAKAAKNELOKKRLMGAGQLMQNYTARLGDMKDVLQA
McpD	125	P-----EKTIDDSGISAGVVTIAPLPLDKSG--KAAGYIVTNWSV----
R.I. McpA	125	G-----AAIAVGRGATSEAEAMVQQLYLTLQC--PAFAAISPRFTSV---
C.c. McpA	132	R-----SAIDTVSGMIS-VDFNMAAG-FIAPFE--EQYVKMTGQLDQVVAA
Tsr	133	HNALAEELIQLLGAGKINEFFDQPTQGYQD--GFEKQYV-----AYMEQ
McpB	251	SGNIRQTQLSVLAPKISGGFKDLQATVTGAQKTLDGSDVATVASATSTTL
McpD	165	--E-----KIAAEVRQK-----VLISLLTQ-
R.I. McpA	166	-TE-----KLASDAEQKRADVSSV-----VRDTSVLSLGTAI
C.c. McpA	174	ANQ-----RIESESARKQAQATAA-----MSVTIIMSLLT--
Tsr	174	NDRLHDIASV-----DNMSYSQAMWILVG-----
McpB	301	IISGLLIVIGLVLSYFVGRLISSAVRNMAQSMEQLARGLERIVITGVEHR
McpD	183	SVITALAVIAFLLA--MRSLVGRPIRVISERISALQKGDLASPVTYKENG
R.I. McpA	197	AALFAVSCFCGFL-A--IRAWLVKPIKQMVTTMKVIADGDLTSTVEGTIRR
C.c. McpA	204	--LGAVGALAFLL-T--VMT-TRKSINDIAAATDKLSKGDNSIDLEKMTRG
Tsr	199	-VMIVVLAVIEFAVWFGIKASLVAPMNRLLIDSIRHIAAGDLVKPIE-VDGS
McpB	351	HELGAARSLSKVFFQETGRAKLIAEANAERARLAAEEERLRLQEAERLSDAQ
McpD	231	DEIGFLARALEVFRHEAIAKVEREQAAAEQSASLDAERARNALFTEEASN
R.I. McpA	244	DEIGSMARAYQIFKDNELRADLGKDAETSRGANERARLAETARQRRAR
C.c. McpA	248	DELGGIYVTAALKVERDNQVHLEQLRADQEKSAALTADERRSKEAAAAAAQ
Tsr	247	NEMGQLAESTR-----
McpB	401	VMEHAFRQISVGLDALSKGDLTVRVGE-VDHRYVRI RDHFNNNSVASLLEEA
McpD	281	TQRLVMTALANSLEKLAAGDFSILHAD-LGPEFDKLRQDFNNMVEAVAAA
R.I. McpA	294	DMAEATSGLAEGLRHLADGNLVFSLDDKFAEDFEPLRANFNAAVAQLAES
C.c. McpA	298	EASLVVSNLAEGLEKLASGDLTFRVTADFPGDYRKLDDENAAMGSLQET
Tsr	258	-----HMQGEILMRT
McpB	450	VDAVIRAVGTIRSGLAETSTASNDLARRTEQQAASLEETWAALGEVTRGV
McpD	330	LTEIKTASVAVEGGSSELASSADQLARRTEQQAALALEQTAAALDEVTTTV
R.I. McpA	344	LRAVSNAATESIDDGAQEISLSAQDLSRRTEHQQAASLEQTAAALDQITQNV
C.c. McpA	348	MKVIAASTDGLSTGADEIAHASDDLSSRRTEQQAASLEETAAALDELTTATV
Tsr	267	VGDVRNGANAIYSGASETATGNNDLSRRTEQQAASLEETAAASMEQLTATV
McpB	500	NGTAEGLASRAQGVVATARTNAEKKGELVARAIDAMTEIQNSSSKIGNIIS
McpD	380	RTSSQRLENAGKLVETKRSAHVSAITVVRDAIGAMDRIQTSSSQIGRIIG
R.I. McpA	394	ASSSKRTAEARHVAIEANKSARHSGEVVSSAVVAMQRIERSSSRISIIIG
C.c. McpA	398	RRTAAGARQASDVVSTTRGEATHSGQVHQAIVSAMGEIEKSSGQISQIIG
Tsr	317	KQNAENARQLASHLALSASETAQRGCKVVDNVVQTMRDISTSSQKTADITIS
McpB	550	VIDEIAFQTNLLALNAGVEAARAGEAGKGFVAVVAQEVRELAQRSANAARE
McpD	430	VIDEIAFQTNLLALNAGVEAARAGEAGKGFVAVVAQEVRELAQRSANAARE
R.I. McpA	444	VIDEIAFQTNLLALNAGVEAARAGEAGKGFVAVVAQEVRELAQRSANAARE
C.c. McpA	448	VIDEIAFQTNLLALNAGVEAARAGEAGKGFVAVVAQEVRELAQRSANAARE
Tsr	367	VIDGIAEQTNLLALNAGVEAARAGEAGKGFVAVVAQEVRELAQRSANAARE

