The *rkp-3* Gene Region of *Sinorhizobium meliloti* Rm41 Contains Strain-Specific Genes that Determine K Antigen Structure

Ernő Kiss,¹ Attila Kereszt,¹ Fatime Barta,¹ Samuel Stephens,² Bradley L. Reuhs,² Ádám Kondorosi,^{1,3} and Péter Putnoky^{1,4}

¹Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary; ²Complex Carbohydrate Research Center, University of Georgia, Athens 30602, U.S.A.;

³Institut des Sciences Végétales, CNRS, Avenue de la Terrasse, F-91198 Gif-sur-Yvette Cedex, France;

⁴Department of Genetics and Molecular Biology, University of Pécs, P.O. Box 266, H-7601 Pécs, Hungary
Submitted 9 April 2001; Accepted 26 July 2001.

The *rkp-3* region is indispensable for capsular polysaccharide (K antigen) synthesis in *Sinorhizobium meliloti* Rm41. Strain Rm41 produces a K antigen of strain-specific structure, designated as the K_{R5} antigen. The data in this report show that the *rkp-3* gene region comprises 10 open reading frames involved in bacterial polysaccharide synthesis and export. The predicted amino acid sequences for the *rkpL-Q* gene products are homologous to enzymes involved in the production of specific sugar moieties, while the putative products of the *rkpRST* genes show a high degree of similarity to proteins required for transporting polysaccharides to the cell surface. Southern analysis experiments using gene-specific probes suggest that genes involved in the synthesis of the precursor sugars are unique in strain Rm41, whereas sequences coding for export proteins are widely distributed among *Sinorhizobium* species. Mutations in the *rkpL-Q* genes result in a modified K antigen pattern and impaired symbiotic capabilities. On this basis, we suggest that these genes are required for the production of the K_{R5} antigen that is necessary for *S. meliloti* Rm41 *exoB* (AK631)–alfalfa (*Medicago sativa*) symbiosis.

Additional keywords: pseudaminic acid.

Gram-negative bacteria of the family *Rhizobiaceae* (rhizobia) associate with specific legume host plants to form a nitrogen-fixing symbiosis. In the course of infection, the bacteria undergo morphological changes resulting in the inclusion of highly differentiated cells (bacteroids) in specialized host plant cells. Symbiotic infection is initiated and maintained by an exchange of signal molecules between the host plant and the microsymbionts (Long 1996; Schultze et al. 1994). For example, flavonoids from the host plant, in conjunction with bacterial regulators, activate bacterial nodulation (*nod*) genes, which then direct the synthesis of lipochitooligosaccharides

(Nod factors) that induce root hair curling and cortical cell division (Schultze and Kondorosi 1998).

Exopolysaccharides (EPS), capsular polysaccharides (K antigens [KPS]), and lipopolysaccharides (LPS) also are important for infection in certain symbiotic systems. For example, EPS production is necessary for successful nodule invasion in *Sinorhizobium meliloti* Rm1021–alfalfa (*Medicago sativa*) symbiosis (Leigh and Coplin 1992; Walker 1992), because *exo* (EPS) mutants are unable to infect. However, in *S. meliloti* Rm41, the K antigen compensates for the EPS, suggesting that K antigen can provide the same functions as the EPS during nodule development (Petrovics et al. 1993; Putnoky et al. 1990). A recent report showed that the *S. meliloti* Rm41 K antigen elicits rapid induction of host genes in the isoflavonoid biosynthetic pathway, indicating an active mechanism for recognition of *S. meliloti* by alfalfa (Becquart-de Kozak et al. 1997).

It was also shown that K antigen production by rhizobia is widespread and that they are strain-specific antigens (Reuhs et al. 1997). Thus far, three gene clusters required for K antigen production have been identified in *S. meliloti* Rm41; these include *rkp-1* (formerly *fix-23*), *rkp-2*, and *rkp-3* (Kereszt et al. 1998; Putnoky et al. 1990). In the *S. meliloti* Rm41 *exoB* (AK631) background, strains carrying mutations in these gene clusters yield aborted infection threads and empty nodules lacking nitrogen-fixing bacteroids, indicating that the mutants are not able to invade the host plants (*Inf*[−] phenotype). Moreover, most of the *rkp* mutants are not able to adsorb the strain-specific bacteriophage 16-3, demonstrating that the mutations also lead to a change in the cell surface properties. In most cases, biochemical and immunological analysis of the mutants demonstrated the partial or complete loss of the K antigen from the cell surface (Kereszt et al. 1998; Kiss et al. 1997; Petrovics et al. 1993).

The *rkp-1* region contains 10 genes, encoding proteins with a high degree of similarity to enzymes involved in fatty acid synthesis (*rkpA-F*) as well as to proteins participating in the modification and transfer of lipophilic molecules (*rkpG-J*). These gene products appear to be involved in the production of a specific lipid carrier required for the biosynthesis of the K antigen (Kiss et al. 1997; Petrovics et al. 1993). The *rkp-2*

Corresponding author: Péter Putnoky; Telephone: +36 (72) 503 600; Fax: +36 (72) 501 527; E-mail: putnoky@ttk.pte.hu

Current address of B. L. Reuhs: Whistler Center for Carbohydrate Research, Department of Food Science, Purdue University, West Lafayette, IN 47907 U.S.A.

region contains two genes, both of which are required for wild-type LPS production; however, only the second open reading frame (ORF) (*rkpK*) is also involved in the synthesis of the K antigen. The predicted protein products of the two ORFs (LpsL and RkpK) exhibited a high degree of similarity to enzymes necessary for the metabolism of nucleotide-diphospho-sugars (Kereszt et al. 1998).

Here we report the genetic analysis of the 10 *rkp* genes identified in the *rkp-3* region and the biochemical characterization of the K antigen produced by the *rkp* mutant derivatives. We found that mutations in *rkpL-Q* resulted in modified K antigen production and impaired symbiotic capabilities, showing that these genes are required for the production of the wild-type K_{R5} antigen and infection in *S. meliloti* Rm41 *exoB-M. sativa* symbiosis. In addition, an analysis of representative *rkp* genes from other strains and genera showed that *rkp-1* and *rkp-2* genes are conserved within the genus *Sinorhizobium*, whereas the *rkp-3* gene region is unique to strain Rm41.

RESULTS

Genetic analysis of the *rkp-3* region.

