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Identification and Mutational Analysis of *rfbG,* the Gene Encoding CDP-D-Glucose-4,6-Dehydratase, Isolated from Free Living Soil Bacterium *Azotobacter vinelandii*

Narasaiah Gavini,[1] Bryan S. Hausman, Lakshmidevi Pulakat, Ryan P. Schreiner, and Jeffrey A. Williamson
Department of Biological Sciences, Bowling Green State University, Bowling Green, Ohio 43403

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We have identified the *rfbG* from a non-symbiotic and non-pathogenic soil bacterium, *Azotobacter vinelandii.* The nucleotide sequence analysis of the *rfbG* revealed an open reading frame that encodes a peptide of 360 amino acids. This deduced peptide shares 57% homology with the RfbG of *Synechocystis* and 47% homology with the RfbG of *Yersinia pseudotuberculosis.* The previously identified short-chain dehydrogenases/reductases family signature sequence is conserved in the sequence of the RfbG of *A. vinelandii.* Southern blotting analysis of *A. vinelandii* chromosome by probed with 1.1kb *Pst*I DNA fragment corresponding to *rfbG* revealed that it is present as single copy on *A. vinelandii* chromosome. Disrupting the *rfbG* present on the chromosome of *A. vinelandii,* by insertion of kanamycin resistance marker via homologous recombination, resulted in drastic changes in the growth characteristics. The *rfbG*-negative *A. vinelandii* grown in liquid medium exhibited agglutination that is characteristic of *rfb*$^-$ mutants of other bacteria, suggesting that we have cloned the functional copy of the *rfbG* of *A. vinelandii.*

Azotobacter vinelandii is an aerobic, non-symbiotic, gram-negative soil bacterium that belongs to the family *Azotobacteriaceae* and is widely known for its ability to fix nitrogen aerobically (1-3). It was also speculated that this bacterium contains multiple chromosomes per cell with unique biology of cell maintenance and growth (4, 5). To date there are neither reports suggesting that *A. vinelandii* associates with eukaryotic organisms in a symbiotic or parasitic manner nor studies to suggest that it carries functional *rfb* genes needed for O-antigen biosynthesis.

[1] To whom correspondence should be addressed. Fax: 419 372 2024. E-mail: ngavini@bgnet.bgsu.edu.

The O-antigen is an integral part of the lipopolysaccharide (LPS) -a major constituent of the outer membrane in many gram-negative bacteria (6, 7). LPS is a complex molecule which consists of an O-specific polysaccharide chain attached to lipid A through the core polysaccharide. The O-specific polysaccharide chain is a polymer of oligosaccharide repeat units which extends outside the surface of the cell and is responsible for the infectious abilities many enteric bacteria exhibit in host organisms (8). The structural variations in this region of the LPS result in serological heterogeneity of O-antigens. The presence of this region of the cell surface may enhance virulence and may increase the resistance of bacterial cells to complement-mediated killing. The sugar residues commonly found within the LPS are unique and require specific genes for synthesis and assembly. The genes required for the biosynthesis of the O-antigen-specific polysaccharides, designated *rfb,* have been identified in common pathogenic bacterial genera of Enterobacteriaceae, such as *Salmonella, Shigella,* and *Yersinia* (9-16)

The *rfb* genes are normally found in clusters in most organisms studied (6). In *E. coli* and *Salmonella* the *rfb* locus is mapped on the linkage map at 44 and 42 minutes, respectively (17, 18). The role of various *rfb* genes in the synthesis of 6-Deoxy and 3,6-Dideoxyhexose biosynthesis had been studied extensively (6). The genes *rfb*BDAC are involved in dTDP-L-rhamnose biosynthesis and the genes *rfb*IFGHJ are involved in CDP-abequose biosynthesis. Mutations in *rfb* genes often result in the inability of the organism to produce O-antigens. For example, in *E. coli* K12 which lacks an O- antigen, it was found that its *rfb* genes carry specific mutations. In the mutant *rfb*50, the mutation was located in the *rfb* cluster as an IS5 insertion (19, 20). Whereas in the mutant *rfb*51, the mutation was a deletion of TDP-rhamnose synthesis genes (19, 20). In summary, *rfb* genes play a critical role in determining the ability of an organism to produce an O-antigen and the