Fig. 3. Continued on facing page.

membrane domain extends from residue 179 to 201. Based on these observations, McpD should contain a periplasmic domain of approximately 147 aa.

The sequence alignment in Fig. 3 shows the strong similarity of the McpB and McpD protein sequences to the *C. crescentus* McpA sequence (Alley *et al.*, 1992), the *R. leguminosarum* McpA (Brito *et al.*, 1996), and the *E. coli* Tsr sequence (Boyd *et al.*, 1983). The strongest similarities occur within the methylation and signalling domains of the MCPs.

Mutagenesis of the *mcp* genes

mcpB, *mcpC*, *mcpD* and *mcpE* were mutated via insertional mutagenesis, using antibiotic-resistance cassettes as the selectable markers (Prentki & Krisch, 1984). Restriction endonuclease sites used for insertions of antibiotic resistance cassettes were ascertained from the sequence data generated in this study. Figs 1 and 2 indicate the location of gene disruption sites in each *mcp* clone. Insertion of the Ω Tc fragment into the *NotI* site of *mcpB* disrupted the gene within the putative second methylation site. The Ω Sp fragment was inserted just downstream of the highly conserved domain of *mcpC* using a *XhoI* site. In *mcpD* the Ω Km cassette inserted at the *HindIII* site ahead of the first methylation region. With *mcpE*, insertional mutagenesis also occurred using a *SmaI* site located slightly upstream of the first methylation region. In addition to the single gene knockouts created, an *mcpC*, *mcpD* double mutant was obtained. The mutant was created by mating

MCPD Ω Nm into VF-MCPC⁻, and then selecting for double recombinants.

Phenotypic analysis of the mutants was performed using VMM swarm plates to assess chemotactic behaviour towards various carbon sources (adonitol, arabinose, arginine, aspartate, erythritol, galactose, glutamate, glycerol, histidine, maltose, manitol, melibiose, ornithine, raffinose, ribose, rhamnose, serine, sorbitol and trigonelline). To date, phenotypes for mutations in *mcpC*, *mcpD* and *mcpE* have not been identified. However, a mutation in *mcpB* inhibited chemotaxis to all carbon sources tested. This phenotype does not conform to the *E. coli* model whereby a MCP is responsive to a specific set of ligands. Fig. 4 provides examples of the swarming patterns of the mutants. VF-MCPC⁻ is impaired in its ability to swarm away from the inoculation point. Cells accumulate in a localized area, resulting in a denser circle of growth relative to the wild-type and the other mutant strains. This is particularly evident in plate (a). In addition, the swarm diameter of VF-MCPC⁻ is smaller relative to the other strains. This behaviour is most pronounced in the raffinose swarm plate.

Possible effects on the ability of the *mcp* mutants to interact with legume host plants were assayed by nodulation competition tests. The principle of this type of study is that if a plant is co-inoculated with two strains of rhizobia, formation of more nodules by one strain indicates greater 'competitiveness'; this may be due to a variety of factors, including faster growth in the

