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The 32-Kilobase *exp* Gene Cluster of *Rhizobium meliloti* Directing the Biosynthesis of Galactoglucan: Genetic Organization and Properties of the Encoded Gene Products

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Proteins directing the biosynthesis of galactoglucan (exopolysaccharide II) in *Rhizobium meliloti* Rm2011 are encoded by the *exp* genes. Sequence analysis of a 32-kb DNA fragment of megaplasmid 2 containing the *exp* gene cluster identified previously (J. Glazebrook and G. C. Walker, Cell 56:661–672, 1989) revealed the presence of 25 open reading frames. Homologies of the deduced *exp* gene products to proteins of known function suggested that the *exp* genes encoded four proteins involved in the biosynthesis of dTDP-glucose and dTDP-rhamnose, six glycosyltransferases, an ABC transporter complex homologous to the subfamily of peptide and protein export complexes, and a protein homologous to *Rhizobium* NodO proteins. In addition, homologies of three Exp proteins to transcriptional regulators, methyltransferases, and periplasmic binding proteins were found. The positions of 26 Tn*5* insertions in the *exp* gene cluster were determined, thus allowing the previously described genetic map to be correlated with the sequence. Operon analysis revealed that the *exp* gene cluster consists of five complementation groups. In comparison to the wild-type background, all *exp* complementation groups were transcribed at a substantially elevated level in the regulatory *mucR* mutant.

The soil bacterium *Rhizobium meliloti* is capable of fixing molecular nitrogen in a symbiotic interaction with alfalfa plants. Bacterial nitrogen fixation takes place within root nodules resulting from a coordinated bacteria-plant interaction which requires the exchange of signals between both symbiotic partners (11, 29). *R. meliloti* is able to produce two acidic exopolysaccharides (EPSs), succinoglycan (EPS I) and galactoglucan (EPS II). At least one of these EPSs is required for invasion of *Medicago sativa* nodules by *R. meliloti* (3, 32, 36, 61, 86).

EPS I is composed of repeating units containing one galactose and seven glucose molecules joined by β-1,4-, β-1,3-, and β-1,6-glycosidic linkages (63) and is decorated by acetate, pyruvate, and succinyl groups. EPS II consists of alternating glucose and galactose residues joined by α-1,3 and β-1,3 linkages (38). It is acetylated and pyruvylated. In the Rm1021 and Rm2011 strain backgrounds, the production of EPS II was observed only at low phosphate concentrations (87) or in the presence of a mutation in either the *expR* (32) or *mucR* (44, 86) gene. The corresponding gene products are thought to negatively regulate the expression of genes directing the biosynthesis of EPS II.

The *exo* gene cluster, directing the biosynthesis of EPS I (50), and the *exp* gene cluster, responsible for the synthesis of EPS II (32), are separated by about 200 kb on megaplasmid 2 (15). Whereas the *exo* gene cluster was extensively studied and functions were assigned to most of the *exo* gene products (4–8, 12, 33, 34, 58, 64), little is known about the organization of the *exp* gene cluster and the functions of *exp* gene products.

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A 23-kb DNA region involved in EPS II biosynthesis was cloned and characterized by Tn*5* mutagenesis (32). Six *exp* complementation groups required for EPS II biosynthesis in the *expR101* mutant background were identified in this region. Here, we report on the 32-kb sequence of the *R. meliloti* Rm2011 *exp* gene cluster comprising 25 genes and on the inferred properties of the encoded *exp* gene products.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. *Escherichia coli* strains were grown in Penassay broth (Difco Laboratories) or in LB medium (65) at 37°C. *R. meliloti* strains were grown in TY medium (10), Vincent minimal medium (79), M9 medium (56), or LB medium (65) at 30°C.

Antibiotics were supplemented as required at the following concentrations (micrograms per milliliter): for *R. meliloti*, spectinomycin, 200; streptomycin, 600; nalidixic acid, 8; tetracycline, 8; gentamicin, 40; and neomycin, 120; for *E. coli*, tetracycline, 10; ampicillin, 100; gentamicin, 10; and kanamycin, 50.

DNA biochemistry. Preparation of plasmid DNA, DNA restriction, agarose gel electrophoresis, cloning, and transformation of *E. coli* cells were carried out according to established protocols (65). Southern hybridizations were performed as described by Kessler (45). Total DNA from rhizobia was isolated as described by Meade et al. (55).

Isolation of the *R. meliloti* Rm2011 *exp* gene cluster. Subfragments from each border of the 9.495-kb *Kpn*I fragment of the *exp* gene cluster isolated previously (44) were cloned into plasmid pK18mob (67). The resulting plasmids were integrated into the genome of *R. meliloti* Rm2011, and total DNA was isolated from the merodiploid strains. Cleavage of total DNA by using enzymes not restricting the integrated plasmid, subsequent ligation, and transformation of *E. coli* XL1-Blue resulted in the isolation of plasmids pARIII and pARIIa (Table 1), carrying fragments of the *exp* gene cluster which overlapped the 9.495-kb *Kpn*I fragment. Plasmids pARIV, pARV, pAB57-1, and pAB58-2 (Table 1), carrying fragments which span a contiguous 19.298-kb region overlapping pARIIa, were isolated accordingly.

DNA sequencing. DNA sequences were obtained either from defined restriction fragments cloned into the sequencing vector pK18, pK19 (62), pUK21, pUC21 (78), pHIP1, or pHIP2 or by using overlapping nested deletion clones generated by exonuclease III digestion as described by Henikoff (37). Sequencing reactions were carried out with an AutoRead sequencing kit (Pharmacia, Uppsala, Sweden), using a protocol devised by Zimmermann et al. (89). Sequence data were obtained and processed by using an A.L.F. DNA Sequencer (Pharmacia) according to the manufacturer's instructions.

TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant properties	Source or reference
R. meliloti		
Rm1021	Wild type, Nx^r Sm^r	F. Ausubel
Rm2011	Wild type, Nx^r Sm^r	14
Rm0540	Rm2011, *exoY0540*::Tn*5*	57
Rm101	Rm2011, Spc^r cassette of pHP45Ω inserted into the *Pmac*I site of *mucR* (*mucR101*-Spc)	This work
Rm3131	Rm2011, *mucR31*::Tn*5*	44
E. coli		
XL1-Blue	*recA1 lac* [F′ *proAB lacI*^{q} ZΔM15 Tn*10* (Tet^r)] *thi*	13
S17-1	*E. coli* 294, *thi* RP4-2-Tc::Mu-Km::Tn*7* integrated into the chromosome	69
Plasmid		
pK18	Sequencing vector, pUC18 derivative, *lacZ*α Km^r	62
pK19	Sequencing vector, pUC19 derivative, *lacZ*α Km^r	62
pUK21	Sequencing vector, *lacZ*α Km^r	78
pUC21	Sequencing vector, *lacZ*α Ap^r	78
pHIP1	Sequencing vector, pK18 derivative, *lacZ*α Gm^r	This work
pHIP2	Sequencing vector, pK19 derivative, *lacZ*α Gm^r	This work
pK18mob	pUC18 derivative, *lacZ*α Km^r, *mob* site	67
pK19mob	pUC19 derivative, *lacZ*α Km^r, *mob* site	67
pAB2001	Promoterless *lacZ*-Gm interposon	9
pHP45Ω	pHP45 carrying an Spc^r cassette	25
pAR1	pK18mob containing a 9.495-kb *Kpn*I fragment of the *exp* gene cluster	44
pARIIa	pUC19 containing a 4.249-kb *Eco*RI-*Bgl*II fragment of the *exp* gene cluster	This work
pARIII	pUC19 containing a 5.250-kb *Bgl*II-*Eco*RI fragment of the *exp* gene cluster	This work
pARIV	pUC19 containing a 4.973-kb *Bgl*II-*Hin*dIII fragment of the *exp* gene cluster	This work
pARV	pUC19 containing a 4.350-kb *Kpn*I fragment of the *exp* gene cluster	This work
pAB57-1	pUC18 containing a 10.879-kb *Hin*dIII-*Kpn*I fragment of the *exp* gene cluster	This work
pAB58-2	pK18 containing a 4.435-kb *Bgl*II-*Kpn*I fragment of the *exp* gene cluster	This work
pIForf24	pK18mob containing an internal 0.320-kb fragment of orf24 (nucleotides 469–788)	This work
pIForf25	pK19mob containing an internal 0.257-kb *Xho*I-*Sst*I fragment of orf25	This work
pKH4081	pK19mob containing a 4.081-kb *Kpn*I-*Hin*dIII fragment of the *exp* gene cluster	This work
pHE842	pK19mob containing a 0.842-kb *Hin*dIII-*Eco*RI fragment of the *exp* gene cluster	This work
p6414	pK18mob containing a 1.203-kb fragment of the *exp* gene cluster (nucleotides 1654–2856)	This work
p6415	pK18mob containing a 0.854-kb fragment of the *exp* gene cluster (nucleotides 1654–2517)	This work

Junctions of transposon Tn*5* insertions were sequenced by using the primer GAGAACACAGATTTAGCCCAG, complementary to the inverted repeats of Tn*5*.

Analysis of nucleotide and protein sequences. The nucleotide and amino acid sequences were analyzed by using the computer programs of Staden (70). Coding probabilities were calculated by the codon usage method (71), using an *R. meliloti* codon usage table as described by Buendia et al. (12). The amino acid sequences deduced from the nucleotide sequence were compared to the GenBank database, using the BLAST algorithm (1). Deduced amino acid sequences were analyzed for hydrophobicity as described by Eisenberg et al. (23) and for the presence of potential signal peptide sequences as described by von Heijne (80), using the PC/Gene software package (release 6.80; IntelliGenetics).

Interposon mutagenesis. Using appropriate restriction sites, a promoterless *lacZ*-Gm interposon (9) was inserted in both orientations into fragments of the *exp* gene region, which subsequently were subcloned into the mobilizable suicide vector pK18mob or pK19mob (67). Resulting hybrid plasmids were transferred from the broad-host-range mobilizing strain *E. coli* S17-1 (69) to *R. meliloti* 2011 as described by Simon (68). Homogenotization of the *lacZ*-Gm insertions was carried out as described by Masepohl et al. (54). All homogenotes were verified by Southern hybridization.

EPS production. Precipitation by cetyl pyrimidinium chloride or ethanol and quantification of EPS were performed as described by Müller et al. (57) after 10 days of cultivation in Vincent minimal medium (79) and morpholinepropanesulfonic acid (MOPS)-buffered medium containing 0.1 mM phosphate (60).

β-Galactosidase assay of *R. meliloti* strains carrying *lacZ*-Gm insertions. *R. meliloti* strains were grown to an optical density (600 nm) of 0.6 to 0.8 in LB medium. β-Galactosidase activity was assayed and relative β-galactosidase units per cell number was calculated as described by Miller (56).

Analysis of transcriptional organization. Fragments of the *exp* gene cluster were subcloned into the mobilizable suicide vectors pK18mob and pK19mob (67). The *exp* genes encoded by the cloned fragments were transcribed opposite the orientation of the *lacZ*α promoter of pK18mob or pK19mob. Hybrid plasmids were transferred to *R. meliloti* 2011 *exp-lacZ*-Gm homogenotes. Integration of hybrid plasmids into the *R. meliloti* genome by single crossover events was selected by the vector-encoded antibiotic resistance. The recombination event between the homologous *exp* sequences of the plasmids and the *exp* gene cluster of the *R. meliloti* genome was verified by Southern hybridization. Transconjugants were assayed for β-galactosidase activities.

Nucleotide sequence. The nucleotide sequence of the *exp* gene cluster was deposited in the GenBank database under accession number Z79692.

RESULTS

Sequence analysis of a 32-kb DNA region carrying the *R. meliloti exp* gene cluster. Previously, a 23-kb DNA region carrying *R. meliloti exp* genes directing the biosynthesis of galactoglucan was isolated and genetically characterized (32). To analyze the corresponding gene cluster from *R. meliloti* 2011 in more detail, overlapping subfragments spanning a contiguous 32-kb DNA region which contained the 23-kb *exp* gene cluster mentioned above were subcloned and sequenced. Open reading frames (ORFs) were identified as described in Materials and Methods, and their locations were correlated with the locations of the *exp* complementation groups previously defined by genetic analysis of Tn*5*-generated *exp* mutations (32).

Twenty-five ORFs were identified on the 32-kb fragment (Fig. 1C; Table 2). Data on an insertional mutagenesis of these ORFs allowing the identification of 22 *exp* genes will be presented below. The orientations and positions of 10 ORFs corresponded to the location of complementation group *expA*. Accordingly, these ORFs were designated *expA1* to *expA10*. The coding regions of *expA5* and *expA6* overlapped by 22 bp,