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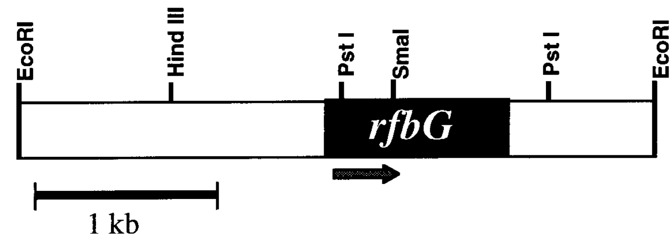
a



b

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                                            10                                          20
 M   E   T   M   V   T   S   V   R   H   A   A   S   V   N   F   W   Q   G   K
ATG GAA ACA ATG GTG ACA TCC GTG CGC CAT GCC GCC AGT GTC AAC TTT TGG CAA GGG AAG
TAC CTT TGT TAC CAC TGT AGG CAC GCG GTA CGG CGG TCA CAG TTG AAA ACC GTT CCC TTC

                                            30                                          40
 R   V   L   L   T   G   H   T   G   F   K   G   G   W   L   A   L   W   L   Q
CGG GTT CTG CTG ACC GGC CAC ACC GGC TTT AAG GGC GGT TGG CTG GCA TTG TGG CTG CAG
GCC CAA GAC GAC TGG CCG GTG TGG CCG AAA TTC CCG CCA ACC GAC CGT AAC ACC GAC GTC

                                            50                                          60
 R   L   G   A   V   V   T   G   I   A   L   P   P   Q   T   R   P   N   L   F
AGG CTG GGA GCG GTG GTA ACG GGA ATC GCT TTG CCC CCC CAG ACT AGG CCA AAC CTG TTC
TCC GAC CCT CGC CAC CAT TGC CCT TAG CGA AAC GGG GGG GTC TGA TCC GGT TTG GAC AAG

                                            70                                          80
 E   L   A   R   V   A   D   G   M   H   S   H   F   A   D   I   R   D   A   A
GAG TTG GCC CGA GTT GCC GAT GGT ATG CAC AGT CAT TTC GCC GAT ATT CGC GAT GCA GCA
CTC AAC CGG GCT CAA CGG CTA CCA TAC GTG TCA GTA AAG CGG CTA TAA GCG CTA CGT CGT

                                            90                                         100
 K   L   M   E   I   V   Q   A   A   Q   P   E   V   V   F   H   L   A   A   Q
AAA CTT ATG GAA ATA GTG CAA GCC GCG CAG CCA GAA GTG GTG TTC CAT CTA GCG GCC CAA
TTT GAA TAC CTT TAT CAC GTT CGG CGC GTC GGT CTT CAC CAC AAG GTA GAT CGC CGG GTT

                                           110                                         120
 P   L   V   R   A   S   Y   R   E   P   V   A   T   F   A   S   N   V   M   G
CCG CTG GTA CGT GCA AGC TAC CGC GAG CCG GTA GCC ACC TTT GCC TCT AAT GTG ATG GGC
GGC GAC CAT GCA CGT TCG ATG GCG CTC GGC CAT CGG TGG AAA CGG AGA TTA CAC TAC CCG

                                           130                                         140
 T   V   H   V   L   D   S   M   R   G   L   D   S   V   R   V   A   V   M   V
ACG GTA CAT GTG CTG GAT TCC ATG CGT GGC TTG GAT AGC GTG CGT GTG GCG GTG ATG GTG
TGC CAT GTA CAC GAC CTA AGG TAC GCA CCG AAC CTA TCG CAC GCA CAC CGC CAC TAC CAC

                                           150                                         160
 T   T   D   K   V   Y   R   N   L   E   H   P   Y   P   Y   R   E   D   D   V
ACC ACC GAC AAG GTG TAC CGC AAT CTG GAA CAT CCC TAT CCC TAT CGT GAA GAC GAT GTC
TGG TGG CTG TTC CAC ATG GCG TTA GAC CTT GTA GGG ATA GGG ATA GCA CTT CTG CTA CAG

                                           170                                         180
 L   G   G   H   D   P   Y   S   A   S   K   A   A   S   E   I   V   I   A   S
TTG GGT GGC CAC GAT CCC TAC AGT GCC AGC AAG GCG GCG AGT GAA ATC GTC ATT GCC AGT
AAC CCA CCG GTG CTA GGG ATG TCA CGG TCG TTC CGC CGC TCA CTT TAG CAG TAA CGG TCA