McpB	600	I K Q L I S T S S A Q V K T G V Q L V G E S G L S L E Q I V E Q V T A M N A T V A E I A V A A R E Q
McpD	480	I K N L I N V S G Q E V A A G V G L V N E T G D A L L K I E E Q I N R I S D S I A S I V Q S Y R E Q
R.l. McpA	494	I K D L I L N S V D E V S S G V K L V R D T G E A L K I I V D Q I V L I N T Q L D A V T A A S N E Q
C.c. McpA	498	I K A L I S S S T Q Q V S Q G V S L V G Q T G E A L Q R I V T K V G E I D A L V T E I A A S A A E Q
Tsr	417	T K S L T E D S V G K V D V G S T L V E S A G E T M A E I V S A V T R V T D I M G E T A S A S D E Q
		~~~~~
McpB	650	A T S L R E V S A A G D Q M D K V T Q Q N A A M V E E T T A A A Q S L T H E T E S L A E L L R R F R
McpD	530	A T G L Q E I N G A I N Q M D Q A T Q Q N A A M V E E T N A A C Q E L L Q Q G R L L Q D S A G R F V
R.l. McpA	544	S A T L F E V N R T V N Q M D Q V T Q Q N A A M S E E S T A A S T A L A I E V K Q L R G I V A E F Q
C.c. McpA	548	A T G L N E V N T A V N Q M D Q V T Q Q N A A M V E Q S T A A T H S L K G E T A E L V R L M A R F Q
Tsr	467	S R G I D Q V G I A V A E M D R V T Q Q N A A I V E E S A A A A A I E E Q A S R L L T E A V A V E R
		~~~~~
McpB	700	T G S G R V S E H R H - - - - - Y A M A S - - - - -
McpD	580	V G A S S A S Q P R P M - - - - - Q P A R Q S P P E P R A - - - - - F V Q - - R H A G N A A V A A
R.l. McpA	594	I D A G D K L H G S V I L F D - - R E P P A - - A M T L T Q A D K D P F D A P - R K V G S S V K G G
C.c. McpA	598	V G S G S S S Y A R P A V A D A G H A P A R N P V A E Q Q A R L N T F A R P G R S S G S A A L A Q
Tsr	517	I - - - - - Q Q Q R E T S A V - - - - - V K T V T P A A P R K M A V
		~~~~~
McpB		- - - - -
McpD	617	A P G - - A W E E F
R.l. McpA		- - - - -
C.c. McpA	648	A P A S D G W E E F
Tsr	542	A D S E E N W E T F

Fig. 3. Amino acid sequence alignment of McpB and McpD with *R. leguminosarum* McpA, *C. crescentus* McpA, and *E. coli* Tsr. The shaded rectangular boxes denote the approximate location of the transmembrane domains for each MCP. The hatched rectangular boxes indicate the methylation regions of each MCP. The boxed residues indicate identical amino acids among the five chemotaxis proteins. McpB and McpD have high homology to the methylation domains and signalling domain of both of the McpA proteins and of Tsr. The sequence alignment was performed using the CLUSTAL V program, and displayed using the SeqVu program (Garvan Institute).

Fig. 4. Swarm plates showing the chemotactic behaviour of the various VF39SM MCP mutants. Strains are labelled as follows: A, VF-MCPB⁻; B, VF-MCPC⁻; C, VF-MCPD⁻; D, VF-MCPE⁻; E, VF39SM. (a) Yeast extract swarm plate. (b) VMM raffinose swarm plate. (c) VMM adonitol swarm plate. The swarm plates were prepared as described in Methods.

Fig. 5. Nodulation competition experiments: inoculation and recovery ratios of *mcp* mutants versus the wild-type VF39SM. Trapper peas were co-inoculated with VF39SM and an individual *mcp* mutant strain as described in Methods. The *mcp* mutants co-inoculated were as follows: set 1, VF-MCPB⁻; set 2, VF-MCPC⁻; set 3, VF-MCPD⁻; set 4, VF-MCPC⁻/D⁻. After 5 weeks incubation, 100 nodules were crushed to determine which strain formed each nodule. Since bacteria in each nodule are usually of clonal origin a selectable marker can be used to determine the number of nodules formed by a strain. Ratios are expressed as the *mcp* mutant/VF39SM. An asterisk indicates that the recovery ratio differed significantly from the initial inoculation ratio. Statistical significance was confirmed using the chi-squared test.

wild-type VF39SM, and they appeared just as rapidly. Therefore, the mutants were not impaired in nodulation or nitrogen fixation capacity *per se*. The recovery ratio of VF-MCPD⁻ indicated that it was able to compete successfully with VF39SM for nodule occupancy. The inability of the *mcpC*, *mcpD* double mutant to compete with VF39SM for nodule occupancy is consistent with the result of the *mcpC* mutant competition experiment. *mcpE* mutants were unaltered in competitive ability (data not shown).

Localization of *mcp* genes to VF39SM plasmids