The *rkp-3* region (K antigen production) was recently identified by genetic complementation experiments (Kereszt et al. 1998). In order to delimit the *rkp-3* genes, several Tn5 insertion derivatives of two overlapping cosmid clones (pAT399

and pAT401) were isolated and the mutations were introduced into the *S. meliloti* AK631 genome by homologous recombination. The resulting recombinants were tested for phage 16-3 sensitivity and symbiotic properties (Fig. 1), as well as for K antigen and LPS production (described below). Importantly, phage 16-3-resistant mutants were uniformly ineffective in symbiotic nitrogen fixation (Inf⁻ and Fix⁻), and two phage 16-3-sensitive mutants (Tn5-158 and Tn5-168) also exhibited the same phenotype. The Inf⁻ mutations delimited two *rkp* gene regions separated by a 6-kb DNA fragment in which insertions resulted in no observable phenotype.

Nucleotide sequence analysis.

The nucleotide sequence of overlapping restriction fragments from a 12,821-bp DNA region was determined in order to identify the *rkp* genes in the left part of the *rkp-3* region (Fig. 1). The deduced amino acid sequences of potential ORFs were then compared with protein databases, and they were further analyzed for hydrophobic regions, conserved domains, and protein family signatures (Table 1). Six ORFs were identified and designated as the *rkpL-Q* genes (Fig. 1). Organization of the genes in the same orientation and the very short distances between the coding regions suggest that they constitute a polycistronic operon. Possible transmembrane domains were predicted in RkpM and RkpO by computer-aided structural analysis of the deduced protein sequences, whereas the other four protein sequences were primarily hydrophilic.

Fig. 1. Physical genetic map of *rkp-3* region of *Sinorhizobium meliloti* 41. Vertical bars represent the Tn5 insertions. The height of the bars corresponds to the phenotype of the mutation as indicated to the left of the map. The physical map was constructed by *EcoRI* (E), *BamHI* (B), and *HindIII* (H) restriction enzymes. Solid horizontal bars show the DNA regions carried by the complementing cosmid clones (pAT399 and pAT401). Organization of the *rkp-3* genes, as determined by sequence analysis of the 12,821-bp region is shown in the lower part. Vertical bars represent those Tn5 insertions (with strain numbers) where the positions were established by sequencing. The length of the bar and the different shading reflect the phenotype of the insertion as it is applied above.

The *rkpL-Q* gene products had several conserved domains and protein family signatures and, in each case, there was considerable similarity to enzymes associated with polysaccharide biosynthesis in different Gram-negative bacteria (Table 1). The similarities suggest that the protein products of *rkpL-Q* are involved in the biosynthesis of the nucleotide-sugar precursors of the K antigen polymer.

Downstream from *rkpL-Q*, three additional ORFs were identified and designated *rkpR* and *rkpTS* (Fig. 1). The opposite orientation of *rkpR* and *rkpTS* to *rkpL-Q* indicates that they are transcribed independently from *rkpL-Q*. The putative protein products of *rkpR* and *rkpST* exhibited significant homology to transport proteins involved in the translocation of mature K antigen polymer from the cytoplasm to the cell surface (Table 1). RkpR and RkpT had two and five predicted transmembrane domains, respectively. As with their homo-

logues, RkpT and RkpS possessed conserved sequence motifs specific for the ABC transporter family (Table 1). The last ORF on the right side of the *rkp-3* region, designated *rkpZ*, was identical to the *lpsZ* sequence reported earlier (Brzoska and Signer 1991). Although it was originally proposed to function in LPS biosynthesis, a later study showed that LpsZ has an effect on the size-specific expression of capsular polysaccharides and no effect on LPS production (Reuhs et al. 1995); consequently, the gene designation was changed to *rkpZ*. The homology of *rkpZ* to *kpsC* of *Escherichia coli* is further evidence for a function in K antigen expression (Table 1).

K antigen production by the *rkp-3* mutants.

The positions of several Tn5 insertions were determined within the sequenced region using primers constructed for the ends of the IS50 arms, and the mutated genes were identified

Table 1. Homologies of the deduced *rkp-3* gene products to proteins of known functions^a

Rkp		Homologue				
Protein	Protein family signatures (putative function)	Protein	Bacteria	Function	Amino acid I/S ^b (%)	Reference
RkpL	NAD-dependent epimerases/dehydratases (dNDP-hexose dehydratase/epimerase)	Jhp0778	<i>Helicobacter pylori</i>	Sugar nucleotide synthesis	54/70	Alm et al. 1999
		Cap5E	<i>Staphylococcus aureus</i>	UDP-glucose epimerase	42/58	Sau et al. 1997
		FlmA	<i>Aeromonas caviae</i>	O-antigen biosynthesis	53/69	AAD45656
RkpM	DegT/DnrJ/EryC1/StrS (possible amino-sugar biosynthesis protein or aminotransferase)	FlmB	<i>Aeromonas caviae</i>	O-antigen biosynthesis	66/79	AAD45657
		SpsC	<i>Methanococcus jannaschii</i>	Polysaccharide synthesis	35/56	Bult et al. 1996
		WlbF	<i>Bordatella bronchiseptica</i>	Amino-sugar synthesis	35/50	CAA07666
RkpN	(Pse activation)	NeuA	<i>Escherichia coli</i>	O-antigen biosynthesis	59/73	AAD45658
		NeuA	<i>Escherichia coli</i>	CMP-NeuNAC synthetase	26/42	Zapata et al. 1989
		KpsU	<i>Aeromonas caviae</i>	CMP-KDO synthetase	28/54	Pazzani et al. 1993
RkpO	(transferase)	FlmD	<i>Aeromonas caviae</i>	O-antigen biosynthesis	37/52	AAD45659
		FlaR	<i>Caulobacter crescentus</i>	Flagellar protein	32/46	U27302
		SpsG/H	<i>Methanococcus jannaschii</i>	Polysaccharide synthesis	27/48	Bult et al. 1996
RkpP	Acetyltransferases (transferase)	FlaG	<i>Caulobacter crescentus</i>	Flagellar protein	38/56	U28867
		SpeG	<i>Escherichia coli</i>	Acetyltransferase	26/48	Fukuchi et al. 1994
RkpQ	(KDO synthesis)	NeuB	<i>Aeromonas caviae</i>	O-antigen biosynthesis	64/78	AAD45660
		NeuB	<i>Helicobacter pylori</i>	Sialic acid synthetase	42/59	Tomb et al. 1997
		NeuB	<i>Escherichia coli</i>	NeuNAC condensation	32/50	Annunziato et al. 1995
RkpR	(KPS export throughout the periplasmic space)	KpsE	<i>Escherichia coli</i>	KPS export protein	28/50	Russo et al. 1998
		BexC	<i>Haemophilus influenzae</i>	KPS export protein	27/51	Kroll et al. 1990
		CtrB	<i>Neisseria meningitidis</i>	KPS export protein	27/50	Frosch et al. 1991
RkpS	ABC transporters (KPS export throughout the cell membrane)	KpsT	<i>Escherichia coli</i>	KPS export protein	48/67	Pavelka et al. 1991
		BexA	<i>Haemophilus influenzae</i>	KPS export protein	44/65	Kroll et al. 1990
		CtrD	<i>Neisseria meningitidis</i>	KPS export protein	47/66	Frosch et al. 1991
RkpT	ABC-2 type transporters (KPS export throughout the cell membrane)	KpsM	<i>Escherichia coli</i>	KPS export protein	31/56	Pavelka et al. 1991
		BexB	<i>Haemophilus influenzae</i>	KPS export protein	25/57	Kroll et al. 1990
		CtrC	<i>Neisseria meningitidis</i>	KPS export protein	24/55	Frosch et al. 1991
RkpZ	(chain-length determination)	LpsZ	<i>Rhizobium meliloti</i>	LPS processing protein	100/0	Brzoska and Signer 1991
		KpsC	<i>Escherichia coli</i>	KPS export protein	40/56	Pazzani et al. 1993
		LipA	<i>Neisseria meningitidis</i>	KPS modification protein	37/53	Frosch and Muller 1993