                                           190                                         200
 Y   R   D   A   F   L   A   Q   Q   G   V   A   I   A   S   A   R   A   G   N
TAC CGC GAT GCC TTT TTG GCG CAG CAG GGG GTG GCG ATT GCC TCT GCC CGG GCG GGA AAT
ATG GCG CTA CGG AAA AAC CGC GTC GTC CCC CAC CGC TAA CGG AGA CGG GCC CGC CCT TTA

                                           210                                         220
 V   I   G   G   G   D   W   S   E   D   R   L   I   P   D   A   L   R   A   W
GTA ATT GGT GGC GGC GAC TGG TCG GAA GAC CGC TTG ATT CCC GAT GCG CTG CGA GCC TGG
CAT TAA CCA CCG CCG CTG ACC AGC CTT CTG GCG AAC TAA GGG CTA CGC GAC GCT CGG ACC

                                           230                                         240
 Q   S   G   Q   L   L   E   V   R   R   P   E   A   I   R   P   W   Q   H   V
CAG TCC GGT CAG TTG CTG GAG GTC CGC CGG CCC GAG GCC ATC CGT CCC TGG CAG CAT GTG
GTC AGG CCA GTC AAC GAC CTC CAG GCG GCC GGG CTC CGG TAG GCA GGG ACC GTC GTA CAC
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FIG. 1. (**a**) Schematic illustration of the 3.8kb *Eco*RI-*rfb*-homologous fragment from *A. vinelandii.* The arrow indicates the direction of transcription of the putative gene. (**b**) Nucleotide sequence and deduced amino acid sequence of the putative RfbG from *A. vinelandii.*

significance of these genes in pathogenic enterobacteria is well documented (18). Here we report identification of a functional *rfbG* homologue in the free-living soil bacterium *A. vinelandii.* The *rfbG* gene is known to encode an enzyme, CDP-D-glucose-4,6-dehydratase, that is involved in converting CDP-D-glucose to CDP-6-deoxy-L-threo-D-glycero-4-hexulose.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. vinelandii* strains were grown at 30°C in modified Burk nitrogen-free (BN^-) medium (21). When it was necessary to include fixed nitrogen in the medium, ammonium acetate ($NH_4OAc.\ H_2O$) was added to a final concentration of 400μg/ml. *E. coli* strains were normally grown at 37°C in Luria broth or 2YT (22). When required the antibiotics ampicillin and kanamycin were used at final concentrations of 50μg/ml and 25μg/ml, respectively in *E. coli.* Kanamycin was used at a final concentration of 2.5μg/ml for selection in *Azotobacter.*

General molecular techniques. Oligonucleotides used for sequencing were purchased from GIBCO BRL Life Technologies, Inc. (Gaithersbureg, MD). Radio-labeled material for sequencing ([^{35}S]-dATP) was obtained from Dupont NEN (Boston, MA). Restriction enzymes were purchased either from Boehringer Mannheim (Indianapolis,

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                                     250                                     260
 L   E   P   L   S   G   Y   L   V   L   A   E   M   L   W   H   E   P   A   R
CTG GAG CCG CTC AGT GGT TAT CTA GTA CTG GCA GAA ATG CTC TGG CAT GAA CCT GCG CGG
GAC CTC GGC GAG TCA CCA ATA GAT CAT GAC CGT CTT TAC GAG ACC GTA CTT GGA CGC GCC

                                     270                                     280
 A   G   A   Y   N   F   G   P   F   T   H   E   A   A   T   V   R   K   V   I
GCT GGC GCT TAC AAT TTC GGC CCG TTC ACC CAT GAG GCG GCC ACC GTG CGG AAG GTC ATT
CGA CCG CGA ATG TTA AAG CCG GGC AAG TGG GTA CTC CGC CGG TGG CAC GCC TTC CAG TAA

                                     290                                     300
 E   M   A   R   E   A   Y   G   Q   G   D   V   R   Y   G   Y   G   A   G   C
GAG ATG GCC CGC GAG GCT TAC GGC CAG GGT GAT GTG CGT TAC GGC TAT GGA GCC GGA TGT
CTC TAC CGG GCG CTC CGA ATG CCG GTC CCA CTA CAC GCA ATG CCG ATA CCT CGG CCT ACA

                                     310                                     320
 P   M   K   R   V   G   A   L   E   T   S   K   T   R   V   A   L   G   V   V
CCC ATG AAG CGG GTT GGG GCG CTG GAG ACC AGT AAG ACG CGA GTG GCA TTA GGC GTG GTG
GGG TAC TTC GCC CAA CCC CGC GAC CTC TGG TCA TTC TGC GCT CAC CGT AAT CCG CAC CAC

