JOURNAL OF BACTERIOLOGY, Jan. 2004, p. 535–542 Vol. 186, No. 2
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.2.535–542.2004
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An Evolutionary Hot Spot: the pNGR234*b* Replicon of *Rhizobium* sp. Strain NGR234

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Received 22 July 2003/Accepted 8 October 2003

***Rhizobium* sp. strain NGR234 has an exceptionally broad host range and is able to nodulate more than 112 genera of legumes. Since the overall organization of the NGR234 genome is strikingly similar to that of the narrow-host-range symbiont *Rhizobium meliloti* strain 1021 (also known as *Sinorhizobium meliloti*), the obvious question is why are the spectra of hosts so different? Study of the early symbiotic genes of both bacteria (carried by the SymA plasmids) did not provide obvious answers. Yet, both rhizobia also possess second megaplasmids that bear, among many other genes, those that are involved in the synthesis of extracellular polysaccharides (EPSs). EPSs are involved in fine-tuning symbiotic interactions and thus may help answer the broad- versus narrow-host-range question. Accordingly, we sequenced two fragments (total, 594 kb) that encode 575 open reading frames (ORFs). Comparisons revealed 19 conserved gene clusters with high similarity to *R. meliloti*, suggesting that a minimum of 28% (158 ORFs) of the genetic information may have been acquired from a common ancestor. The largest conserved cluster carried the *exo* and *exs* genes and contained 31 ORFs. In addition, nine highly conserved regions with high similarity to *Agrobacterium tumefaciens* C58, *Bradyrhizobium japonicum* USDA110, and *Mesorhizobium loti* strain MAFF303099, as well as two conserved clusters that are highly homologous to similar regions in the plant pathogen *Erwinia carotovora*, were identified. Altogether, these findings suggest that ≥40% of the pNGR234*b* genes are not strain specific and were probably acquired from a wide variety of other microbes. The presence of 26 ORFs coding for transposases and site-specific integrases supports this contention. Surprisingly, several genes involved in the degradation of aromatic carbon sources and genes coding for a type IV pilus were also found.**

The *Proteobacteria* contain many bacterial species that associate with plants either as symbionts or pathogens. Complete sequencing of the genomes of several of these microbes, including *Agrobacterium tumefaciens* (20, 49), *Bradyrhizobium japonicum* (28), *Mesorhizobium loti* (27), and *Rhizobium* (*Sinorhizobium*) *meliloti* (18), has made comparative genome analyses possible. *Rhizobium* sp. strain NGR234 belongs to the α-proteobacteria and is able to establish nitrogen-fixing symbiosis with many different legumes. Despite extensive study, the molecular mechanisms behind this broad host range are not fully apparent (6). Although *R. meliloti* has a very limited host range (5), it is phylogenetically close to NGR234 and the organization of both genomes is similar (14a, 31a). In both cases, the genome comprises three replicons (14a). Most symbiotic genes are carried on SymA plasmids of 0.54 Mb in strain NGR234 (17) and 1.35 Mb in *R. meliloti* (1). Both bacteria also possess a second group of plasmids, the so-called exo- or megaplasmids (pSymB). pNGR234*b* is estimated to be 2.2 Mb (31a), and the size of *R. meliloti* is 1.68 Mb (14). Chromosomes in both bacteria are about the same size (3.5 Mb in NGR234, 3.34 Mb in *R. meliloti*) (7, 31a). Also snapshot sequencing suggested that housekeeping and many metabolic genes are similar (48). Sequencing data also suggested that NGR234 differs in its gene content from *R. meliloti*, however.

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We sequenced two large contigs of pNGR234*b*, one of which contains loci involved in extracellular polysaccharide (EPS) synthesis and, thus, in fine-tuning symbiosis. Altogether 575 open reading frames (ORFs) were identified, of which 222 appear to be organized into clusters of more than four genes. Comparative analyses indicated that NGR234 may have acquired large parts of the genetic content of pNGR234*b* from other soil- and plant-associated microbes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* was grown at 37°C on Luria-Bertani medium (42) supplemented with appropriate antibiotics, and *Rhizobium* sp. strain NGR234 (46) was grown on TY medium (0.5% tryptone, 0.25% yeast extract, and 10 mM $CaCl_2$ [pH 7.0]).