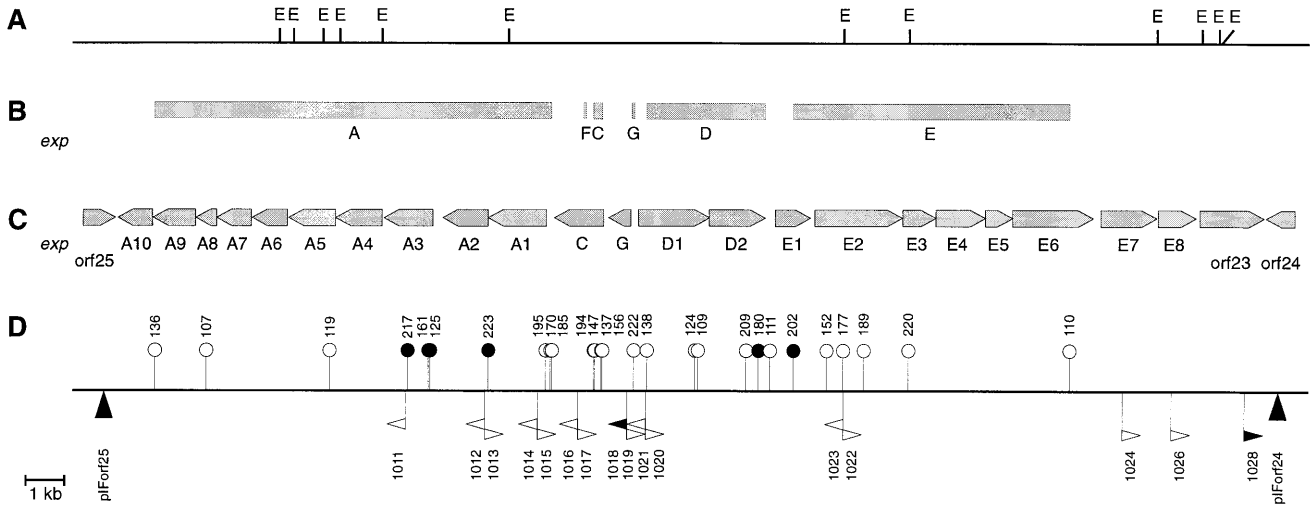

FIG. 1. Genetic organization of the *exp* gene cluster of *R. meliloti*. (A) *Eco*RI (E) restriction map of the *exp* gene cluster. (B) Positions and extents of *exp* complementation groups (32). (C) Positions and extents of ORFs identified by sequence analysis of the 32-kb fragment. (D) Positions of transposon Tn*5* (32), the *lacZ*-Gm interposon, and plasmid insertions. Transposons indicated by open hairpins blocked EPS II production in the *expR101* mutant background, whereas transposons denoted by filled hairpins prevented EPS II production in the *expR101* mutant background and plasmid induced EPS II production in the wild-type background (32). All Tn*5* insertion sites except insertion site 110 were determined by DNA sequencing. Interposon insertions denoted by open flags blocked EPS II production in the *mucR* mutant background, whereas filled flags indicate interposon mutations which did not prevent EPS II production in the *mucR* mutant background. Filled triangles indicate plasmid integration mutations that did not block EPS II production in the *mucR* mutant background.

whereas the coding regions of *expA7* and *expA8* overlapped by 5 bp. The overlap of the *expA7* and *expA8* coding regions might be indicative of a coupled translation of this pair of genes. Two ORFs belonging to complementation groups *expC* and *expG* were found upstream of *expA1*. Complementation group *expD* consists of two ORFs oriented opposite *expC*, and complementation group *expE* is comprised of at least six ORFs designated *expE1* to *expE6*. The start and stop codons of *expD1* and *expD2*, of *expE2* and *expE3*, and of *expE5* and *expE6* overlapped (**ATG**A). In these cases as well, the translation of these pairs of genes might also be coupled. Three further ORFs oriented in the same direction as *expE* were located downstream of Tn*5*

TABLE 2. Positions of genes and features of the predicted gene products encoded by *exp* genes

ORF	Putative ribosome-binding site[a]	Longest potential coding region		Largest deduced gene product	
		Start at nt[b]:	Stop at nt:	Length (amino acids)	Molecular mass (kDa)
orf23	ACTGGAGCGCACGTTTCA**ATG**GAG	2719	1108	535	58.7
expE8	CTCGGGAATTCAC**ATG**CTC	3807	2833	325	36.1
expE7	GACGAAAGACTGTTTTCGGCCG**ATG**GGC	5225	3855	457	50.9
expE6	GCCGGAGC**ATG**AGGAGTACC**ATG**GCC	7394	5364	677	72.5
expE5	AAGGGGAAATCG**ATG**GCT	8095	7394	234	24.8
expE4	GCCGAGGAGGCGGAGC**GTG**AGC**ATG**CGC	9369	8110	420	46.7
expE3	AACGAGGACCGCCGC**ATG**AGC	10211	9369	281	30.8
expE2	GTCAGAGGAGTGGCGT**GTG**ACG	12457	10211	749	82.6
expE1	CACGAAGAGGAGAAGCAG**ATG**GCC	13247	12591	219	22.1
expD2	CGCGAAGGTACCGCATC**ATG**AAC	14943	13525	473	51.5
expD1	ATTAGAAGATCGCCCC**ATG**CGT	16709	14943	589	63.3
expG	ATTGGAAGTACGTTCCCA**ATG**GAGAGAGGG**ATG**AAC	16906	17487	194	21.8
expC	ACTGACAAGTGGCTT**ATG**AAG**ATG**CAT	17628	18878	417	46.0
expA1	TGCAAGAGTTG**ATG**TCT	19065	20534	490	52.9
expA2	AATAAGGAAAACTCGGACCTGATC**GTGGTG**GCA**ATG**	20557	21669	371	41.6
expA3	CCCAGCAGGTG**ATG**ATC	21800	23203	468	51.1
expA4	TTCAAGAAGAAGGTCGATTGC**ATG**CGT	23241	24416	392	43.7
expA5	GCGAGGTGTAATCG**ATG**CGT	24462	25631	390	44.0
expA6	TGTGAGACCAACTTCCGCA**ATG**CTC	25609	26790	394	43.0
expA7	ACCGGAGTGGGCGC**ATG**AAG	26816	27694	293	32.3
expA8	GTCAGGGAGCTCTCGG**ATG**CTT	27690	28250	187	20.8
expA9	CGTGGAGAAACC**ATG**CGA	28265	29332	356	40.2
expA10	CATAGGCGGTCGGGA**ATG**GAG	29406	30230	275	28.9
orf24	CCTGCAAAGCAT**GTG**AAC	336	1034	233	24.7
orf25	AACAGGAGCGACC**ATG**AAG	31097	30282	272	31.3

[a] Putative ribosome-binding sites are underlined, and potential start codons are in boldface.
[b] nt, nucleotides.