                                     330                                     340
 P   C   W   S   L   A   E   S   V   S   R   T   I   A   W   H   R   A   Q   H
CCA TGC TGG TCA CTG GCC GAA AGT GTG AGT CGC ACC ATT GCT TGG CAT CGA GCA CAG CAC
GGT ACG ACC AGT GAC CGG CTT TCA CAC TCA GCG TGG TAA CGA ACC GTA GCT CGT GTC GTG

                                     350                                     360
 G   G   A   D   A   R   G   L   C   E   A   E   I   K   A   H   E   T   R   V
GGA GGG GCC GAT GCG CGG GGC TTG TGC GAA GCC GAA ATA AAG GCA CAT GAG ACG CGA GTA
CCT CCC CGG CTA CGC GCC CCG AAC ACG CTT CGG CTT TAT TTC CGT GTA CTC TGC GCT CAT

STOP
TGA
ACT
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FIG. 1—*Continued*

IN) or from Promega (Madison, WI). Nucleotide sequencing was performed using a T7 Sequenase version 2.0 DNA sequencing kit purchased from USB-Amersham Life Sciences Inc (Cleveland, OH). DNA sub-cloning, plasmid DNA isolations, restriction enzyme digestions, agarose gel electrophoresis, ligations and *E. coli* transformations were carried out as described in laboratory manuals (22, 23) or as suggested in the manufacturers instructions. Nucleotide and amino acid sequence analysis was performed using MacVector 5.0 software and sequence homology searches were conducted using NCBI BLAST search analysis (24). Amino acid alignments were done using the Clustal V multiple protein sequence alignment program via Netscape (25, 26). Amino acid sequences were obtained through Swiss Protein Database and MacVector 5.0. *A. vinelandii* transformations were done by the method of Page and von Tigerstrom (27). Isolation of chromosomal DNA from *A. vinelandii* was carried out using the procedure described previously (28). Southern blotting was carried out according to standard methods. The DNA hybridizations were performed with digoxigen-labeled DNA probes (Boehringer Mannheim Corporation, Indianapolis, IN). The DNA was labeled with digoxigenin-11-UTP using the random primed method (Boehringer Mannheim Corporation, Indianapolis, IN). Detection of digoxigenin-11-UTP probe DNA was accomplished using "Lumi-Phos 530" and repeated exposures to Kodak-XAR-5 films.

Construction of A. vinelandii rfbG mutant strain. To construct an *A. vinelandii* strain that contains an insertion mutation caused by the insertion of a kanamycin resistance gene within the *rfbG* gene, a gene replacement technique previously described in detail was used (29, 30). *A. vinelandii* strains have a very efficient recombination system that allows homologous recombination between the newly delivered sequence and the host chromosome. The plasmid pBG225, a derivative of pUC18 and pUC19 carrying the mutated version of the *rfbG* gene, which contains a kanamycin resistance gene insert in the ORF of the RfbG, was used in this experiment to transform *A. vinelandii* and the transformants were selected on BN agar supplemented with kanamycin. Since plasmids such as pUC18 and pUC19 can not replicate in *A. vinelandii* cells, and are lost during cell division, the kanamycin resistant transformants obtained have acquired the mutated *rfbG* at the original position of the wild type *rfbG* on their chromosome.

Scanning electron microscopy. The *A. vinelandii* cells (wild type and *rfbG* mutant) were grown in BN^{+} medium. Samples of 100μl were processed for SEM as described previously (3). The samples were visualized and photographed using Hitachi S-2700 Scanning Electron Microscope.