Manipulation of DNA and construction of an ordered cosmid library. On the basis of hybridization and sequence data, a minimal set of cosmids of the canonical ordered library of the NGR234 genome (37a) was selected for further analysis. Selected cosmids were partially digested with *Sau*3A. Fragments of 0.5 to 3.5 kb were isolated after electrophoretic separation on agarose gels, cloned into pTZ19R (Amersham, Essex, United Kingdom), and sequenced with standard primers. Sequencing was performed by using dye terminator technology on a model 377 sequencer (Applied Biosystems, Foster City, Calif.) or on capillary sequencers from Amersham. The GC-Phrap software package (http://www.jgi.doe.gov/Docs/JGI_Seq_Quality.html#_SeqQ.I) was used to assemble the sequences. Editing and finishing was facilitated by the Staden software package (http://www.mrc-lmb.cam.ac.uk/pubseq/staden_home.html). Sequencing of PCR-generated fragments was used to close single- and double-stranded gaps. ORFs were initially identified by the programs Glimmer (http://www.tigr.org/software/glimmer/) and GeneMarkS (4). The cutoff limit for ORFs without database homologues was 150 bp. Predicted ORFs and intergenic regions were used to interrogate nonredundant protein databases with Blast programs via the website http://www.ncbi.nlm.nih.gov/blast. ORFs were entered into the ERGO

Integrated Genomics, Inc. (Chicago, Ill.) bioinformatics suite for genome annotation and metabolic reconstruction. The predicted ORFs were subjected to two initial rounds of annotation (one automatic and one manual). Proteins were categorized with a modified Riley classification (40). Analysis of the sequenced region resulted in the identification of 575 ORFs, which were arbitrary assigned identification numbers with the specific prefix *ngr*. The names of ORFs of pNGR234*a* that are also found on pNGR234*b* are followed by a superscript b; thus, the cysteine synthase *cysM* (y4xP) has a homologue *y4xP*^{b} on pNGR234*b* and so on.

Nucleotide sequence accession numbers. The nucleotide sequences were deposited in GenBank under accession numbers AY316746 and AY316747.

RESULTS AND DISCUSSION

General. In total, 357,655 bp of contig1 and 234,455 bp of contig2 were sequenced, and 575 ORFs were identified (354 on contig1 and 221 on contig2). At 61.8 mol%, the average G+C content for both contigs is similar to that found for the entire genome (5a) but is also significantly higher than the value of 58.5 mol% calculated for pNGR234a (17). The gene density on both contigs was almost identical at one gene per 1.03 kb. Possible functions were assigned to 403 ORFs, which were grouped into eleven categories (Fig. 1 and Table 1).

Transporters and genes involved in protection responses. Altogether, 79 ORFs were classified as encoding transporters or proteins related to transport processes, a majority of which are ABC-type transporters. Surprisingly, a relatively high number of ORFs (11) were linked to the transport of spermidine or putrescine, possible osmoprotectants. Other predicted solute transporters include those for iron, amino acids, peptides (and oligopeptides), rhizopines, taurine, and other small molecules. This density of transporters is similar to that found on the *R. meliloti* pSymB (14, 18), but pNGR234*b* carries at least one component of a phosphotransferase system (*ngr563*) (18).

Other interesting features included 14 ORFs that play roles in protection responses. Among these are three genes encoding proteins involved in resistance to acriflavine (*ngr226*, *ngr288*, and *ngr289*), genes involved in detoxification of other small molecules (e.g., *ngr065*, *ngr334*, two copies of the multidrug resistance protein B), CopC (copper resistance protein C), and Ngr174, a macrolide glycosyltransferase. *ngr153* encodes a homoserine lactone efflux protein, which belongs to the family of the RhtB proteins, that can confer resistance to elevated levels of exogenous L-threonine, L-homoserine, and analogues (53). Homologues of this protein are also found in *A. tumefaciens* and *B. japonicum* as well as several other gram-negative bacteria (e.g., *Salmonella enterica* serovar Typhi, *E. coli* O157:H7 Sakai, and *Brucella melitensis*) but not in *R. meliloti*.