TABLE 3. Homologies of the proteins predicted from the *exp* genes

Exp protein	Homologous protein	Degree of homology[a] (% identity/% similarity)	Function of the homologous protein	Organism	Reference(s)
ExpA7	RfbA	63/89	Glucose-1-phosphate thymidylyltransferase[b]	*S. typhimurium*	42
ExpA8	RfbC	49/80	dTDP-4-dehydrorhamnose 3,5-epimerase[b]	*S. typhimurium*	42
ExpA9	RfbB	56/88	dTDP-glucose 4,6-dehydratase[b]	*S. typhimurium*	42
ExpA10	RfbD	42/83	dTDP-4-dehydrorhamnose reductase[b]	*S. typhimurium*	42
ExpA2	ExoO	Partial	Glucosyltransferase[b]	*R. meliloti*	5, 33
ExpE2	ExoO	Partial	Glucosyltransferase[b]	*R. meliloti*	5, 33
ExpA3	RfbF	15/53	Galactosyltransferase[c]	*Serratia marcescens*	75
ExpC	RfpB	18/85	Galactosyltransferase[b]	*Shigella dysenteriae*	35
ExpE4	RfpB	16/58	Galactosyltransferase[b]	*Shigella dysenteriae*	35
ExpE7	RfbF	17/56	Galactosyltransferase[b]	*Klebsiella pneumoniae*	16, 75
ExpD1	PrtD	40/74	ABC transporter protein of a proteinase export complex[b]	*E. chrysanthemi*	48
ExpD2	PrtE	24/70	MFP-like protein of a proteinase export complex[b]	*E. chrysanthemi*	48
ExpE1	NodO	39/63	Secreted Ca^{2+}-binding protein[b]	*Rhizobium* sp. strain BR816	77
ExpE3	GerC2	21/59	Methyltransferase[c]	*H. influenzae*	28
	GerC2	19/59	Protein involved in spore germination and vegetative growth,[b] methyltransferase[c]	*B. subtilis*	84, spP31113 [d]
ORF23	OphA	36/77	Periplasmic binding protein[c]	*A. tumefaciens*	30
ORF24	YHR043c	31/71	2-Deoxyglucose-6-phosphate phosphatase[b]	*Saccharomyces cerevisiae*	43
ExpA4	ExpA5	46/82	Unknown	*R. meliloti*	This work
ExpA5	ExpA4	47/82	Unknown	*R. meliloti*	This work
ExpG	PapX	17/61	Potential transcriptional regulator of P-pilus biosynthesis[b]	*E. coli*	53

[a] Alignments of complete protein sequences were carried out as described by Myers and Miller (59). Percent identities and similarities were calculated in relation to the deduced gene products of the *exp* gene cluster. "Partial" indicates that short regions of the amino acid sequences displayed the characteristic signature of β-glycosyltransferases defined by Saxena et al. (66).
[b] Experimental evidence for the protein function is available.
[c] The protein function was proposed on the basis of homology data.
[d] SwissProt database accession number.

insertion 110 (Fig. 1D), which defined the minimal length of the *expE* complementation group (32). Since operon analysis revealed that the first two ORFs belong to the *expE* transcriptional unit (see below), they were named *expE7* and *expE8*. The third ORF, which does not belong to the *expE* complementation group, was designated orf23. No ORF corresponding to complementation group *expF* was identified. The *exp* gene region was flanked by two ORFs (orf25 and orf24) oriented opposite *expA10* and orf23, respectively.

The predicted molecular weights of the deduced amino acid sequences of the *exp* gene products are listed in Table 2. Results of database searches for homologous proteins and secondary structure analysis for the deduced Exp amino acid sequences are listed in Table 3. Exp proteins without significant homologies were not included in Table 3.

Homologies of deduced amino acid sequences of *exp* genes to proteins of known function. Strong homologies of ExpA7, ExpA8, ExpA9 and ExpA10 were found to proteins involved in the biosynthesis of dTDP-rhamnose of several bacteria (42, 46). The four genes *rfbA*, *rfbB*, *rfbC*, and *rfbD*, encoding enzymes of this biosynthetic pathway in *Salmonella typhimurium* LT2, are organized in an operon (42). In comparison to the gene order of *S. typhimurium* LT2, the order of the *exp* genes homologous to *rfbB* and *rfbC* was interchanged.

ExpA2 and ExpE2 displayed characteristic features of β-glycosyltransferases that use α-linked nucleoside diphosphate (NDP)-sugars as donors for the transfer of the sugar to an acceptor to form a β-linked product (66). Figure 2 compares these features of ExpA2 and ExpE2 to those of five β-glucosyltransferases that are involved in the synthesis of EPS I by *R. meliloti*. The conserved sequence motif is located at the same place in the N-terminal part on the ExpA2 amino acid sequence and on the sequences of the glucosyltransferases (Fig. 2). Whereas the molecular weights of ExpA2 and the glucosyltransferases are similar, ExpE2 is much larger and the conserved sequence motif is located in the C-terminal part (Table 2; Fig. 2). ExpA3, ExpC, ExpE4, and ExpE7 displayed similarities to glycosyltransferases, such as several galactosyltransferases (Table 3), mannosyltransferases (72), an *N*-acetylglucosamine transferase (52), and a glycogen synthase (76), which transfer the sugar from α-linked NDP-sugars to an acceptor, forming an α-glycosidic linkage. The highest degrees of homology over the entire lengths of the protein sequences were found between these four Exp proteins and several galactosyl-

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RmExpE2    IIYVLDSPEIQDETEHLLGGLHLLHGLPMKLVVMNRNSGY--ARACNAG---ARFARGSVVVMLNSDVVPSAPGWLQK  570
RmExpA2    VVVSVDGDPRQETFDQLLLYA--AAHPAV-HVLFGANAGPGGARNRAIDYVLANLPEAEAVYFLDADNRVLPGTIETL  106
RmExoM     RVIVADNDAEPSARALVEGLRPEMPFDILYVHCPHSNIS--IARNCCLD-----NSTGDFLAFLDDDETVSGDWLTRL  110
RmExoA     RVVIADGGSTDGTREIARRLA-TEDPRVLFLDNPKRIQSAAVNRAVAE--  --LGAGSDYLIRIDAHGTYPDDYCERL  113
RmExoO     EVVVVDDCSADATPALVAAI---PDPRVRLIAL-DRNRGPGGARNAGIG-----AARGRWIAVLDSDDTVRPDRLRRM  110
RmExoW     HVLVIDDESPYPIADELAGLA--QEERERITVIRQPNGGPGGARNTGLD---NVPADSDFVAFLDSDDVWTPDHLLNA  107
RmExoU     EVVVIDDGSTDDSASVARAADDGT-GRLN-VVRFEENRGPAAARNHAIA-----ISHSPLIGVLDADDFFFPGRLGQL  108

Consensus  VVV D  S                R          N GP  ARN          A       LD D    PD L  L
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FIG. 2. Comparison of partial sequences of the *R. meliloti* ExpA2 and ExpE2 (RmExpA2 and RmExpE2) proteins and β-glucosyltransferases from *R. meliloti* (5, 6, 33, 64). Amino acid positions conserved in at least four of the protein sequences are in boldface.

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                                                    * *
RmExpE1     FLLGGRGNDVLLGGAGNDVIDGGAGSDLLAGGQGSDIFVF  141
R1NodO      IPHSGEGDDVLYAGPGSDILVAGDGADVLTGGDDGDAFVF  132
EchPrtB     ILIGNGADNILQGGAGDDVLYGSTGADTLTGGAGRDIFVY  385
EchHlyA     VLSGGKGNDKLYGSEGADLLDGGEGNDLLKGGYGNDIYRY 1010
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FIG. 3. Comparison of partial sequences of the *R. meliloti* ExpE1 protein and putative Ca^{2+}-binding proteins. Amino acid positions conserved in at least three of the protein sequences are in boldface. Nonapeptide repeats are marked by arrows; asterisks denote conserved aromatic residues after the last repeat. Abbreviations: RmExpE1, *R. meliloti* ExpE1; R1NodO, *Rhizobium leguminosarum* bv. viciae NodO (22); EchPrtB, *E. chrysanthemi* PrtB (20); EcHlyA, *E. coli* HlyA (26).