Rhizobia characteristically contain large plasmids (Hynes *et al.*, 1989; reviewed by Mercado-Blanco & Toro, 1996). Some are involved in symbiosis, while the function of other plasmids has been less well studied. VF39SM contains six plasmids, ranging in size from 150 kb to over 600 kb (Hynes *et al.*, 1988). To investigate whether any putative *mcp* genes reside on VF39SM plasmids, a mixture of probes derived from the six indigenous plasmids of VF39SM was used to probe a blot containing the putative *mcp* genes (data not shown). The results of this experiment indicated that *mcpC* is plasmid localized. When a typical Eckhardt gel of VF39SM and the plasmid-cured derivative strains developed by Hynes & McGregor (1990) was probed with a fragment from MCPC.B, *mcpC* was found to reside on pRleVF39f (Fig. 6). Additionally, three of the 15 cosmid clones which hybridized to the *mcpD* probe were of plasmid origin (data not shown).

DISCUSSION

Our results provide evidence for the existence of a family of *mcp*-like genes in *R. leguminosarum* VF39SM. Positive hybridization to a probe containing DNA corresponding to the conserved signalling domain of MCPs, and sequence similarities between MCPs and the deduced amino acid sequences for McpB through McpF, suggest that these genes do code for MCP-like proteins.

root environment, faster infection rates, or superior ability to migrate towards suitable infection sites (Ames & Bergman, 1981; Gulash *et al.*, 1984; Dharmatilake & Bauer, 1992). The results of the nodule competition experiments (Fig. 5) indicate that VF-MCPB⁻ and VF-MCPC⁻ were unable to compete with VF39SM in nodulation frequency. On control plants where VF-MCPB⁻ and VF-MCPC⁻ were inoculated alone, the number and appearance of nodules were the same as for

Fig. 6. (a) Typical Eckhardt gel showing VF39SM and the plasmid-cured derivative strains prepared by Hynes & McGregor (1990). The strain in each lane is as follows: A, VF39SM; B, LRS39201 (cured of pRleVF39b); C, LRS39301 (cured of pRleVF39c); D, LRS39401 (cured of pRleVF39d); E, LRS39501 (cured of pRleVF39e); F, LRS39601 (cured of pRleVF39f). (b) Southern blot of an Eckhardt gel with the same strain order as that in (a) probed with the 1.5 kb *Eco*RI fragment of MCP.C.B. A band is present in all lanes except that (F) containing LRS39601. The letters A–F indicate the positions of the six plasmids of strain VF39 (pRleVF39a, pRleVF39b, etc.) as described by Hynes *et al.* (1988).

In addition, the identification of 15 further clones showing homology to the highly conserved domain of *mcp* genes strongly suggests that, as in *Desulfovibrio* (Deckers & Voordouw, 1994) and *Halobacterium* (Zhang *et al.*, 1996), *Rhizobium* spp. may contain numerous genes encoding proteins showing similarity to MCPs. Further evidence for this comes from recent publications showing that genes homologous to the *mcp* genes we have identified are present on the nodulation plasmids of another strain of *R. leguminosarum* (Brito *et al.*, 1996) and of *Rhizobium* sp. NGR234 (Freiberg *et al.*, 1997); also, genes encoding proteins with amino acid sequence similarity to the signalling domain of MCPs in *S. meliloti* have been described (Greck *et al.*, 1995). None of these genes appears to be closely related to any of the genes from strain VF39SM for which we have complete or partial sequence data. Further investigation will be required before a role (if any) for these genes in

chemotaxis can be defined. We do believe that the majority of the clones we have identified from VF39SM code for proteins with homology to MCPs for two reasons: (1) Western blots using antibodies against the MCP highly conserved domain reveal the presence of at least 10 proteins that react with the antibody (data not shown); and (2) most clones hybridized at least as strongly to several *mcp*-derived probes as did the five genes for which we have DNA sequence data. Since five clones chosen more or less at random from those hybridizing with *mcp* probes code for MCP-like proteins, it is highly probable that some of the other clones do so as well. However, it is also possible that some of the clones carrying DNA homologous to *mcp* genes are pseudogenes, or code for proteins like the *tcp1* and *acfB* gene products in *Vibrio cholerae* (Harkey *et al.*, 1994; Everiss *et al.*, 1994), which show similarity to the MCP signalling domain, but apparently do not function as chemoreceptors, although they have important roles in other facets of signal transduction.