Catabolic functions. A significant number of genes encoding proteins that could be involved in oxidative metabolism were identified (Table 1). Among them are dehydrogenases, oxidoreductases, and dehydratases. Several predicted sugar kinase genes were also found. A number of ORFs were identified that encode proteins involved in the degradation of complex or aromatic carbon sources, including a protocatechuate 3,4,-dioxygenase (*ngr051*), an opine oxidase (*ngr333* and *ngr334*, α- and β-subunit), and a hydroxyquinol 1,2-dioxygenase (*ngr391*). Other proteins possibly involved in the degradation of complex carbon compounds available to NGR234 include four myoinositol 2-dehydrogenases (*ngr233*, *ngr250*, *ngr251*, and *ngr252*) and two other proteins linked to the degradation of myoinositol (IolD and IolE). Also, one ORF encodes an octopine dehydrogenase subunit B (*ngr446*), two ORFs encode agmatinases (*ngr257* and *ngr540*), and another encodes a metapyrocatechase (*ngr571*). The latter is involved in the degradation of naphthalene in *Bacillus*, *Pseudomonas*, and *Rhodococcus* via the metacleavage pathway (29, 33). A homologue of the trihydroxytoluene oxygenase, which is involved in the catabolism of 2,4-dinitrotoluene, is encoded by ORF *ngr570*, and a protein involved in nitrilotriacetate catabolism (*ngr138*) (50) was found. Both *ngr570* and *ngr138* are part of a conserved cluster in *B. japonicum*, several *Brucella* species, *Burkholderia pseudomallei*, and *Sphingomonas aromaticivorans*. Obviously, pNGR234*b* is important in the catabolism of a remarkably wide spectrum of carbon and energy sources, including loci that are involved in the degradation of aromatic compounds (i.e., *ngr570* and *ngr571*) but are not found in the *R. meliloti* genome.

Regulatory elements. Another 58 ORFs encode possible regulatory proteins: two for possible polymerase sigma factors (*sigJ* and *sigB*), with the rest mostly belonging to the LysR, GntR, and TetR families. Two ORFs (*ngr159* and *ngr160*) encode possible homologues of the two-component regulators NodV-NodW or NwsA-NwsB (Fig. 1. The *nodVW* genes of *B. japonicum* are involved in activation of *nod* gene expression in response to plant-produced isoflavones (20a, 30).

Chaperones and cofactor biosynthesis. Two copies of the heat shock proteins GroES and GroEL, as well as several other ORFs encoding small heat shock proteins, two of which belong to the Hsp20 family (*ngr309* and *ngr311*), were found (Fig. 1 and Table 1). All are required for rapid adaptation to heat stress (34). Although their transcription is commonly activated from sigma-70 promoters, it is also negatively regulated by *cis*-acting elements (ROSE [repression of heat-shock gene expression]) (32, 35). Two possible ROSE elements upstream of the *groES* genes are indicated in Fig. 1.

Other genes encode proteins involved in the biosynthesis of cofactors and vitamins. Examples include proteins involved in the biosynthesis of pyrroloquinone (PqqA to -E), pyridoxal phosphate (PdxA), a riboflavin-specific deaminase (*ngr151*), and the thiamine biosynthesis protein ThiD. Despite their well-known roles as classical cofactors, several of these compounds might be involved in promoting colonization of roots (44, 51). Two proteins involved in the biosynthesis of amino acids (AroC, the shikimate 5-dehydrogenase, and the possible cysteine synthase y4xP^{b} [CysM] homologue) were identified; both, however, may have chromosomal homologues. Nevertheless, the observation that pNGR234*b* encodes pathways involved in cofactor and amino acid biosynthesis, indicates an essential role in cellular processes. The suggestion that pNGR234*b* might be essential was further supported by the discovery of a gene involved in the biosynthesis of the 30S ribosomal protein S21 A (RbsU). Interestingly, in both NGR234 and *R. meliloti*, the *rbsU* gene is close to the cold shock gene *cspA*. In *R. meliloti*, however, the corresponding homologues of *rbsU* and *cspA* are found on the bacterial chromosome and are transcribed as one operon (7, 36). To further verify that the pNGR234*b* is essential for NGR234 cell processes and metabolism, further tests in which the pNGR234*b* is cured from the host strain are necessary, as well as confirming that other genes essential for cell division are present on this replicon (41).

