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Characterization of the *rfaD* Gene Region of *Bradyrhizobium japonicum* 61A101C

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Abstract In our previous studies, we have cloned and characterized a gene region from *Bradyrhizobium japonicum*, which is involved in the synthesis of lipopolysaccharide (LPS). In this study, we have expanded the sequence analysis of the region and found an additional open reading frame (orf), which appeared to be divergently transcribed from the *rfaF* gene. Sequence alignment of the orf revealed a significant similarity with *rfaD* genes of *Salmonella typhimurium*, *Escherichia coli*, and *Neisseria gonorrhoeae*. These genes encode a heptose-6-epimerase, which catalyzes the interconversion of ADP-D-glycerol-D-manno-heptose to ADP-L-glycero-D-manno-heptose. This divergent organization of the *rfaF* and *rfaD* genes is different from that of other Gram-negative bacteria where two genes form an operon. A *rfaD*$^-$ mutant of *E. coli* was successfully transformed with plasmid constructs containing the *rfaD* gene of *B. japonicum*. Novobiocin sensitivity test showed that the *rfaD* gene from *B. japonicum* could complement the *rfaD* mutation in *E. coli*, which confirms the functionality of the cloned *B. japonicum* gene.

Key words: *Bradyrhizobium japonicum*, lipopolysaccharide (LPS), *rfaD*, complementation, novobiocin

B. japonicum is a Gram-negative soil bacterium infecting soybean. Interaction between soybean and *B. japonicum* results in the formation of nodules, in which differentiated *B. japonicum* can actually fix the atmospheric nitrogen into ammonia. Since the nodulation process requires an intimate cell-to-cell interaction, it is evident that cell surface structures such as LPS play important roles [2, 5, 12, 17]. LPS, a major component of the outer membrane of Gram-negative bacteria, is composed of three domains: lipid A; the core which is an oligosaccharide consisting of an inner and outer region; and a distal repeating unit known as O-antigenic chain [8]. The inner core is composed of at least two ketodeoxyoctonate (KDO) units followed by two of heptose units. Several genes for synthesis of the heptose region have been identified in *E. coli* and *S. typhimurium* [1, 4, 7] as well as in *B. japonicum* [9, 16].

In the previous study, we have isolated and characterized a gene region from *B. japonicum* that is involved in LPS synthesis [15]. Sequence analysis of the region has identified the *rfaF* gene encoding heposyltransferase [16]. Since the previously sequenced region showed a partial orf running opposite of the *rfaF*, we decided to expand the sequencing of the region. The dideoxy chain termination method of sequencing was performed at 37°C by using the enzyme Sequenase, version 2.0, that was purchased from the United States Biochemical Corp (Clevelend, OH, U.S.A.). The deoxy- and dideoxynucleotides were purchased from Pharmacia (Piscataway, NJ, U.S.A.), and the radioactive nucleotide [α-^{35}S] dCTP was purchased from Amercham Corp. (Arlington Heights, IL, U.S.A.). In addition, some difficult regions were sequenced at 65°C with a modified Silver sequence™ of DNA sequencing system (Promega, U.S.A.). Computer analysis of the DNA sequence and the predicted amino acid sequence was accomplished through the use of the PC/GENE™. Sequence comparison was accomplished through the internet with database that was provided by National Center for Biotechnological Information, NIH (Bethesda, MD, U.S.A.). Sequence analysis revealed a complete orf having a strong homology with *rfaD* genes of *E. coli*, *S. typhimurium*, and *N. gonorrhoeae* (Fig. 1). The GC content of the orf (984 bp) was 65% and appeared to encode a protein of 326 amino acids having a molecular weight of 41,618 dalton. The *rfaD* gene of *E. coli* encodes ADP-L-glycero-D-manno-heptose-6-epimerase, which converts ADP-D-glycero-D-manno-heptose to ADP-L-glycero-D-manno-heptose [1, 4, 11, 14]. As shown in Fig. 1, all the epimerases have well-conserved cofactor binding finger sequences at their amino terminal region [1, 6, 7, 10, 14]. It was interesting to note that the putative *rfaD* gene of