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RmExpE3    80 VLDIGCGIGRMA  92 --- 178 EVGRLLKPGG 187
ScErg6    123 VLDVGCGVGGPA 134 --- 209 EIYKVLKPGG 218
EcUbiG     60 VLDVGCGGGILA  71 --- 144 ACAQLVKPGG 153
EcCFA     170 VLDIGCGWGGLA 182 --- 252 VVDRNLKPEG 161
HiGerC2    42 VLEVACNMGTTA  54 --- 130 EYFRVLKPNG 139

Consensus     VLDVGCG G  A              E  R LKPGG
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FIG. 4. Comparison of partial sequences of the *R. meliloti* ExpE3 protein and *S*-adenosyl-L-methionine-utilizing enzymes. Amino acid positions conserved in at least three of the protein sequences are in boldface. Abbreviations: RmExpE3, *R. meliloti* ExpE3; ScErg6, *Saccharomyces cerevisiae* Erg6 (41); EcGyrA, *E. coli* UbiG (40); EcCFA, *E. coli* CFA (82); HiGerC2, *H. influenzae* GerC2 (28).

transferases (Table 3). The hydropathic profile (23) of the ExpC amino acid sequence suggested four putative hydrophobic helices which were not classified as transmembrane helices by the hydrophobic moment blot (23) but might mediate an association of ExpC to the inner membrane (data not shown).

The N-terminal part of the deduced amino acid sequence of ExpD1 showed homologies to the membrane-spanning domain of ABC transporters such as PrtD of *Erwinia chrysanthemi* (48), and the C-terminal part of ExpD1 was homologous to ATP-binding cassette (ABC) domains (24). A potential ATP-binding site consisting of the Walker motifs A and B (81) was identified on the C-terminal part of ExpD1. The hydropathic profile (23) of the N-terminal part of ExpD1 predicted six membrane-spanning α-helices typical for ABC transporters. ExpD2 displayed homologies to accessory factors of these exporter systems, such as PrtE of *E. chrysanthemi* (48). Accessory factors are thought to be anchored in the cytoplasmic membrane and span the periplasm. They have therefore been designated membrane fusion proteins (MFPs) (21). Consistent with the possibility of ExpD2 playing such an MFP-like role, a potential membrane-spanning segment which could anchor the N terminus of ExpD2 to the inner membrane was identified by the algorithm of Eisenberg et al. (23). According to the algorithim of Garnier et al. (31), this putative membrane-spanning segment (residues 55 to 75) was separated from a segment with a high probability to form β-strands (residues 326 to 403) by a region which was predicted to be mainly α-helical (residues 125 to 325) (data not shown). By analogy to other MFP-like proteins, the segment predicted to form β-strands might be associated with the outer membrane (21). These exporter complexes consisting of an ABC transporter and an MFP-like protein in conjunction with an outer membrane protein are involved in the signal peptide-independent secretion of proteins which usually contain a C-terminal secretion signal (21).

ExpE1 was found to be homologous to *Rhizobium* NodO proteins involved in nodulation. These proteins have Ca^{2+}-binding and pore-forming activities (22, 74). ExpE1 contained 15 copies of the nonapeptide repeat (X[I/L]X[A/G]GXGXD) which was proposed to be implicated in the binding of Ca^{2+} (51, 83). These repeats were also found in NodO (12 copies) and other secreted proteins such as hemolysin (15 copies) and several proteases (4 copies) (20, 26, 49). After the last nonapeptide repeat, two highly conserved positions containing the aromatic residue tyrosine or phenylalanine were present in ExpE1 and the related proteins (Fig. 3). The structural characteristics of the C-terminal portion of ExpE1 were similar to those of C-terminal secretion signals (39, 47, 85). This finding suggests that ExpE1 might be secreted by a signal peptide-independent mechanism. Since ExpE1 is located immediately downstream of the *expD1-expD2* operon, the putative ExpD1/ExpD2 export system is an attractive candidate to be involved in the export of ExpE1.

ExpE3 shared 19% identical amino acids with the GerC2 protein of *Bacillus subtilis*, involved in spore germination and vegetative growth (84), and contained the characteristic sequence motifs of *S*-adenosyl-L-methionine-utilizing enzymes (82) (Fig. 4).

The gene product of orf23 was found to be homologous to periplasmic binding proteins involved in the import of metabolites. The ORF23 protein and the DppA dipeptide-binding protein of *Haemophilus influenzae* (27) displayed 26% identical amino acids, whereas the OphA protein of *Agrobacterium tumefaciens* (30) and the ORF23 protein were found to be 36% identical. Analysis by the algorithm of von Heijne (80) identified a putative signal peptide at the N terminus of the ORF23 protein.

Weak homologies were found between ExpG and the PapX protein of *E. coli*, which shared 17% identical amino acids. PapX belongs to the MarR family of transcriptional regulators which bind DNA through a helix-turn-helix motif (17, 73) and was proposed to function as a transcriptional regulator of genes directing the biosynthesis of P pili (53). Apart from similarities over the entire length of ExpG to members of this family, the strongest homologies were found to a conserved region of 35 amino acids which was described as the MarR family signature (18) (Fig. 5). Recently, Astete and Leigh (2) identified in *R. meliloti* the gene *mucS*, which is identical to *expG*. *mucS* was found to be required for the induction of EPSII synthesis by low phosphate concentrations and by extra copies of the *exp* gene cluster (2). The authors also showed that *mucS* is involved in the activation of at least the expression of one gene of the *expE* complementation group by phosphate limitation. It is therefore likely that ExpG (MucS) acts as a transcriptional regulator of EPS II biosynthesis.

No homologies of ExpA4 and ExpA5 were found to proteins of known function, but these two proteins were highly homologous to each other, indicating comparable functions. ExpA6, ExpE5, and ExpE6 also displayed no significant homologies to proteins of known function. A signal peptide was predicted at the N terminus of ExpA6, according to the algorithm of von Heijne (80), suggesting that ExpA6 is exported to the periplasm. The hydropathic profile (23) of the ExpE5 amino acid

```
RmExpG      NISYYLKQLADGDYIDRIASQRDKRSARIRLSEKG 144
EcPapX      NITRITDFLEKAGYIKRTDSREDRRTKKISLTSEG 120
EcMprA      NATRIADELEKRGWIERRESDNDRRCLHLQLTEKG 121
EcMarR      ALTRMLDRLVCKGWVERLPNPNDKRGVLVKLTTGG  85

Signature   AAXSXXXXMVXXSWCXXXXXXXNGKXXXXXXTXXA
            NI G    LI  EYV       DQR         G
            TL N     E  GFI        K
            S  R       LL         R
```

FIG. 5. Comparison of partial sequences of the *R. meliloti* ExpG protein and proteins of the MarR family. Amino acid positions identical to the MarR family signature (18) are in boldface. Abbreviations: RmExpG, *R. meliloti* ExpG; EcPapX, *E. coli* PapX (53); EcMprA, *E. coli* MprA (19); EcMarR, *E. coli* MarR (17).