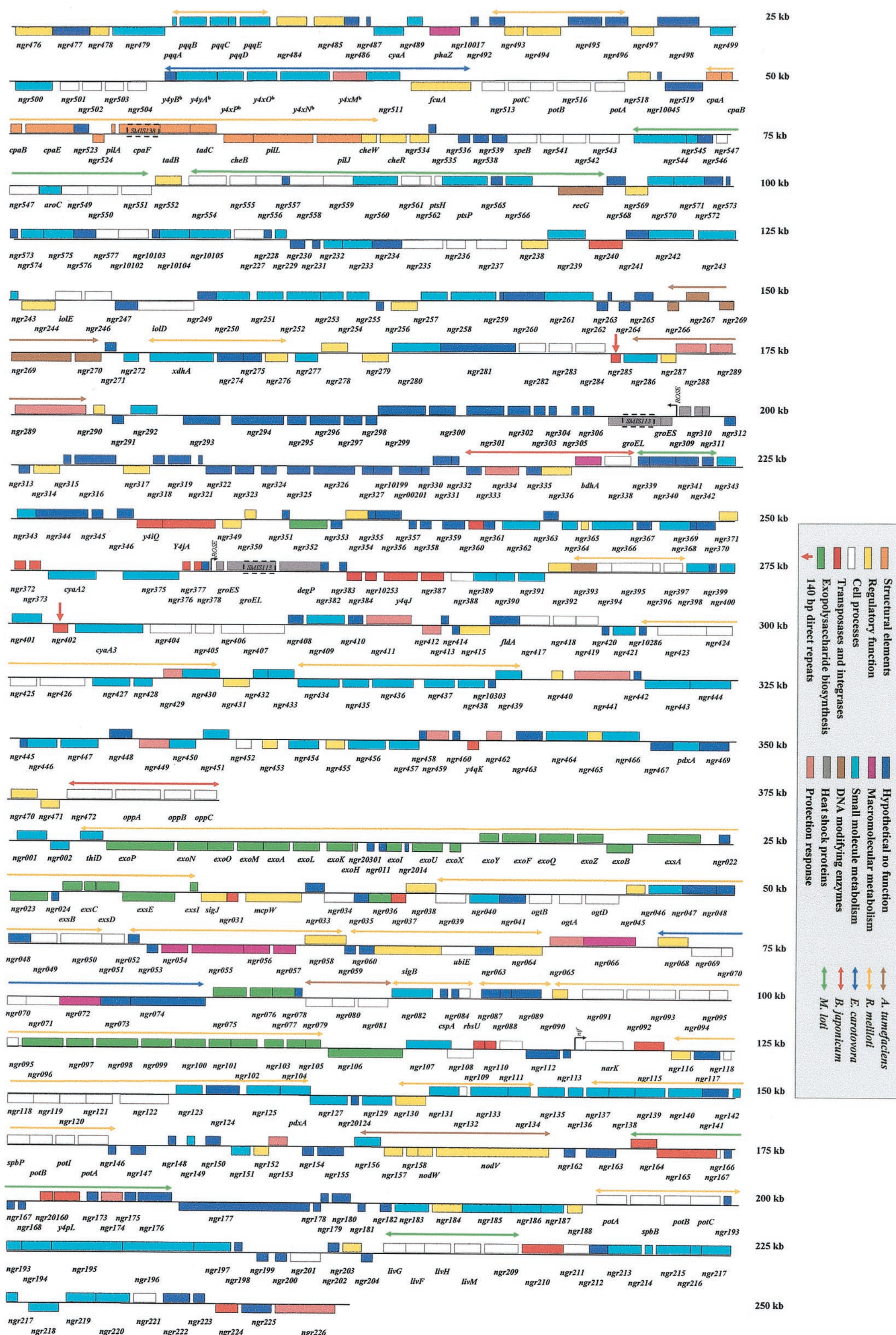


FIG. 1. Physical organization of the ORFs of pNGR234*b*. Coordinates are given in kilobases. Putative genes and ORFs are colored (grey-boxed area) according to putative functions. ORFs were named genes when BLAST-P searches of the National Center for Biotechnology Information database indicated an identity of <E^{-80}. Several insertion sequence elements are indicated as regions boxed with dotted lines. Angled arrows indicate the locations of possible sigma-54-dependent promoters as well as the location of two possible ROSE elements. Conserved clusters of genes are shown as double-headed and horizontal arrows. Conserved clusters (≥3 ORFs) present in identical or highly similar orders in other bacteria are indicated. Colors show the highest similarity to the bacterium indicated in the boxed area. Only the microbe that showed the highest similarity and most conserved gene order over the entire range of the cluster is indicated.

TABLE 1. Genes and ORFs identified on the sequenced contigs of pNGR234*b*[a]

Functional class	No. of ORFs identified
Regulators	58
Cellular processes	106
Transporters	79
Others	27
Small-molecule metabolism	129
Macromolecule metabolism	8
DNA modification	6
Heat shock	7
Transposases and integrases	26
Protection responses	14
Exopolysaccharide synthesis and modification	38
Structural genes	11
Hypothetical	172
No function	128
No similarity and no function	44
Total	575

[a] Functions were assigned by using the ERGO software and the corresponding genome database. Data were obtained by DNA sequence analysis of 594 kb of pNGR234*b* (estimated total size, ≈2.2 Mb).