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B  MFL-VTGGAGFIGSNVVAALNDAGHSDVAVCDVLGQDGKWRNLAKRQLAD----------VVPPAELGDW 59
S  •II•••••••••••I•K••••K•IT•IL•V•N•KDGT•FV••VDLNI••YMDKEDFLIQIMSGE•••••- 68
E  •II•••••••••••I•K••••K•IT•IL•V•N•KDGT•FV••VDLNI••YMDKEDFLIQIMAGE•F••- 68
N  •T•-••••••••••I•K••••R•IT•IVAV•N•TKGE•FK••AECEIAHYLDKHEFIRQBREHILPYQN 70

B  LKGRRLTAVIHLGAISETTATDGDLVIETNFRLSMRLLDWCTANATPFIYASSAATYGDGERGFDDDASV 129
S  -----IE•IF•E••C•S••EW••KYMMDN•YQY•KE••HY•LEREI••L•••••••••GRTSD•-----I 128
E  -----VE•IF•E••C•S••EW••KYMMDN•YQY•KE••HY•LEREI••L•••••••••GRTSD•-----I 128
N  -----IE••F•Q••C•D•MNH••LYMM•N•YQYTLD•••••QDERI••L••••••V••K••I--REEREL 134

B  EALKRLRPMNLYGWSKNLFDLAVAE---RVAKGERLPPQWAGLKFFNVFGPNEYHKGPMMSVLARRFDDI 196
S  •SREYEK•L•V••Y••F•••EY•RQILPEAN------S•IV•FRY•••Y••R•G•••S•A••AFHLNTQL 192
E  •SREYEK•L•V••Y••F•••EY•RQILPEAN------S•IV•FRY•••Y••R•G•••S•A••AFHLNTQL 192
N  •-----K•L•V••Y••F•••QVLRRRMKEGLT-----A•VV•FRY•••Y•QH•Q•••R•A••AFHH•HQY 194

B  KAGRVVQLFKSHREGIADGDQRRDFIYVDDVVRVMMWLIATPRVSGLFNVGTGKARSFRDLIVSAYGA-- 264
S  NN•ESPK••EGSE------NFK•••V••G••AA•NL•FLESGK-••I••L•••R•E••Q-AVAD•TL•-- 252
E  NN•ESPK••EGSE------NFK•••V••G••AD•NL•FLENGV-••I••L•••R•E••Q-AVAD•TL•-- 252
N  REHGY•N••G•N-D•YGN•E•T•••VS•E••AKINLYFFDHPEL••IY•L•••RSQQ•NELAAATVN•CR 263

B  ------------LGLEPNIQYVDLPEQIRGSYQYFTQSAVDRLGRAGYNGGFTRL-EDAVGAYVTGFLDR 321
S  ------------YHKKGS•E•IPF•DKLD•R••A•••ADLTN•RN•••DKP•KTVA•G-•TE•M      303
E  ------------YHKKGQ•E•IPF•DKLD•R••A•••ADLTN•RA•••DKP•KTVA•G-•TE•M      303
N  AAEGKSELSLKELVE•EL•R•IPF•DALK•K••G•••ADITK•RE•••KEE•FDVKAG--NR••KWM•E 331

B  ADRYR 326
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Fig. 1. Multiple sequence alignment of *rfaD* of *B. japonicum* (B), *S. typhimurium* (S), *E. coli* (E), and *N. gonorrhoeae* (N).
Individual sequence identity with B as indicated by dots under the B sequence was found to be 55% for S; 55% for E; 54% for N. Sequences with similarity were highlighted by dots above the B sequence. For maximum sequence alignment, dashed lines were used for blank amino acids. Boxed sequences indicate the cofactor binding finger domain of various epimerases [1, 6, 14].

B. japonicum was running opposite of the *rfaF* gene (Fig. 2). This divergent organization of *rfaF* and *rfaD* genes in *B. japonicum* was different from that of other Gram-negative bacteria where the two genes form an operon and they are cotranscribed [7, 14]. The nucleotide sequence of the putative *rfaD* gene of *B. japonicum* has been submitted to GenBank under the accession number of AF217196.