RESULTS AND DISCUSSION

Cloning and Nucleotide Sequence Analysis of rfbG Gene of A. vinelandii

A genomic library of *Azotobacter vinelandii* was screened using heterologous probes and a λ clone carrying the *rfbG* was isolated (31). Restriction mapping and Southern blot analysis identified a 3.8kb *Eco*RI restriction fragment from this λ clone carrying the *rfbG* (31). This fragment was cloned into the unique *Eco*RI site of pUC18 (32) and the plasmid was designated pBG200. A partial restriction map of this 3.8kb *Eco*RI fragment is shown in the Fig. 1a. This 3.8kb *Eco*RI fragment was inserted into the unique *Eco*RI site of M13 mp18 (32). The recombinant plaques were identified and were used to obtain pure single stranded DNA to determine the nucleotide sequence. We obtained two clones that contained the 3.8kb *Eco*RI fragment in opposite orientations (pBG210 and pBG211). Since the 3.8kb *Eco*RI fragment contained 2 *Pst*I restriction sites (Fig. 1a), we purified the internal 1.1kb *Pst*I restriction fragment and cloned into the unique *Pst*I site of M13 mp18. Two clones carrying the 1.1kb *Pst*I fragment in opposite orientations were identified (pBG212 and pBG213). The M13 mp18 clones carrying the *Eco*RI and *Pst*I fragments were used for the determination of the nucleotide sequence. Initial sequence analysis identified that the majority of the open reading frame corresponding to the *rfbG* homologue was located on the 1.1kb *Pst*I fragment. Further nucleotide sequence