Macromolecular metabolism. When an imbalance between carbon and nitrogen, phosphorus, or biotin occurs, many rhizobia sequester the excess carbon as polyhydroxybutyrate (13, 25, 45). Proteins involved in the degradation of polyhydroxybutyrate, BdhA (poly-3-hydroxybutyrate-dehydrogenase) and PhaZ (poly-3-hydroxybutyrate-depolymerase), were found. Degradation is initiated by the action of a polyhydroxybutyrate depolymerase that releases the monomer 3-hydroxybutyrate. A possible endoglucanase (*ngr054*) and two putative cellulose synthases (*ngr055* and *ngr066*) are encoded on pNGR234*b*. Several putative proteins involved in cell wall biosynthesis as well as the degradation of polygalacturonate (*ngr072*) were discovered. The latter belongs to the family of 28 glycosyl hydrolases (23) that cleave 1,4-α-D-galactosiduronic linkages in pectate and other galacturonans. Pectinases and polygalacturonases are part of the armory of plant pathogens, including *Erwinia chrysanthemi*, where the expression of pectinases is directly linked to pathogenicity (24, 26). No polygalacturonases have been reported in the genome of *R. meliloti*, whereas *A. tumefaciens* (ORF ATU4560) and *B. japonicum* USDA110 (2a) possess putative polygalacturonases. The identification of polygalacturonases in NGR234 is thus intriguing and may suggest a role during the infection process.

Transposases and integrases. Twenty-six ORFs encoding integrases and transposases were found. Their presence is probably linked to the high frequency of DNA rearrangements found in soil bacteria (21, 22). Short repeats (the largest is 140 bp) (positions are indicated in Fig. 1) are interspersed between the integrases and transposases. Interestingly, the G+C content of the DNA fragment framed by both repeats has a significantly lower G+C content (60.4%) than the remaining part of the contig1 (61.7%). These data suggest that lateral transfer of genetic material has occurred.

ORFs without assigned functions. Finally, 128 ORFs were identified for which similarities were observed but functions could not be assigned, and 44 ORFs had no known homologues in the databases.

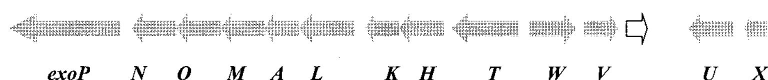
Comparative analyses of loci found in other plant-associated species. Possible horizontal transfer of all the identified ORFs was examined by comparing pNGR234*b* with the genomes of other plant-associated microbes. All potential operons and gene clusters (≥3 ORFs), were compared with the genes of pNGR234*a* (17) and the complete genomes of the following members of the *Rhizobiaceae*: *A. tumefaciens* (20, 49), *B. japonicum* USDA110 (28), *M. loti* MAFF303099 (27), and *R. meliloti 1021* (18). Available information on the plant pathogen *Erwinia carotovora* (http://www.sanger.ac.uk/Projects/E_carotovora/) was also included in the analysis.

Initial analyses indicate that at least 176 ORFs are part of paralogous clusters and 291 ORFs are part of 62 chromosomal clusters. Use of the ERGO suite to examine a number of these loci showed that 29 conserved gene clusters (comprising 222 ORFs) have similar or identical gene orders to those found in one or several plant-associated microbes (Fig. 1). Of these, 158 ORFs were identified in clusters or operons that are highly similar to clusters or operons of pSymB of *R. meliloti*.

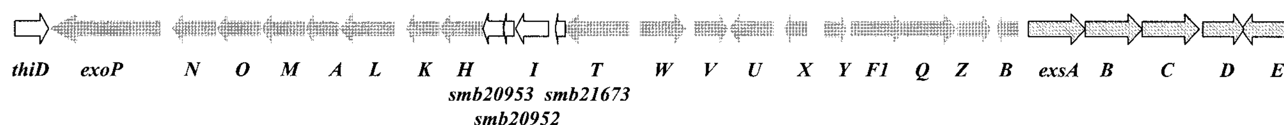
Analysis of the *exo-exs* cluster. Obvious structural similarities were seen among the *exo* and *exs* genes of pNGR234*b* and p*Rm*SymB. This cluster contains 31 ORFs stretching from the *thiD* gene to the *exsI* gene (Fig. 1 and 2). DNA identities of about 80% extend across the cluster, and the orientation of the genes is the same. *exo* and *exs* genes are involved in the synthesis of low-molecular-weight EPSs, which are essential for nodule invasion (2, 37). Thus, Exo mutants of NGR234 are ineffective on the host plant *Leucaena leucocephala* (8). Profiling with restriction enzymes and comparison with previously sequenced *exoX* and *exoY* genes (GenBank accession number X16704) showed that the core of this cluster has been previously mapped (9). In addition, the pNGR234*b exo* cluster is similar to comparable loci in *A. tumefaciens* C58 and *M. loti* MAFF303099. The *exoPNOMAL* fragment is present in all four species (Fig. 2). The most striking differences between NGR234 and *R. meliloti* were found on both sides of the conserved *exoI* region. ORFs corresponding to *exoH* and the genes *exoTWV* were not found in the sequenced regions of pNGR234*b*, suggesting that two deletions occurred (Fig. 2). This possibility is supported by the identification of a 37-bp *exoH* fragment of NGR234 (upstream of *exoK*), which forms the left border of the deleted *exoH* region.