^a CMP = cytidine 5'-monophosphate, KDO = 3-deoxy-D-manno-2-octulosonic acid, KPS = capsular polysaccharide, LPS = lipopolysaccharides, and Pse = pseudaminic acid.

^b I/S = identity/similarity.

(Fig. 1). The cell surface polysaccharides (K antigen and LPS) from the specific mutants were then extracted with hot phenol-water and analyzed by polyacrylamide gel electrophoresis (PAGE) (Fig. 2A). The banding patterns of the K antigens from the *Fix⁻* mutants were altered in each case, relative to the parent strain (AK631), whereas the LPS migration patterns were unchanged. The phage-sensitive and *Inf⁻* mutant (*rkpO*) produced some low-molecular-weight (LMW) K antigen and minor amounts of high-molecular-weight (HMW) K antigen. In contrast, no K antigen production was seen with the phage-resistant and *Inf⁻* strains affected in the sequenced region. Interestingly, the *rkpR* mutant displayed wild-type K antigen expression (Fig. 2A), as did the *rkpT* mutant (data not shown), and these mutants were also *Inf⁺ Fix⁺* in the nodulation assays. Thus, despite the homology of RkpR and RkpT to

K antigen export proteins in other bacteria, the corresponding genes did not appear to be essential for normal capsule expression in laboratory culture or in symbiosis.

We next performed an immunoblot analysis of the phenol-water extracts from the parent strain AK631, the *rkpO* mutant, and the *rkpQ* mutant, using anti-Rm41 polyclonal antisera (Fig. 2B). The results confirmed the production of HMW K antigen by the *rkpO* mutant. A subsequent immunoblot analysis of the AK631 extract, using antisera that had been preadsorbed with whole cells of the same three strains, showed that the HMW K antigen produced by the *rkpO* mutants was, in fact, translocated to the cell surface (Fig. 2C). In addition, the fact that each strain removed all the antibodies to the LPS showed that there was no LPS defect associated with the insertions in the *rkp-3* region.

Fig. 2. A, Polyacrylamide gel electrophoresis analysis of the phenol-water extracts from *Sinorhizobium meliloti* AK631 (Rm41 *exoB*) and representative *rkp-3* mutants. The gels were Alcian blue-silver stained. Lanes: 1, *rkpL*; 2, *rkpM*; 3, *rkpO*; 4, *rkpQ*; 5, *rkpP*; 6, *rkpR*; and 7, AK631. B, Immunoblot analysis of the phenol-water extracts from strain AK631 (lane 1), *rkpO* (lane 2), and *rkpQ* (lane 3), using anti-Rm41 polyclonal antisera. C, Immunoblot analysis of the phenol-water extract from strain AK631, using anti-Rm41 polyclonal antisera that was preadsorbed with whole cells of strain AK631 (lane 1), *rkpO* (lane 2), and *rkpQ* (lane 3). HMW, high-molecular-weight K antigen; LMW, low-molecular-weight K antigen; S-LPS, smooth lipopolysaccharide; and R-LPS, rough lipopolysaccharide.

Table 2. Identification of *rkp-3* homologous sequences by Southern blot analysis in different bacteria

Bacterium	Gene-specific hybridization probes ^a								
	<i>rkpL</i>	<i>rkpM</i>	<i>rkpN</i>	<i>rkpR</i>	<i>rkpS</i>	<i>rkpZ</i>	<i>rkpK</i>	<i>rkpE</i>	<i>rkpJ</i>
<i>Mezorhizobium loti</i>	—	—	—	—	—	—	+	—	—
<i>Agrobacterium tumefaciens</i>	—	—	—	—	—	—	+	—	—
<i>Agrobacterium rhizogenes</i>	—	—	—	—	—	—	+	—	—
<i>Rhizobium galagae</i>	—	—	—	—	—	—	+	—	—
<i>Sinorhizobium meliloti</i> NRG185	+	—	—	++	++	+	+	++	++
<i>Sinorhizobium meliloti</i> NRG247	+	—	—	++	++	++	+	++	++
<i>Sinorhizobium meliloti</i> 1021	—	—	—	+++	+++	+	+++	+++	+
<i>Sinorhizobium meliloti</i> Rm41	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>Sinorhizobium fredii</i> USDA205	—	—	—	++	+++	++	+	++	—
<i>Sinorhizobium fredii</i> USDA208	—	—	—	+	++	+	++	+	—
<i>Sinorhizobium fredii</i> USDA257	+	—	—	+	++	++	++	+	—
<i>Rhizobium</i> sp. NGR234	++	+	—	+	+++	+	+	+	—
<i>Rhizobium leguminosarum</i>	—	—	—	—	—	—	+	—	—
<i>Rhizobium trifolii</i> ANU843	—	—	—	—	—	—	+	—	—
<i>Agrobacterium caulinodans</i>	—	—	—	—	—	—	—	—	—
<i>Rhizobium spheroides</i>	—	—	—	—	—	—	—	—	—
<i>Xanthomonas campestris</i>	—	—	—	—	—	—	—	—	—

^a — = No hybridization found (that is, the gene is not present or not conserved), and + = weak, ++ = middle, and +++ = strong hybridization signal was obtained (that is, a similar sequence is present in the genome).

Detailed phenotypic analysis of *rkpZ* (*lpsZ*) was published earlier (Reuhs et al. 1995; Williams et al. 1990a).