TABLE 4. Positions of Tn*5* insertions

Tn*5* insertion	Position
136	1,033 bp downstream of the *expA9* start codon
107	228 bp downstream of the *expA8* start codon
119	204 bp downstream of the *expA5* start codon
217	639 bp downstream of the *expA3* start codon
161	104 bp downstream of the *expA3* start codon
125	74 bp downstream of the *expA3* start codon
223	5 bp upstream of the *expA2* start codon
195	29 bp downstream of the *expA1* start codon
170	89 bp upstream of the *expA1* start codon
185	129 bp upstream of the *expA1* start codon
194	297 bp downstream of the *expC* start codon
147	268 bp downstream of the *expC* start codon
137	79 bp downstream of the *expC* start codon
156	37 bp downstream of the *expC* start codon
222	94 bp upstream of the *expG* start codon
138	204 bp downstream of the *expD1* start codon
124	1,435 bp downstream of the *expD1* start codon
109	1,492 bp downstream of the *expD1* start codon
209	923 bp downstream of the *expD2* start codon
180	1,235 bp downstream of the *expD2* start codon
111	151 bp upstream of the *expE1* start codon
202	448 bp downstream of the *expE1* start codon
152	340 bp downstream of the *expE2* start codon
177	766 bp downstream of the *expE2* start codon
189	1,245 bp downstream of the *expE2* start codon
220	149 bp downstream of the *expE3* start codon

sequence predicted a transmembrane α-helix at the C terminus, which might anchor this protein to the cytoplasmic membrane. The hydropathic profile of the ExpE6 protein sequence revealed the presence of five putative membrane-associated α-helices. The hydrophobic moment blot (23) classified the N-terminal helix as transmembrane, indicating that ExpE6 might be located in the inner membrane.

The *exp* genes are organized in five complementation groups. To determine the *exp* genes affected by the Tn*5* mutations and to verify the assignment of the complementation groups described previously (32) to the genes identified, the junctions of 26 representative Tn*5* insertions were sequenced. All of these Tn*5* insertions blocked the biosynthesis of EPS II in the *expR101* mutant background (32). The precise positions of these Tn*5* insertions are shown in Fig. 1D and are listed in Table 4.

These Tn*5* insertions were found to be located in 13 of the 22 *exp* genes and were grouped into five complementation groups, *expA*, *expC*, *expG*, *expD*, and *expE* (Fig. 1). The insertions within the *expA* group disrupted genes corresponding to *expA1*, *expA2*, *expA3*, *expA5*, *expA8*, and *expA9*. The other *exp*::Tn*5* insertions disrupted the coding regions or the potential promoter sequences of *expC*, *expG*, *expD1*, *expD2*, *expE1*, *expE2*, and *expE3*. *exp*::Tn*5* insertion 111, which had apparently defined the *expF* complementation group, was not located between *expA* and *expC* as originally reported (32) but was instead located 151 bp upstream of the *expE1* start codon. It seems mostly likely that Tn*5* insertion 111 was mistakenly mapped to the wrong end of an *Eco*RI restriction fragment and thus incorrectly appeared to define a new complementation group because of the way that the complementation tests were performed (32). In the original genetic analysis, all *exp*::Tn*5* mutations blocked the synthesis of EPS II in a strain carrying the *expR101* mutation (32). In the same study, it was noted that plasmids carrying the *exp* gene cluster also caused increased production of EPS II although not to the same extent as seen in an *expR101* mutant. Interestingly, Tn*5* insertions in only a few *exp* loci blocked this exp^+ plasmid-stimulated EPS II synthesis under these circumstances (32). The Tn*5* insertions we mapped that had this characteristic (217, 161, 125, 233, 180, and 202) were located in the *expA2*, *expA3*, *expD2*, and *expE1* genes.

In addition to the *exp*::Tn*5* mutants described above, defined interposon mutants were constructed in genes *expA1*, *expA2*, *expA3*, *expC*, *expG*, *expE2*, *expE7*, *expE8*, and orf23 (Fig. 1D) by insertion of a *lacZ*-Gm interposon (9). Insertions in *expA3*, *expE7*, *expE8*, and orf23 were oriented in the sense direction, whereas in all other cases the *lacZ*-Gm interposons were inserted in the sense and antisense orientations. Insertions in the antisense orientation resulted in polar mutations, whereas insertions in the sense orientation were nonpolar due to the constitutive promoter in front of the gentamicin resistance gene of the interposon, which directs transcription of the *exp* genes located downstream. The phenotypes of the *exp-lacZ*-Gm mutants are indicated in Fig. 1D. All interposon insertions except mutations *expG*#1018 and orf23#1028 prevented EPS production in the background of the *mucR101*-Spc *exoY0540*::Tn*5* double mutant, which otherwise produces EPSII as the sole EPS.

The coding regions of orf24 and orf25 were mutated by the integration of plasmids pIForf24 and pIForf25, carrying internal fragments of orf24 and orf25, respectively (Fig. 1D). In the background of the *mucR101*-Spc *exoY0540*::Tn*5* double mutant, disruption of orf24 or orf25 by the integrated vector did not block the production of EPS.

***expE7* and *expE8* belong to the *expE* transcriptional unit.** Tn*5* insertion 110 defined only the minimal length of the *expE* complementation group. Since three ORFs were located downstream of this Tn*5* insertion site in the direction of the first six *expE* genes, we used *exp-lacZ* transcriptional fusions in conjunction with plasmid integration mutagenesis as described previously (5) to determine whether these ORFs belong to the *expE* operon as well.

exp-lacZ transcriptional fusions were created in *expE7*, *expE8*, and orf23 by insertion of the *lacZ*-Gm interposon in the sense orientation. These mutants were subjected to plasmid integration mutagenesis in the *mucR101*-Spc mutant background. Plasmid pKH4081, carrying a 1,707-bp DNA segment upstream of the *expE1* start codon, the *expE1* gene, and the incomplete *expE2* coding region, was integrated into the genomes of the *mucR101*-Spc *expE7*#1024-*lacZ*, *mucR101*-Spc *expE8*#1026-*lacZ*, and *mucR101*-Spc *orf23*#1028-*lacZ* strains (Fig. 6). The β-galactosidase activities of the resulting strains did not differ significantly from the activities of the recipients. In contrast, integration of plasmid pHE842, carrying parts of the *expE2* and *expE3* coding regions, blocked transcription of the *expE7-lacZ* and *expE8-lacZ* fusions (Fig. 6), indicating that *expE7* and *expE8* are exclusively transcribed from a promoter upstream of *expE1*.