In *R. meliloti*, the ExoH protein is a succinyl transferase that is required to produce succinoglycan. It seems likely that a common rhizobial ancestor produced succinoglycans, as *R. meliloti* does, whereas NGR234 evolved symbiotically active EPSs that lack succinyl groups. The repeating subunit of the NGR234 polysaccharide is a nonasaccharide that consists of a main chain [Gal-$(Glu)_5$] and a side chain [$(GlcA)_2$-Gal] decorated by an acetyl and a pyruvyl group on the terminal galactose (11). Based on the known function of the *exo* genes of *R. meliloti* (39), the *exo* cluster of pNGR234*b* is probably involved in the synthesis of the main chain [Gal-$(Glu)_5$]. ExoF and ExoY are required for the first step of synthesis of the oligosaccharide subunit (transfer of galactose to a lipid carrier), and ExoA, ExoL, ExoM, ExoO, and ExoU are glycosyl transferases which subsequently add glucose units, forming the hexasaccharide [Gal-$(Glu)_5$]. In *R. meliloti*, elongation of the oligosaccha-

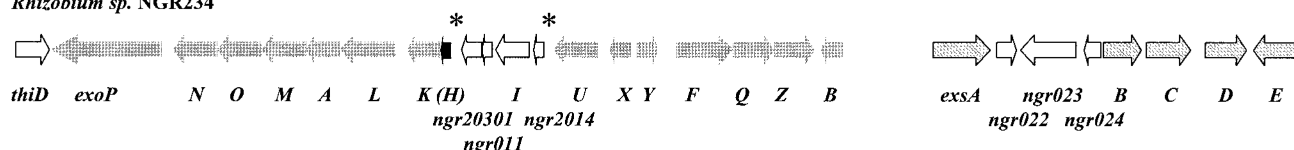
***A. tumefaciens* C58**



***Rhizobium meliloti* 1021**



***Rhizobium sp.* NGR234**



***M. loti* MAFF303099**

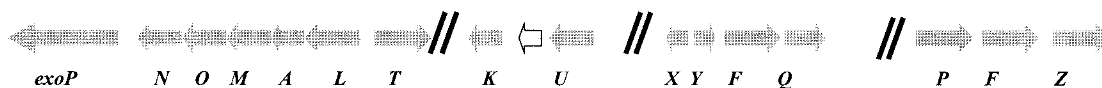


FIG. 2. Physical organization of the *exo-exs* cluster of *Rhizobium* sp. strain NGR234 compared to that of *A. tumefaciens* strain C58, *M. loti* strain MAFF303099, and *R. meliloti* strain 1021. Asterisks mark deletions of *exoH* and *exoTWV* (see text).

ride chain is continued by the glucosyl transferase ExoW. Based on the results obtained in *R. meliloti*, one can assume that in the absence of *exoW*, NGR234 is unable to link an additional glucose unit and completes the synthesis of its subunit by using two glucuronyl transferases and a galactosyl transferase, enzymes that are outside the sequenced regions.

ExoV modifies the terminal glucose of the *R. meliloti* succinoglycan subunit with a pyruvyl group. Although the NGR234 *exo* gene cluster lacks *exoV*, the nonreducing galactose of the subunit is also pyruvylated (11), suggesting that a nonidentified pyruvyl transferase must exist in the NGR234 genome. Interestingly, NGR234 also harbors the acetyltransferase *exoZ*. ExoZ of *R. meliloti* acts on the trisaccharide Gal-$(Glu)_2$ (39), whereas the subunit of NGR234 is acetylated at the nonreducing galactose of the side chain [$(GlcA)_2$-Gal] and at sites that have not been determined (10, 11). Thus, it is possible that the NGR234 ExoZ gained the ability to acetylate the third sugar of the side chain, thereby conserving the specificity for trisaccharides.