Conservation of *rkp* genes in rhizobia.

In order to determine which *rkp* genes were conserved in the genus *Sinorhizobium* and which were strain specific (i.e., specific to a given K antigen structure), selected genes were tested in Southern blot analyses. DNA fragments (200 to 700 bp) from *rkpL*, *M*, *N*, *R*, *S*, and *Z* (*rkp-3* region), *rkpK* (*rkp-2* region), and *rkpE* and *J* (*rkp-1* region) were radio-labeled and hybridized to *Eco*RI-digested genomic DNA from different genera of legume symbionts and plant pathogens (Table 2). The occurrence of *rkp* homologues in different *Rhizobiaceae* spp. was in agreement with the phylogenetic tree based on 16S rRNA sequences (Martinez-Romero and Caballero-Mellado 1996). Hybridization signals were weakly detectable or absent in the analyses of the *Azorhizobium*, *Mezorhizobium*, *Agrobacterium*, and *Rhizobium* genomes. The majority of the sequences were present in the representatives of the genus *Sinorhizobium*, except *rkpL*, *rkpM*, and *rkpN*.

In a second series of hybridization experiments, DNA samples of 20 *S. meliloti* strains were tested for the presence of the *rkpC* and *rkpN* genes, representing the *rkp-1* and the *rkp-3* regions, respectively. Although the *rkpC* sequences could be clearly detected in all cases, the *rkpN* probe showed hybridization to only one total-DNA sample (*S. meliloti* Rm1322) in addition to the control (strain Rm41; data not shown).

Based on the strength and pattern of the hybridizations, genes with general functions such as transport (*rkpR* and *S*), size determination (*rkpZ*), and lipid carrier synthesis (*rkpE*) exhibited broad-range conservation in the genus *Sinorhizobium*. Genes proposed to be involved in the synthesis of strain-specific sugar precursors (*rkpM* and *rkpN*) were absent in the majority of the *S. meliloti* strains. One exception was *rkpK*, which showed weak hybridization to DNA from almost all bacteria tested. This was likely due to the fact that *rkpK* encodes a UDP-glucose dehydrogenase (Kereszt et al. 1998), leading to the formation of glucuronic acid, which is present in a wide range of bacteria.

G+C content and codon usage of *rkp* genes.

Although the average G+C content of the *Rhizobium* genome is approximately 62 to 64% (Broughton et al. 1972), the G+C content of many genes involved in Nod factor biosynthesis is lower (Perret et al. 1997). Therefore, we compared the G+C content of the *S. meliloti* *rkp* genes with "housekeeping" and symbiotic genes. As expected, both *nif-fix* (58.4%) and *nod* (56.9%) genes had a lower G+C content, whereas the ancestral, nonsymbiotic *ndv* (62.4%) and *exo* (62%) genes were within the genomic average. Interestingly, the G+C content of the *rkp-3* genes was also lower (55.2%), while the G+C content of the *rkp-1* and *rkp-2* genes proved to be 63.4 and 62.5%, respectively. In addition, there were no significant differences between the G+C content of *rkp-1* and *rkp-2* and their flanking sequences, whereas the G+C content of *rkp-3* was significantly lower than that of its flanking DNA (the DNA sequences upstream of *rkpL* and downstream from *rkpZ*).

Analysis of several coding sequences, using the CODON-FREQUENCY and CODONPREFERENCE programs, showed a clear difference in G+C bias of some *rkp* genes when compared with other *S. meliloti* sequences (Fig. 3). The house-

keeping genes tended to contain codons that carried G or C at the third position, whereas the symbiotic genes formed a second group in which this preference was decreased. Some *rkp* genes, such as *rkpOP*, *rkpZ* (this study), and *rkpY* (P. Putnoky, unpublished data), represented a third group, showing a significantly lower preference for codons with G or C at the third position. For example, there were six codons for arginine and three of them contained G or C at the third position. Although 85% of the arginine codons of the housekeeping genes and 75% of the arginine codons for the symbiotic genes carried G or C at the third position, the percentage dropped to 55% for *rkpOPZY* (i.e., G or C not preferred). This tendency was found for all amino acids determined by at least two codons, except tyrosine. These results suggest that several genes in the *rkp-3* region have different origins and that the genes are acquired by the *S. meliloti* Rm41 genome relatively recently on an evolutionary scale.

DISCUSSION

The production of the K_{R5} antigen is required for symbiotic infection by the *S. meliloti* Rm41 *exoB* (AK631) (Petrovics et al. 1993; Putnoky et al. 1990). Thus, the *rkp-3* region represents genes of both general importance (required for capsule production and protection against dehydration, phage adsorption, and so on) and symbiotic significance (needed for infection in the absence of EPS or under specific environmental circumstances). As such, the diversity in capsule structure is derived from multiple demands. This may explain, in part, the fact that each strain of *S. meliloti* produces unique K antigens, yet most are composed of similar disaccharide repeats. The importance of the K antigens in symbiosis extends beyond *S. meliloti* Rm41; recently, we have found that six of eight *S. meliloti* *exoB* (EPS⁻) derivatives of wild-type strains tested are capable of infecting alfalfa (G. R. O. Campbell, T. Ojaneu-Reuhs, S. B. Stephens, and B. L. Reuhs, unpublished data). Thus, K antigen activity in alfalfa is common, and the inability of strain Rm1021 *exoB* to infect (Williams et al. 1990a)

Fig. 3. Example for the codon usage of the *rkpOP*, *rkpZ*, and *rkpY* genes representing the fractions of the Arg and Thr codons and the total G+C content at their third positions. The housekeeping, symbiotic, and this group of *rkp* genes show different GC bias.

may be exceptional. The identification of the *rkp-3* gene region offers an opportunity to delineate the structural requirements for K antigen activity in the host plant.

Current data indicate that the K_R5 antigen consists of disaccharide repeating units of glucuronic acid (GlcA) and 5,7-diamino-5-*N*-β-hydroxybutyryl-7-*N*-acetyl-3,5,7,9-tetra-deoxy nonulosonic acid (pseudaminic acid; Pse), Pse5NAc7N(β-OH-But), with minor *O*-acetylation (Reuhs et al. 1993, 1998; B. L. Reuhs and J. Glushka, *unpublished data*). The biosynthesis of the GlcA from glucose (Glc) requires UDP-glucose dehydrogenase, which is encoded by *rkpK* in the *rkp-2* gene region (Kereszt et al. 1998); however, the *rkpK* mutant is defective in the synthesis of both the K antigen and LPS.