In order to confirm the functionality of the orf, we performed a series of complementation experiments with a

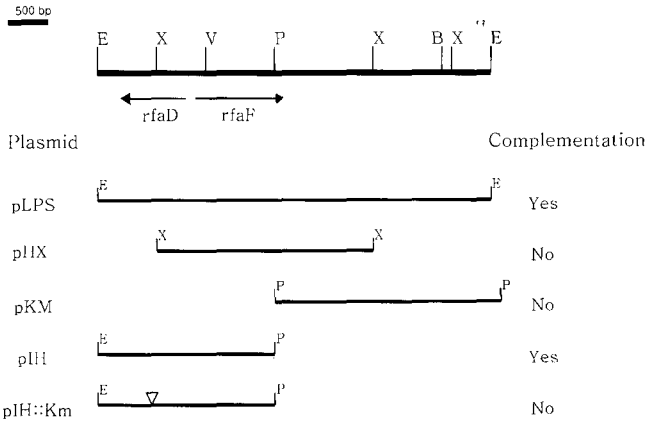

Fig. 2. Genetic organization of the 5.5 kb LPS gene region and the complementation of *rfaD*⁻ mutant strain of *E. coli* CL89 with various subclones.
Arrows indicate the divergent orientation of *rfaF* and *rfaD*. The open arrowhead in pIH::Km indicates the insert of the Km^r cassette. Abbreviations: E, *Eco*RI; X, *Xho*I; V, *Eco*RV; P, *Pst*I; B, *Bam*HI; S, *Sal*I; NT, not tested.

rfaD⁻ mutant strain of *E. coli*. It is well known that *E. coli* and *S. typhimurium rfaD*⁻ mutants are hypersensitive to hydrophobic agents such as novobiocin [1, 11, 14]. Based on the sensitivity level of the *rfaD*⁻ mutants to a hydrophobic antibiotic, novobiocin, we were able to show the functionality aspect of the orf. As shown in Fig. 2, various DNA fragments containing the orf were subcloned by using combinations of several restriction enzymes prior to being transformed into a *rfaD*⁻ mutant strain of *E. coli*, CL89. *E. coli rfaD*⁻ mutant strain CL89 was obtained from the *E. coli* genetic stock center at Yale University. For subcloning and transformation, standard procedures were employed [13]. In addition, the smallest fragment containing the *rfaD* gene (pIH) was inactivated by inserting the kanamycin resistance (Km^r) cassette to construct the pIH::Km, as previously described [3]. Briefly, 1.3 kb Km^r cassette flanked by duplicate multiple cloning sites was digested with *Kpn*I and ligated with pIH which had been linearized by *Kpn*I digestion. Insertion of the Km^r cassette was confirmed by comparing the amplification products obtained by polymerase chain reactions (PCRs) of two plasmids, pIH and pIH::Km, using the primer sets designed from the *rfaD* internal sequence. Resulting PCR products were size fractionated by an agarose gel electophoresis to find the size difference of 1.3 kb. Compared with the *rfaD*⁻ mutant, the complemented strain completely regained the resistance level to novobiocin (Fig. 3). In the presence of novobiocin (100 μg/ml), the *rfaD*⁻ mutant strain that was transformed with pIH (the smallest subclone containing the orf) grew as successfully as the wild-type strain, while the mutants transformed with pGEM-3zf(+) vector DNA and other subclones or pIH::Km did not. Further studies will be conducted to determine the overall organization and regulation of *rfa* gene clusters in *B. japonicum*.

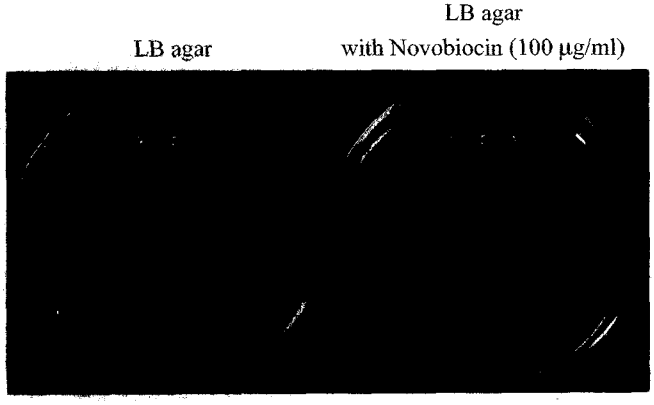

Fig. 3. Complementation of the *E. coli rfaD*⁻ mutant with the *B. japonicum rfaD* gene.
Lane 1, JM109; lane 2, CL89 (the *rfaD*⁻ mutant); lane 3, CL89/pGEM-3zf(+); lane 4, CL89/pIH::Km; lane 5, CL89/pIH. According to the direction of arrowhead, individual cultures were diluted 10-fold prior to being spotted onto the plates.

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