Once the succinoglycan subunits of *R. meliloti* are synthesized, they are polymerized and exported by ExoP, ExoQ, and ExoT (19). The pNGR234*b exo* cluster lacks *exoT*. This finding suggests that *exoT* is not required for acidic EPS synthesis in NG234 or that a functional *exoT* exists at another position in the genome. The symbiotically active succinoglycan of *R. meliloti* consists of low-molecular-weight succinoglycan, which is released from the polymer by the extracellular glycanases ExoK and ExsH (52). We identified a sequence encoding a putative ExoK glycanase, but *exsH* was not found. A promoter is located 211 bp upstream of the *exoK* start codon of *R. meliloti* (3). The corresponding sequence in NGR234 is located within the mutated region upstream of *exoK*. It is possible, therefore, that the promoter of *exoK* is not functional in NGR234. Low extracellular glycanase activity would explain why NGR234 produces relatively low amounts of low-molecular-weight EPS (10).

In summary, the organization of the *exo* cluster suggests that acquisition and deletion of genetic information has extensively shaped pNGR234*b*. It is tempting to speculate that the *exoKHTWV* is the original sequence of succinoglycan-producing members of the *Rhizobiaceae*. In *A. tumefaciens*, this arrangement has been maintained. Then a common ancestor of *R. meliloti* and NGR234 acquired a 2-kbp fragment containing *ngr20301* (also known as *smb20953*), *ngr011* (also known as *smb20952*), *exoI*, and *ngr2014* (also known as *smb21673*). These imported DNA sequences seem not to be involved in the synthesis of EPS. Finally, two deletions (perhaps first *exoTWV* and later the now useless *exoH*) resulted in the organization of pNG234*b* shown in Fig. 2.

Genes encoding type IV pili. Among other interesting loci identified was a cluster encoding a type IV pilus. Type IV pili are unique structures on the bacterial surface that are found in many gram-negative bacteria (Fig. 3A), where they play an important role in bacterial adhesion to host cells, biofilm formation, conjugative DNA transfer, motility, and infection by bacteriophages (12). Pili are secreted through the inner and outer membranes. In *Caulobacter crescentus*, at least seven

FIG. 3. (A) Physical organization and comparative analysis of the type IV pilus cluster of *Rhizobium* sp. strain NGR234. Same colors indicate similar predicted functions of the depicted ORF. No coloring indicates that no link to the pili biosynthesis cluster or to the larger gene cluster was observed. ORFs *rsm4251* and *rsm4262* encode transposases. (B) Physical organization and comparative analysis of the *y4yB-y4xM* clusters of *Rhizobium* sp. strain NGR234. Same colors indicate similar predicted functions of the depicted ORF.

genes are required for pilus assembly, including *pilA*, *cpaA*, *cpaB*, *cpaC*, *cpaD*, *cpaE*, and *cpaF*.

Use of the ERGO suite highlighted clusters of similarly organized genes encoding type IV pili in plant-associated bacteria including *A. tumefaciens*, *B. japonicum*, *M. loti*, and *R. meliloti* (Fig. 3A). Surprisingly, *B. japonicum* and *R. meliloti* both carry two copies of most of the genes necessary for pili biosynthesis. One copy in *R. meliloti* is located on pSymA while the second is on the chromosome. Comprising at least 16 ORFs, the pSymA copy is strikingly similar to the copy identified on pNGR234*b* (Fig. 3A). Interestingly, *cpaB* and *cpaC* are apparently separated in both organisms on the copies found on the megaplasmids. A putative *cpaC* homologue was identified directly upstream of the *pilA* gene in NGR234 (ORF *ngr524*) and upstream of the *cpaA* gene on pRmSymA (ORF *rsm1839*). Nevertheless, both putative *cpaC* homologues encoded much shorter proteins than the *cpaC* copy on the *R. meliloti* chromosome, but a similar ORF was observed in several of the other rhizobial clusters (Fig. 3A). CpaC belongs to the PulD/pIV family of proteins, which are commonly referred to as secretins. CpaC might be involved in outer membrane channel formation and pilus secretion (43). A function has yet to be assigned to the *cpaD* gene product.