In contrast, many of the genes directing Pse synthesis reside in the *rkp-3* region, and they are specific for the K antigen. Although the pathway to Pse has not been elucidated, it may be similar to the biosynthesis of 3-deoxy-D-manno-2-octulosonic acid (Kdo), in which arabinose is condensed with phosphoenolpyruvate (PEP). One possible pathway would involve the oxidation of an activated hexosamine to 2,6-dideoxy-2-amino-hexopyranose-4-ulose by an NAD⁺-dependent 4,6-dehydratase (RkpL), the conversion of the 4-keto moiety to an amino group by a coenzyme B₆-dependent transaminase (RkpM), and the condensation of the 2,4,6-trideoxy-2,4-diamino-hexose with PEP to form Pse (RkpQ). The Pse may be activated by a pyrophosphorylase (RkpN), prior to polysaccharide polymerization, and *O*-acetylation requires an acetyltransferase (RkpP).

Formation of the disaccharide subunits and polymerization of the polysaccharide would require additional genes, and other *S. meliloti* Rm41 K antigen mutants have been identified that could not be complemented by the known *rkp* regions (Campbell et al. 1998). However, the degree of polymerization is regulated by RkpZ, encoded in *rkp-3*, and acting as a chain-length determinant, and a functional copy of *rkpZ* (*kpsC*) is required for K antigen activity in alfalfa (Reuhs et al. 1995). Interestingly, mutations in *rkpR* and *rkpST*, with homology to *kpsE* and *kpsTM* encoding export proteins, did not

disrupt K antigen expression, suggesting that there may be other export systems with overlapping functions in *S. meliloti* Rm41. For example, the *exsA* gene of *S. meliloti* encodes a component of an ABC transporter for EPS export, but a mutation in *exsA* does not result in complete loss of EPS secretion. Current evidence suggests that the NdvA protein, which is involved in cyclic β-(1,2) glucan export, functionally replaces ExsA (Becker et al. 1995). These overlapping functions could be important in maintaining symbiotic viability.

The production of exported K antigen by the *rkpO* mutants is an unexpected phenotype for two reasons. First, sequence homology suggests that RkpO may mediate the addition of Pse residues to the oligomer and the lack of this function should completely block the KPS production. Either RkpO has another function or there is an alternative enzyme catalyzing the same reaction. It is possible that redundant polymerization pathways exist, one in which disaccharide repeats are block-transferred to the growing polysaccharide and another in which the polysaccharide is produced by the sequential addition of monosaccharides. Both are found in bacterial systems (Whitfield and Valvano 1993). Such a redundant system would allow the bacterium to quickly and efficiently respond to environmental factors, such as host signals, with a modified capsule expression. Second, the Tn5 insertions in *rkpO* should display a polar effect, resulting in the same phenotype as the *rkpPQ* mutants, which is not the case. It appears, therefore, that there is an additional promoter for the distal genes.

Hybridization analyses using representative *rkp* sequences showed that the *rkp-1* and *rkp-2* genes are conserved within the genus *Sinorhizobium*, except *rkpJ*, which was only found in *S. meliloti*. In contrast, most of the genes in the *rkp-3* region are unique to *S. meliloti* Rm41 and, therefore, to the K_R5 antigen. This reflects the structural difference in the respective polysaccharides. For example, the K_R6 antigen from *S. meliloti* NRG247 consists of glucose and *N*-acetylneuraminic acid (NeuNAc) [-α-Glc→α-NeuNAc]_n and the K_R7 antigen of *S. meliloti* NRG185 contains *N*-acetylglucosamine and Kdo [-β-GlcNAc→β-Kdo]_n. Consequently, these latter strains

Table 3. Bacterial strains and plasmids used

Strain/plasmid	Relevant genotype	References
Strains		
<i>Escherichia coli</i> JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 (lac-proAB) (F'⁺traD36 proAB lacIqZdM15)</i>	Yanisch-Perron et al. 1985
<i>Sinorhizobium meliloti</i>		
AK631	Rm41 <i>exoB</i>	Putnoky et al. 1990
1021	Wild type, Sm ^R derivative of SU47	F. M. Ausubel
NRG185	Wild type	Olsen et al. 1994
NRG247	Wild type	Olsen et al. 1994
<i>Rhizobium leguminosarum</i>		
TOM	Wild-type strain nodulates cv. Afghanistan	Lie 1978
ANU843	<i>R. leguminosarum</i> bv. <i>trifolii</i>	Rolfe and Shine 1980
<i>Sinorhizobium fredii</i>		
USDA205	...	Keyser et al. 1982
USDA208	...	Keyser et al. 1982
USDA257	...	Keyser et al. 1982
Other rhizobia		
<i>Rhizobium</i> sp. NGR234	...	Trinick 1980
<i>Agrobacterium tumefaciens</i> C58	...	Hamilton and Fall 1972
<i>Agrobacterium rhizogenes</i> 1724	...	Isogai et al. 1988
Plasmids		
pUC19	Cloning vector, Ap ^R	Yanisch-Perron et al. 1985
pBluescript SK(+)	Cloning vector, Ap ^R	Stratagene, La Jolla, CA
pAT399 and pAT401	Overlapping cosmid clones harboring the <i>rkp-3</i> region	Kereszt et al. 1998

would be expected to possess unique genes in the *rkp-3* region. Importantly, both of these strains are able to infect alfalfa in the *exoB* background. Interestingly, *Sinorhizobium* sp. NGR234 produces a K antigen that has the same primary sequence as K_{R5}, with Pse5NAc7NAc instead of Pse5NAc7N(β-OH-But) and a higher degree of *O*-acetylation (B. L. Reuhs and S. B. Stephens, *unpublished data*), and strain NGR234 was the only other strain tested to hybridize *rkpM*. However, *rkpN* was not detected.

In contrast to the linked organization of the *kps* gene clusters in *E. coli* (Roberts 1996; Whitfield and Roberts 1999), the *rkp-1* and *rkp-2* regions of *S. meliloti* Rm41 were mapped to separate regions of the chromosome (Capela et al. 1999; Putnoky et al. 1990), while *rkpZ* (Williams et al. 1990b) and the *rkp-3* region are located on a symbiotic megaplasmid (pRm41c). This segregation of gene regions may act to increase variability, by allowing for the acquisition of structural genes independently of housekeeping K antigen genes. In addition, the genes governing the production of common monosaccharides are already present in the genome of a given strain; therefore, the acquisition of a K antigen-specific transferase may be adequate for the production of a distinct K antigen. Moreover, the blocks of functionally related *rkp* genes have different G+C content and codon usage, which indicate that rhizobia have acquired these gene sets from different sources.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, plasmids, and growth conditions.