Plasmid p6414 was integrated into the genome of the *mucR101*-Spc mutant carrying the orf23-*lacZ* fusion. In the resulting mutant, only 144 bp were located downstream of the integrated vector upstream of the orf23 start codon. The β-galactosidase activity of this plasmid integration mutant did not differ significantly from the activity of the recipient (Fig. 6), indicating that a promoter is located on the 144-bp fragment. Integration of plasmid p6415 carrying an internal fragment of orf23 resulted in a β-galactosidase activity of the plasmid integration mutant that was comparable to the β-galactosidase activity of the *mucR101*-Spc recipient.

These observations indicate that *expE7* and *expE8* are transcribed from a promoter upstream of *expE1* and therefore

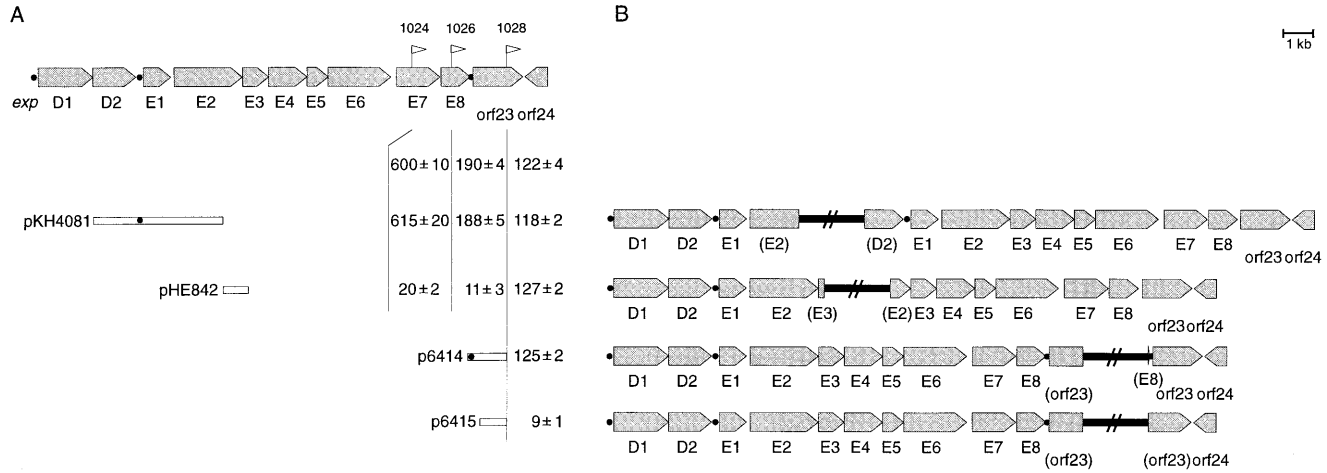

FIG. 6. Assignment of *expE7* and *expE8* to the *expE* transcriptional unit. (A) Gene structure and plasmids used for integration mutagenesis of the *expE* complementation group. Insertion sites of *lacZ*-Gm interposons are indicated by flags; inserts of plasmids used for integration mutagenesis are indicated by bars; potential promoters are denoted by dots. The relative β-galactosidase activities of the *mucR101*-Spc *expE7-lacZ*-Gm, *mucR101*-Spc *expE8-lacZ*-Gm, and *mucR101*-Spc orf23-*lacZ*-Gm double mutants before plasmid integration mutagenesis are given. The background activity of the *R. meliloti mucR101*-Spc mutant was 8 ± 2. The relative β-galactosidase activities determined after plasmid integration mutagenesis are listed; the values are averages of at least five independent assays. (B) Genomic structures resulting from plasmid integration mutagenesis. Heavy lines denote the vector part of the plasmids integrated. Incomplete *exp* genes are shown in parentheses.

belong to the *expE* transcriptional unit. An additional promoter directs the transcription of orf23.

Transcription of the *exp* genes is negatively regulated by MucR. The *exp* gene cluster consists of five complementation groups. We previously reported that the transcription of two *expA-lacZ* fusions (*expA3#*1011 and *expA2#*1012) was induced by the *mucR31*::Tn*5* mutation (44). To determine whether the transcription of the other five *exp* complementation groups is also affected by a *mucR* mutation, the β-galactosidase activities of the *lacZ* transcriptional fusions shown in Fig. 1D were determined in the wild-type background and in the background of the *mucR31*::Tn*5* or *mucR101*-Spc mutation (Table 5). All *exp-lacZ* transcriptional fusions in the sense orientation were found to be expressed at a substantially elevated level in the *mucR* mutant background. Transcription of the orf23-*lacZ* fusion was not stimulated in the *mucR* mutant background.

DISCUSSION

Sequence analysis of the *R. meliloti exp* gene cluster, directing the biosynthesis of EPS II in association with interposon mutagenesis, revealed that the 23-kb *exp* gene cluster previously characterized by transposon mutagenesis (32) contains another 7 kb and comprises at least 22 *exp* genes. These *exp* genes were flanked by orf23, orf24, and orf25. Since mutations in these genes did not prevent EPS II biosynthesis and since the transcription of orf23 was not stimulated in the *mucR* mutant background, these genes might not be involved in EPSII biosynthesis and might mark the termini of the *exp* gene cluster. Six *exp* complementation groups were previously identified by transposon mutagenesis and complementation analysis (32). Mapping of these Tn*5* mutations allowed the assignment of all *exp* genes to five of the six complementation groups. Since Tn*5* insertion 111 was mapped to the region between *expD2* and *expE1*, no *exp* gene corresponded to complementation group *expF*. An analysis of the operon structure of three *exp* genes located on the additional 7-kb *exp* region identified in this study revealed that two of these genes belong to and extend the *expE* transcriptional unit.

Based on homologies or similarities of the deduced amino acid sequences to database sequences, potential functions were proposed for 17 gene products. The Exp proteins might be involved in the biosynthesis of nucleotide sugar precursors, the polymerization of sugars, the export of EPS II, and the regulation of *exp* gene expression. The sequence and homology data presented as well as the analysis of the operon structure of the *exp* gene cluster from a basis for the construction of defined mutations in individual *exp* genes and for biochemical experiments to assign functions to the *exp* gene products.