Two clusters with high similarity to loci of *E. carotovora*. Two other clusters are highly homologous to loci of the plant pathogen *E. carotovora*. A contig2 cluster stretching from ORF *ngr068* to *ngr074* encodes genes probably involved in modification and/or degradation of plant cell walls. A second cluster

on contig1 includes the ORFs *y4yB*[b] (*ngr505*) to *fcuA*, which encode genes important in iron transport and subsequent metabolism. Seven of the ORFs, *y4yB*[b] to *ngr511*, are found on the same strand with overlapping stop and start codons, suggesting they could be transcribed as an operon. *fcuA* is located approximately 300 bp downstream of *ngr511* and on the opposite strand (Fig. 3B). *y4yA*[b] may encode a decarboxylase, whereas y4xP[b] (CysM) has homology to cysteine synthases. Both enzyme families require pyridoxal phosphate as a cofactor, and one of the genes (*pdxA*) required for the synthesis of this cofactor are located on the same contig. y4xO[b] shows weak homology to octopine dehydrogenases, y4xN[b] belongs to the IucA-IucC family of siderophore biosynthetic enzymes, *y4xM*[b] encodes a possible permease and possesses 11 predicted transmembrane domains, *ngr511* has homology to iron(III) dicitrate binding proteins, the *fcuA* gene codes for a possible ferric siderophore receptor, and y4yB[b] is homologous to many hypothetical bacterial proteins of unknown function.

Interestingly, several of the ORFs within this cluster (*y4yB*[b] to *y4xM*[b]) are duplicated (85% identity and in the same order) on pNGR234*a* (Fig. 3B). Upstream of *y4yB* on pNGR234*a* is a 40-bp repeat that is highly similar to *y4yB*[b] on pNGR234*b*. Downstream of the pNGR234*a* *y4xM* ORF there is no sign of *ngr511* nor any indication of sequences originating from pNGR234*b*. Interestingly, the duplicated ORFs on pNGR234*a* are found within a region containing the genes encoding a functional type III protein secretion system. Furthermore, they lie between *nopX* and *nopL*, two genes that encode proteins secreted by this system (47). It is possible that *y4yB* to *y4xM* could be coregulated with *nopX*, as there are no obvious transcriptional termination signals in the 185-bp (*nopX* to *y4yB*) intergenic region. Transcriptional analysis of pNGR234*a* showed that *y4yB* to *y4xM* are all strongly induced 24 h after flavonoid addition, indicating possible symbiotic functions (38) (it should be cautioned that some of the transcripts could have come from the other duplicated genes, however). Since *y4yB* to *y4xM* are within the type III secretion system cluster, a polar mutation in *y4yB* was generated, but it did not have any obvious symbiotic phenotype or effect on protein secretion (R. Dieckmann, C. Marie, X. Perret, W. J. Broughton, and W. J. Deakin, unpublished data). Obviously, the discovery of second copies of *y4yB* to *y4xM* suggests that a double mutant should now be created to answer this question.

Both *B. japonicum* USDA110 and *M. loti* MAFF303099 possess type III secretion systems, yet neither has homologues of *y4yB* to *y4xM*, perhaps suggesting that this locus is not essential for protein secretion. Several of the ORFs have homology to proteins involved in iron transport, including a siderophore receptor, implying that they may monitor the iron status of the environment. It is noteworthy that iron often limits bacterial virulence and, particularly, type III protein secretion. The role of iron in virulence of *Erwinia* has been well documented (15, 16), although homologues of the *y4yB* cluster have not been implicated. Type III protein secretion by the plant pathogen *Ralstonia solanacearum* is controlled by an outer membrane receptor (PrhA) with homology siderophore receptors (31). *R. solanacearum* also contains homologues of many of the genes in the *y4yB* cluster. Several genes of the *y4yB-y4xM* cluster are also found in other plant pathogens as well as the human pathogen *Staphylococcus epidermidis* ATTC 14990.

In summary, these observations suggest that 40% of the genes and operons identified on pNGR234*b* are not strain specific and may have been acquired from other related bacteria. This would explain why NGR234 is so successful in nodulating different legumes; symbiotic competence arises from a flexible genome that is able to efficiently integrate foreign DNA from other bacteria.

ACKNOWLEDGMENTS

We thank G. Gottschalk and G. C. Walker for many helpful comments on the manuscript and D. Gerber for general support.

Research in LBMPS is financed by the Fonds National Suisse de la Recherche Scientifique (project 31-63893.00) and the Université de Genève. Research in Göttingen was funded by the Genomik Network of the BMBF and the FCI.

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