Bacterial strains and plasmids used in this study are described in Table 3. Derivatives of strain AK631 carrying Tn5 insertions in the *rkp-3* region (Fig. 1) were isolated by directed Tn5 mutagenesis (described below). *E. coli* JM109 was used in the cloning procedures (Yanisch-Perron et al. 1985). Media, antibiotic concentrations, culture conditions for *E. coli* and *S. meliloti* strains, and phage 16-3 sensitivity tests have been described previously (Putnoky and Kondorosi 1986; Putnoky et al. 1990).

Directed Tn5 mutagenesis.

Tn5 mutagenesis of the cosmid clones pAT399 and pAT401 was carried out as described earlier (Putnoky et al. 1990). Positions of the insertions were determined by restriction mapping, and several representative Tn5 insertions (Fig. 1) were introduced into the AK631 genome via homologous recombination by the method of Ruvkun and Ausubel (1981). The exact positions of selected insertions were determined by subcloning and sequencing the Tn5-flanking regions (Fig. 1).

DNA manipulations.

Standard procedures, including DNA isolation, restriction enzyme digestion, radioactive labeling of DNA, agarose gel electrophoresis, DNA ligation, and transformation of *E. coli*, were performed using conventional methods (Sambrook et al. 1989) or as recommended by the suppliers. For sequencing, appropriate restriction fragments were subcloned into the vectors pUC19 or pBluescript II SK(+) and their nucleotide sequences were determined by the dideoxy-nucleotide-chain-termination method (Sanger et al. 1977), using [³⁵S]-dATP

and the T7 Sequencing kit from Pharmacia (North Peapack, NJ, U.S.A.). Part of the sequencing was performed on an Applied Biosystems 373A sequencer (Perkin Elmer, Wellesley, MA, U.S.A.) using the BigDye Terminator Kit. Additional sequence information was obtained from sequencing the Tn5-flanking regions of different insertion mutants using a Tn5-specific oligonucleotide primer (5'-GCAAAACGGGAAAGGTTCCG-3').

Southern hybridizations.

Southern hybridization filters were prepared by the downward-alkaline-capillary-transfer method (Chomczynski 1992). For probe preparation, appropriate DNA fragments from *rkp* genes were purified from agarose gels and labeled in the presence of [α-³²P]dCTP with the Oligolabelling Kit (Amersham-Pharmacia Biotech., Uppsala, Sweden). Hybridizations were performed at 65°C in G+C buffer for 16 to 20 h; the filters were then washed in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate three times for 30 min according to the standard protocols (Sambrook et al. 1989).

Computer-assisted sequence analysis.

PCGene software (designed by Amos Bairoch, Intelligenetics Corp., Mountain View, CA, U.S.A.) and the UWGCG program package for VAX (Devereux et al. 1984) was used for basic DNA sequence analysis and assembly. Amino acid homology searches were performed against the "nonredundant" database of NCBI BLAST Network Service using the BLASTX program (Altschul et al. 1990). Protein family and domain predictions were performed using different proteomics tools from ExPASy Molecular Biology Server. Transmembrane domains were calculated with the TopPred2 program at the Stockholm University Server.

The CODONPREFERENCE program of the GCG software package was used to identify ORFs showing similar codon usage to an average *S. meliloti* gene. The characteristics of some *rkp* genes proved to be unusual; therefore, a detailed analysis was performed as follows. Three independent codon usage tables were constructed with the CODONFREQUENCY program for the housekeeping, symbiotic, and *rkp* genes, respectively. The first group was represented by the following DNA sequences of the EMBL data library (accession numbers are in parentheses): *cheAB* (U13166), *flaAB* (M57565), *ftsA* and *ftsZ* (AF024660), *gltA* (U75365), *ndvA* (M20726), *ndvB* (J05219), *ntrA* (M16513), *ntrC* (M15810), *pckA* (U15199), *rpsA* (X07528), *tatA* (L05065), *trpE* (M22983), *phaA* and *phaD* (X93358), and *nuoD1* and *nuoF1* (AJ245398). The symbiotic gene group was represented by the following: *nifA* (X02615), *nifB* (M15544), *nifH* (J01781), *fixABC* (M15546), *fixD* (X03065), *fixF* (M18272), *fixGHI* (M24144), *fixLJ* (J03174), and *fixK* (X15079). The "unusual" *rkp* genes were the following: *rkpOP*, *rkpZ* (AJ245666; this study), and *rkpY* (AJ249130; P. Putnoky and A. Kereszt, *unpublished data*). The frequency of each codon in the three groups was analyzed as described above.

Preparation and analysis of polysaccharides.

Extraction of the surface polysaccharides by a modified hot phenol-water method and deoxycholic acid (DOC)-PAGE analysis of the samples were carried out as previously described (Kiss et al. 1997; Reuhs et al. 1993). For immuno-

chemical analyses, polysaccharides separated on 18% PAGE gels were transferred to nitrocellulose or Nytran⁺ (Schleicher and Schuell, Keene, NH, U.S.A.), using a Trans-Blot SD (Bio-Rad, Richmond, CA, U.S.A.), incubated with anti-Rm41, and developed with goat anti-rabbit/alkaline phosphatase and substrate. Rabbit-anti-Rm41 was donated by Dale Noel (Marquette University, Milwaukee, WI, U.S.A.). Preadsorption of the anti-Rm41 sera is described in detail elsewhere (Petrovics et al. 1993).

Plant tests.

Assays for the symbiotic properties of the mutant *R. meliloti* strains were carried out with alfalfa (*M. sativa*) plantlets, as previously described (Putnoky et al. 1988).

Nucleotide sequence accession number.

A DNA sequence of 12,821 bp has been registered in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession number AJ245666. Coordinates in this publication are identical with those of the database record.

ACKNOWLEDGMENTS

We thank R. W. Carlson for advice in the work and in the preparation of the manuscript. We are grateful to E. Sárai and Z. Liptai for skillful technical assistance. This work was supported by grants OTKA T016674, OTKA T25089, and U.S.-Hungarian Science and Technology Joint Fund JF no. 513. E. Kiss was a recipient of a short-term fellowship from the joint Hungarian Academy of Science-CNRS research program "Jumelage" for the two participating institutes. B. Reuhs was supported by grant MCB-9728564 from the National Science Foundation and by the U.S. Department of Energy-funded Center for Plant and Microbial Complex Carbohydrate Research under grant DE-FG02-93ER-20097.