An unexpected finding was that the ExpA7, ExpA8, ExpA9, and ExpA10 proteins were homologous to enzymes of the dTDP-rhamnose biosynthetic pathway (42, 46). Four enzymes homologous to these four *exp* gene products are known to be

TABLE 5. β-Galactosidase activities expressed by *exp-lacZ* transcriptional fusions in *mucR*$^+$ and *mucR* backgrounds

Fusion	β-Galactosidase activity (Miller units)[a]	
	Wild type	*mucR31*::Tn*5* or *mucR101*-Spc
Sense orientation		
*expA3#*1011-*lacZ*	6 ± 1	102 ± 9
*expA2#*1012-*lacZ*	6 ± 2	158 ± 3
*expA1#*1014-*lacZ*	21 ± 1	277 ± 2
*expC#*1016-*lacZ*	15 ± 1	55 ± 4
*expG#*1018-*lacZ*	8 ± 0	49 ± 4
*expD1#*1020-*lacZ*	10 ± 0	202 ± 5
*expE2#*1022-*lacZ*	14 ± 0	441 ± 10
*expE7#*1024-*lacZ*	20 ± 3	600 ± 10
*expE8#*1026-*lacZ*	9 ± 3	190 ± 4
orf23#1028-*lacZ*	125 ± 10	122 ± 4
Antisense orientation		
*expA2#*1013-*lacZ*	5 ± 1	5 ± 1
*expA1#*1015-*lacZ*	9 ± 2	13 ± 1
*expC#*1017-*lacZ*	7 ± 2	12 ± 3
*expG#*1019-*lacZ*	9 ± 3	7 ± 2
*expD1#*1021-*lacZ*	13 ± 2	10 ± 2
*expE2#*1023-*lacZ*	8 ± 3	7 ± 2

[a] Average of at least five independent assays. The background β-galactosidase activities of the wild-type strain Rm2011 and the *mucR* mutants were 3 ± 1 and 8 ± 2 Miller units, respectively.

required for the conversion of glucose-1-phosphate and dTTP to dTDP-rhamnose (42, 46). The sequence homologies strongly suggest that ExpA8, ExpA9, and ExpA10 are involved in the conversion of dTDP-glucose to dTDP-rhamnose or a related NDP-sugar and that the synthesis of EPS II was blocked in *expA8* and *expA9* mutants. This effect might be due to the disruption of one of these two genes or to a polar effect of the transposon insertions on the following *expA10* gene. This observation demonstrates that at least one of the three gene products (ExpA8, ExpA9, or ExpA10) that are likely to be involved in the synthesis of this NDP-sugar is required for EPSII production. Only glucose and galactose were identified in EPSII isolated from culture supernatants (38). Therefore, EPSII might contain only traces of this yet unknown NDP-sugar, or this NDP-sugar is involved in EPS II biosynthesis but is not contained in the final EPS II polymer. It might be required for the synthesis of a primer to start a new EPS II chain or as a donor of a sugar that might be added to the terminus of the EPSII polymer to stop chain extension. Such a role in the initiation of a growing polysaccharide chain was proposed for 3-deoxy-D-manno-octulosonic acid in the synthesis of the group II capsular polysaccharides in *E. coli* K5 (27). A similar finding was reported for the *rfb* gene cluster of *Yersinia enterocolitica*, involved in O-antigen biosynthesis (88). Although this O antigen does not contain rhamnose, four genes encoding proteins homologous to enzymes of the dTDP-rhamnose biosynthetic pathway were identified in this gene cluster.

We identified six putative *exp* gene products whose homologies to known glycosyltransferases suggest that they too might function as glycosyltransferases. Two of these (ExpA2 and ExpE2) might form β-glycosidic linkages, whereas the four other putative glycosyltransferases (ExpA3, ExpC, ExpE4, and ExpE7) might form α-glycosidic bonds. EPS II contains glucose and galactose joined by β-1,3 and α-1,3 linkages. Therefore, at least two glycosyltransferases should be required for EPS II biosynthesis. Nonpolar mutations in *expA2* and *expE2*, encoding proteins homologous to β-glycosyltransferases, and in *expA3*, *expC*, and *expE7*, coding for proteins homologous to α-glycosyltransferases, prevented EPS II production. This finding indicates that at least the five putative glycosyltransferases ExpA2, ExpE2, ExpA3, ExpC, and ExpE7 fulfill essential functions in EPS II biosynthesis.

The results of the initial genetic study of the *exp* gene region (32), together with the results described here, indicate that the *expA3*, *expD2*, and *expE2* genes must be present on the cosmid carrying an extra copy of the *exp* gene cluster to stimulate EPSII synthesis. A simple interpretation of this finding is that the putative glycosyltransferases encoded by the *expA3* and *expE2* genes are required in larger amounts than the products of many of the other *exp* genes. Although the mutation in *expA2* caused a similar phenotype, such an explanation cannot be proposed for this gene, since a mutation in *expA2* might have a polar effect on *expA3*.

ExpE1 displayed homologies to *Rhizobium* NodO proteins. The *R. leguminosarum* bv. viciae NodO protein was shown to have pore-forming activity in lipid bilayers (74). This finding suggests that the homologous ExpE1 protein might be involved in the export of EPS II. As for NodO, the C-terminal part of ExpE1 also displayed similarities to proteins secreted by a signal peptide-independent secretory pathway (22). Homology data suggest that ExpD1 and ExpD2, whose genes are located just upstream of *expE1*, represent components of a complex involved in such a signal peptide-independent secretion of proteins containing a C-terminal secretion signal. ExpE1 could therefore be secreted by a protein complex containing ExpD1 and ExpD2.

ExpG, which had limited local homologies with proteins of the MarR family of transcriptional regulators (18), was found to be identical to MucS (2). The *mucS* gene was required for the activation of expression of at least one gene of the *expE* complementation group by low phosphate concentrations (2). This finding indicates that ExpG may function as a transcriptional activator of *exp* gene expression. Genes *expG* and *expC* (which is located just downstream of *expG*) were shown to belong to different complementation groups, and the phenotype of the *expG*::Tn*5* *expR101* double mutant demonstrated that this gene is essential for EPS II biosynthesis (32). This is different from the phenotype of the nonpolar *expG*#1018 interposon mutant, which produced EPS II in a *mucR* mutant background. Since a constitutive promoter of the interposon directed transcription of *expC* in the nonpolar *expG* interposon mutant, these different phenotypes might be explained if ExpG is required to activate the expression of *expC* in the *mucR* mutant and the constitutive promoter supplemented for the function of the *expG* gene product. On the other hand, it is possible that MucR acts downstream of ExpG in a regulatory cascade; thus, the effect of a mutation in *expG* might be overcome by the *mucR* mutation. We therefore cannot exclude the possibility that the *mucR* and *expR* mutant backgrounds have different effects on the production of EPS II by *expG* mutants.

No homologies suggesting that certain *exp* gene products are involved in the decoration of EPS II were found, although it cannot be excluded that Exp proteins sharing no homologies to database sequences are required for the acetylation and pyruvylation of EPS II.

In *R. meliloti*, production of EPS II is switched on by the *expR101* or a *mucR* mutation (32, 44). Previously, it was reported that the transcription of *expA-lacZ*, *expD-lacZ*, and *expE-lacZ* fusions was induced by the *expR101* mutation (32) and that the transcription of *expA-lacZ* fusions was induced in a *mucR* mutant background (44). The induction of *exp-lacZ* transcriptional fusions situated in the five *exp* complementation groups in a *mucR* mutant background demonstrated that the regulation of *exp* gene expression in relation to the *mucR* gene product takes place at the transcriptional level and encompasses the five *exp* operons of the 32-kb gene cluster.

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