All the putative *mcp* genes for which DNA sequencing (complete or partial) was carried out had high homology to the methylation domains characteristic of MCPs. The amino acid sequences deduced from the DNA sequences of *mcpB*, *mcpC* and *mcpD* show all the characteristic features of MCPs, such as transmembrane domains, methylation sites, and the signalling domain. The genes also code for proteins of a size typical for MCPs. Of the other putative MCP-encoding genes so far identified in the rhizobia, those from *S. meliloti* (Greck *et al.*, 1995) appear unusual in that the methylation sites and transmembrane regions are missing, whereas those from NGR234 (Freiberg *et al.*, 1997) and *R. leguminosarum* bv. *viciae* UPM791 (Brito *et al.*, 1996) appear to code for fairly typical MCPs.

Our results suggest that some of the *mcp* genes we have identified play a role in chemotaxis. While mutations in *mcpC*, *mcpD* and *mcpE* conferred no alteration in chemotactic properties towards a variety of sugars, amino acids and other carbon sources, mutations in *mcpB* appeared to create a general chemotactic block. The reason for this remains unclear, but *mcpB* mutations resemble *mcpA* mutants in *Rhodobacter sphaeroides* (Ward *et al.*, 1995), which lost chemotaxis to a wide range of carbon sources, rather than a discrete set. In addition, this phenotype was apparent only under aerobic conditions. This suggests that the role of certain MCPs in chemotaxis of members of the alpha sub-group of the proteobacteria may deviate significantly from the *E. coli* paradigm.

Further evidence that several of the *mcp* genes we have identified have a biological function comes from our nodulation competition experiments. It has long been speculated that chemotaxis may play a role in competition between *Rhizobium* strains in the legume rhizosphere, and indeed some studies (Ames & Bergman, 1981; Caetano-Anollés *et al.*, 1988; Bauer & Caetano-Anollés, 1990) have shown that non-motile and some non-chemotactic strains cannot compete success-

fully with their wild-type counterparts. Our results establish not only that chemotaxis *per se* is important in competition for nodulation, but that subtle, and as yet uncharacterized, effects on chemotactic behaviour may also affect competitiveness. Not only was the *mcpB* (chemotaxis minus) mutant greatly reduced in its ability to compete, but the mutations in *mcpC*, which caused no apparent chemotactic phenotype in laboratory assays, also had drastic effects on competition for nodule occupancy. This suggests that these genes may code for proteins that sense highly specific compounds in the plant environment, and that chemotaxis towards these compounds may be an important aspect of signal exchange between plant and bacterial partners in the symbiosis.

Identifying ligands for a large family of putative chemoreceptors could prove to be a daunting task, given the great metabolic diversity of *Rhizobium* species (Stowers, 1985; Parke & Ornston, 1984), and the wide diversity of compounds that can serve as chemo-attractants for rhizobia (Parke *et al.*, 1985). Adding to the difficulty, some of the potential chemoattractants may be compounds from root exudates that have not yet been identified. The finding that certain putative *mcp* genes are located on plasmids, both in strain VF39SM, as is the case for *mcpC* and at least three other cosmid clones, and in other rhizobia (Brito *et al.*, 1996; Freiberg *et al.*, 1997) may be of some use in determining functions. It is not unlikely that *mcp* genes located on plasmids code for proteins that sense attractants catabolized by enzymes encoded by those plasmids. Many catabolic genes have been mapped to plasmids in rhizobia (Murphy *et al.*, 1987; Tepfer *et al.*, 1988; Boivin *et al.*, 1991; Charles & Finan, 1991; Baldani *et al.*, 1992), and the complete sequence of the nodulation plasmid from NGR234 suggests the presence of a large number of transport and catabolic genes (Freiberg *et al.*, 1997). Strains cured of particular plasmids (e.g. Hynes & McGregor 1990; Baldani *et al.*, 1992) could be used to aid in identification of specific compounds in root exudates catabolized by plasmid-encoded genes, and these compounds could then be tested as chemo-attractants.

This study has demonstrated that cloning genes based on their homology to DNA sequences encoding highly conserved regions in MCPs, and then analysing them by mutation and sequencing, provides a valid approach for detecting previously unidentified *mcp*-like genes in bacteria. Further work on each of the clones we have obtained will determine the precise role the putative *mcp* genes play in chemotaxis and other cellular functions, and should establish conclusively whether a large family of such genes exists in rhizobia.

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