LITERATURE CITED

- Alm, R. A., Ling, L. S., Moir, D. T., King, B. L., Brown, E. D., Doig, P. C., Smith, D. R., Noonan, B., Guild, B. C., deJonge, B. L., Carmel, G., Tummino, P. J., Caruso, A., Uria-Nickelsen, M., Mills, D. M., Ives, C., Gibson, R., Merberg, D., Mills, S. D., Jiang, Q., Taylor, D. E., Vovis, G. F., and Trust, T. J. 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* 397:176-180.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Annunziato, P. W., Wright, L. F., Vann, W. F., and Silver, R. P. 1995. Nucleotide sequence and genetic analysis of the *neuD* and *neuB* genes in region 2 of the polysialic acid gene cluster of *Escherichia coli* K1. *J. Bacteriol.* 177:312-319.
- Becker, A., Kuster, H., Niehaus, K., and Puhler, A. 1995. Extension of the *Rhizobium meliloti* succinoglycan biosynthesis gene cluster: Identification of the *exsA* gene encoding an ABC transporter protein, and the *exsB* gene which probably codes for a regulator of succinoglycan biosynthesis. *Mol. Gen. Genet.* 249:487-497.
- Becquart-de Kozak, I., Reuhs, B. L., Buffard, D., Breda, C., Kim, J. S., Esnault, R., and Kondorosi, A. 1997. Role of the K-antigen subgroup of capsular polysaccharides in the early recognition process between *Rhizobium meliloti* and alfalfa leaves. *Mol. Plant-Microbe Interact.* 10: 114-123.
- Broughton, W. J., Dilworth, M. J., and Passmore, I. K. 1972. Base ratio determination using unpurified DNA. *Anal. Biochem.* 46:164-172.
- Brzoska, P. M., and Signer, E. R. 1991. *lpsZ*, a lipopolysaccharide gene involved in symbiosis of *Rhizobium meliloti*. *J. Bacteriol.* 173:3235-3237.
- Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., Blake, J. A., FitzGerald, L. M., Clayton, R. A., Gocayne, J. D., Kerlavage, A. R., Dougherty, B. A., Tomb, J. F., Adams, M. D., Reich, C. I., Overbeek, R., Kirkness, E. F., Weinstock, K. G., Merrick, J. M., Glodek, A., Scott, J. L., Geoghegan, N. S. M., and Venter, J. C. 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science* 273:1058-1073.
- Campbell, G. R. O., Reuhs, B. L., and Walker, G. C. 1998. Different phenotypic classes of *Sinorhizobium meliloti* mutants defective in synthesis of K antigen. *J. Bacteriol.* 180:5432-5436.
- Capela, D., Barloy-Hubler, F., Gatiús, M. T., Gouzy, J., and Galibert, F. 1999. A high-density physical map of *Sinorhizobium meliloti* 1021 chromosome derived from bacterial artificial chromosome library. *Proc. Natl. Acad. Sci. U.S.A.* 96:9357-9362.
- Chomczynski, P. 1992. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. *Anal. Biochem.* 201:134-139.
- Devereux, J., Haeberli, P., and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387-395.
- Frosch, M., and Muller, A. 1993. Phospholipid substitution of capsular polysaccharides and mechanisms of capsule formation in *Neisseria meningitidis*. *Mol. Microbiol.* 8:483-493.
- Frosch, M., Edwards, U., Bousset, K., Krausse, B., and Weisgerber, C. 1991. Evidence for a common molecular origin of the capsule gene loci in Gram-negative bacteria expressing group II capsular polysaccharides. *Mol. Microbiol.* 5:1251-1263.
- Fukuchi, J., Kashiwagi, K., Takio, K., and Igarashi, K. 1994. Properties and structure of spermidine acetyltransferase in *Escherichia coli*. *J. Biol. Chem.* 269:22581-22585.
- Hamilton, R. H., and Fall, M. Z. 1972. The loss of tumor-initiating ability in *Agrobacterium tumefaciens* by incubation at high temperature. *Experientia* 27:229-230.
- Isogai, A., Fukuchi, N., Hayashi, M., Kamada, H., Harada, H., and Suzuki, A. 1988. Structure of a new opine, mikimopine, in hairy root induced by *Agrobacterium rhizogenes*. *Agric. Biol. Chem.* 52:3235-3237.
- Kereszt, A., Kiss, E., Reuhs, B., Carlson, R. W., Kondorosi, A., and Putnoky, P. 1998. Novel *rkp* gene clusters of *Sinorhizobium meliloti* involved in capsular polysaccharide production and the invasion of the symbiotic nodule: *rkpK* gene encodes for a UDP-glucose dehydrogenase. *J. Bacteriol.* 180:5426-5431.
- Keyser, H. H., Bohloul, B. B., Hu, T. S., and Weber, D. F. 1982. Fast-growing rhizobia isolated from root nodules of soyabean. *Science* 215: 1631-1632.
- Kiss, E., Reuhs, B., Kim, J., Kereszt, A., Petrovics, G., Putnoky, P., Dusha, I., Carlson, R. W., and Kondorosi, A. 1997. The *rkpGHI* and *-J* genes are involved in capsular polysaccharide production by *Rhizobium meliloti*. *J. Bacteriol.* 179:2132-2140.
- Kroll, J. S., Loynds, B., Brophy, L. N., and Moxon, E. R. 1990. The *bex* locus in encapsulated *Haemophilus influenzae*: A chromosomal region involved in capsule polysaccharide export. *Mol. Microbiol.* 4:1853-1862.
- Leigh, J. A., and Coplin, D. L. 1992. Exopolysaccharides in plant-bacterial interactions. *Annu. Rev. Microbiol.* 46:307-346.
- Lie, T. A. 1978. Symbiotic specialization in pea plants: The requirement of specific *Rhizobium* strains for peas from Afghanistan. *Proc. Assoc. Appl. Biol.* 88:462-465.
- Long, S. R. 1996. *Rhizobium* symbiosis: Nod factors in perspective. *Plant Cell* 8:1885-1898.
- Martinez-Romero, E., and Caballero-Mellado, J. 1996. *Rhizobium* phylogenies and bacterial genetic diversity. *Crit. Rev. Plant Sci.* 15:113-140.
- Olsen, P., Collins, M. M., and Rice, W. A. 1994. Reactivity of MAbs raised against whole cells to a panel of *Rhizobium* strains. *Appl. Environ. Microbiol.* 60:652-661.
- Pavelka, M. S., Jr., Wright, L. F., and Silver, R. P. 1991. Identification of two genes, *kpsM* and *kpsT*, in region 3 of the polysialic acid gene cluster of *Escherichia coli* K1. *J. Bacteriol.* 173:4603-4610.
- Pazzani, C., Rosenow, C., Boulnois, G. J., Bronner, D., Jann, K., and Roberts, I. S. 1993. Molecular analysis of region 1 of the *Escherichia coli* K5 antigen gene cluster: A region encoding proteins involved in cell surface expression of capsular polysaccharide. *J. Bacteriol.* 175: 5978-5983.
- Perret, X., Viprey, V., Freiberg, C., and Broughton, W. J. 1997. Structure and evolution of NGRS-1, a complex, repeated element in the genome of *Rhizobium* sp. strain NGR234. *J. Bacteriol.* 179:7488-7496.
- Petrovics, G., Putnoky, P., Reuhs, B., Kim, J., Thorp, T. A., Noel, K. D., Carlson, R. W., and Kondorosi, A. 1993. The presence of a novel type of surface polysaccharide in *Rhizobium meliloti* requires a new fatty acid synthase-like gene cluster involved in symbiotic nodule develop-

- ment. *Mol. Microbiol.* 8:1083-1094.
- Putnoky, P., and Kondorosi, A. 1986. Two gene clusters of *Rhizobium meliloti* code for early essential nodulation functions and a third influences nodulation efficiency. *J. Bacteriol.* 167:881-887.
- Putnoky, P., Grosskopf, E., Ha, D. T. C., Kiss, G. B., and Kondorosi, A. 1988. *Rhizobium fix* genes mediate at least two communication steps in symbiotic nodule development. *J. Cell Biol.* 106:597-607.
- Putnoky, P., Petrovics, G., Kereszt, A., Grosskopf, E., Ha, D. T. C., Banfalvi, Z., and Kondorosi, A. 1990. *Rhizobium meliloti* lipopolysaccharide and exopolysaccharide can have the same function in the plant-bacterium interaction. *J. Bacteriol.* 172:5450-5458.
- Reuhs, B. L., Carlson, R. W., and Kim, J. S. 1993. *Rhizobium fredii* and *Rhizobium meliloti* produce 3-deoxy-D-manno-2-octulosonic acid-containing polysaccharides that are structurally analogous to group II K antigens (capsular polysaccharides) found in *Escherichia coli*. *J. Bacteriol.* 175:3570-3580.
- Reuhs, B. L., Williams, M. N., Kim, J. S., Carlson, R. W., and Cote, F. 1995. Suppression of the Fix⁻ phenotype of *Rhizobium meliloti* *exoB* mutants by *lpsZ* is correlated to a modified expression of the K polysaccharide. *J. Bacteriol.* 177:4289-4296.
- Reuhs, B. L., Kim, J. S., and Matthysse, A. G. 1997. Attachment of *Agrobacterium tumefaciens* to carrot cells and *Arabidopsis* wound sites is correlated with the presence of a cell-associated, acidic polysaccharide. *J. Bacteriol.* 179:5372-5379.
- Reuhs, B. L., Geller, D. P., Kim, J. S., Fox, J. E., and Pueppke, S. G. 1998. *Sinorhizobium fredii* and *Sinorhizobium meliloti* produce structurally conserved lipopolysaccharides and strain-specific K antigens. *Appl. Environ. Microbiol.* 64:4930-4938.
- Roberts, I. S. 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annu. Rev. Microbiol.* 50:285-315.
- Rolfe, B. G., and Shine, J. 1980. Rapid screening for symbiotic mutants of *Rhizobium* and white clover. *Plant Sci. Lett.* 19:277-284.
- Russo, T. A., Wenderoth, S., Carlino, U. B., Merrick, J. M., and Lesse, A. J. 1998. Identification, genomic organization, and analysis of the group III capsular polysaccharide genes *kpsD*, *kpsM*, *kpsT*, and *kpsE* from an extraintestinal isolate of *Escherichia coli* (CP9, O4/K54/H5). *J. Bacteriol.* 180:338-349.
- Ruvkun, G. B., and Ausubel, F. M. 1981. A general method for site-directed mutagenesis in procaryotes. *Nature* 289:85-88.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74:5463-5467.
- Sau, S., Bhasin, N., Wann, E. R., Lee, J. C., Foster, T. J., and Lee, C. Y. 1997. The *Staphylococcus aureus* allelic genetic loci for serotype 5 and 8 capsule expression contain the type-specific genes flanked by common genes. *Microbiology* 143:2395-2405.
- Schultze, M., and Kondorosi, A. 1998. Regulation of symbiotic root nodule development. *Annu. Rev. Genet.* 32:33-57.
- Schultze, M., Kondorosi, E., Ratet, P., Buire, M., and Kondorosi, A. 1994. Cell and molecular biology of *Rhizobium*-plant interactions. *Int. Rev. Cytol.* 156:1-75.
- Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Glodek, A., McKenney, K., Fitzgerald, L. M., Lee, N., Adams, M. D., and Venter, J. C. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* 388:539-547.
- Trinick, M. J. 1980. Relationships amongst the fast-growing rhizobia of *Lablab purpureus*, *Leucaena leucocephala*, *Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial groups. *J. Appl. Bacteriol.* 49:39-53.
- Walker, G. C. 1992. Role of exopolysaccharides in nodulation. Pages 55-60 in: *Nodulation and Nitrogen Fixation in Rice*. G. S. Khush and J. E. Bennett, eds. Int. Rice Res. Inst., Manila, Philippines.
- Whitfield, C., and Roberts, I. S. 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. *Mol. Microbiol.* 31:1307-1319.
- Whitfield, C., and Valvano, M. A. 1993. Biosynthesis and expression of cell-surface polysaccharides in Gram-negative bacteria. *Adv. Microbiol. Physiol.* 35:136-245.
- Williams, M. N., Hollingsworth, R. I., Klein, S., and Signer, E. R. 1990a. The symbiotic defect of *Rhizobium meliloti* exopolysaccharide mutants is suppressed by *lpsZ*+, a gene involved in lipopolysaccharide biosynthesis. *J. Bacteriol.* 172:2622-2632.
- Williams, M. N. V., Hollingsworth, R. I., Brzoska, P. M., and Signer, E. R. 1990b. *Rhizobium meliloti* chromosomal loci required for suppression of exopolysaccharide mutations by lipopolysaccharide. *J. Bacteriol.* 172:6596-6598.
- Yanisch-Perron, C., Viera, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* 33:103-119.
- Zapata, G., Vann, W. F., Aaronson, W., Lewis, M. S., and Moos, M. 1989. Sequence of the cloned *Escherichia coli* K1 CMP-N-acetylneuraminic acid synthetase gene. *J. Biol. Chem.* 264:14769-14774.