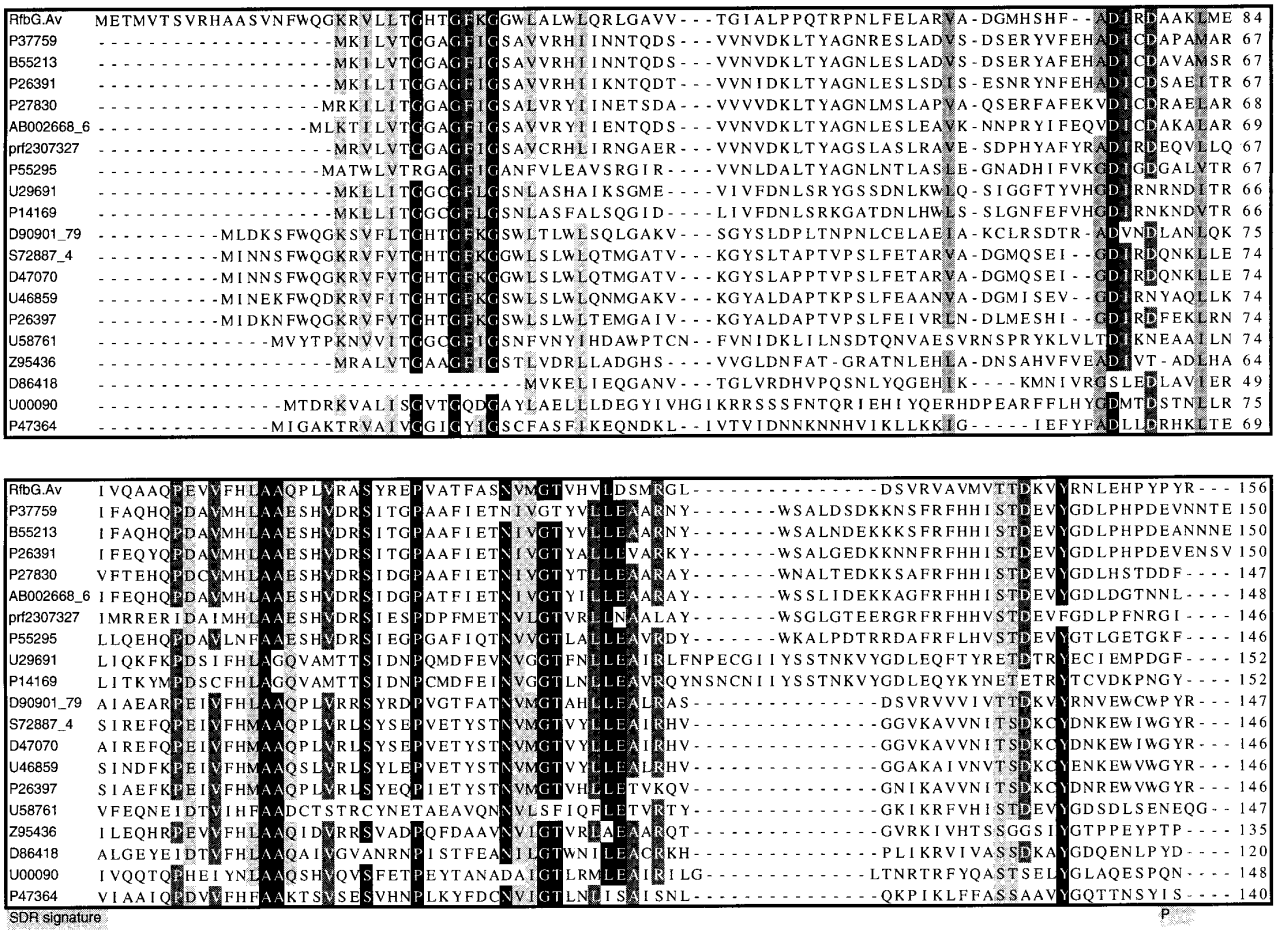

FIG. 2. Multiple Sequence Alignment results adapted from Clustal V 1.7 homology alignment. The amino acid sequences used for the alignment are identified by Accession number when available. The putative and identified dehydratases and epimerases are as follows: RfbG.Av (this study): putative CDP-Glucose 4,6-Dehydratase from *A. vinelandii* strain OP; P37759 (RfbB): dTDP- Glucose 4,6-Dehydratase from E. *coli* strain K12/WG1 (34); B55213 (RfbB): dTDP-D-Glucose 4,6-Dehydratase from *Shigella flexneri* strain 2a (16); P26391 (RfbB): dTDP- Glucose 4,6-Dehydratase from *Salmonella typhimurium* strain LT2 (35); P27830 (RfbG): dTDP- Glucose 4,6-Dehydratase from *E. coli* strain K12/MG1655 (36); AB002668_6: dTDP- Glucose 4,6-Dehydratase from *Actinobacillus actinomycetemcomitans* (37); prf2307327X (expA9): *Rhizobium meliloti* (38); P55295 (RfbB): dTDP- Glucose 4,6-Dehydratase from *Xanthomonas campestris* (39); U29691 (tyv): CDP-Tyvelose Epimerase (formerly RfbE) from *Yersinia pseudotuberculosis* serogroup IVA (40); P14169 (RfbE): CDP-Tyvelose-2-Epimerase from *S. typhi* strain TY2 (41); D90901_79 (RfbG): CDP-Glucose 4,6-Dehydratase from *Synechocystis* sp. strain PCC6803 (42); S72887_4 (AscB): CDP-Glucose 4,6-Dehydratase from *Y. pseudotuberculosis* serogroup VA (43); D47070 (RfbG): CDP-Glucose 4,6-Dehydratase from *Y. pseudotuberculosis* serogroup IIA (44); U46859 (DbhB): putative CDP-Glucose 4,6-Dehydratase from *Y. eneterocolitica* type 0:8 (45); P26397 (RfbG): CDP- Glucose 4,6-Dehydratase from *Salmonella typhimurium* strain LT2 (13); U58761, CO1F1.3(residues 1-358 of 631 aa): similarity to dTDP- Glucose 4,6-Dehydratase from *Caenorhabditis elegans* strain Bristol N2 (46); Z95436 (MTCY15C10.18) unknown, from *Mycobacterium tuberculosis* cosmid SCY15C10 (47); D86418_14 (YfnG): from *Bacillus subtilis* (48); U00090 (RfbE): putative CDP-Glucose 4,6-Dehydratase from *Rhizibium* sp. sp. NGR234 (49); P47364 (GalE or MG118): UDP-Glucose-4-Epimerase from *Mycoplasma genitalium* sp. ATCC33530/ G-37 (50). The darkness of blocks reflects the significance of similarity - with black boxes representing >90% conserved identity, dark gray boxes representing >75% conserved identity and light gray boxes representing >80% conserved functional similarity. Numbers on the right correspond to numbers of amino acid residues. The short-chain dehydrogenases/reductases family (SDR) signature is shown below the second and third panel (above). The consensus for this family is: [LIVS**P**ADNK] - ×(12) - **Y** - [P**S**TAGNCV] - [ST**A**GNQCIVM] - [**S**TAGC] - **K** - **{PC}** - [SAGFR] - [LIVM**S**TAGD] -×(2) - [LI**V**MFYW] - ×(3) - [LIVMF**Y**WGAPTHQ] - [GSACQ**R**HM]. Matching amino acid residues of *A. vinelandii* RfbG are shown in bold.

analysis using standard and sequence-specific primers on the DNA fragments in the M13 mp18 clones was performed by radio-labeled chain termination sequencing with a T7 sequencing kit purchased from USB-Amersham Inc.. Sequences were determined on both strands. The resulting nucleotide sequence was analyzed by sequence data analysis program MacVector 5.0. This analysis identified an open reading frame of 360 amino acids as shown in the Fig. 1b. The predicted molecular mass of this peptide is 39,449 daltons. Homology searches using NCBI Blast indicated that the open reading frame shares significant homology with other *rfbG* genes found in the members of Enterobacteriaceae family. The predicted RfbG protein of *A.*

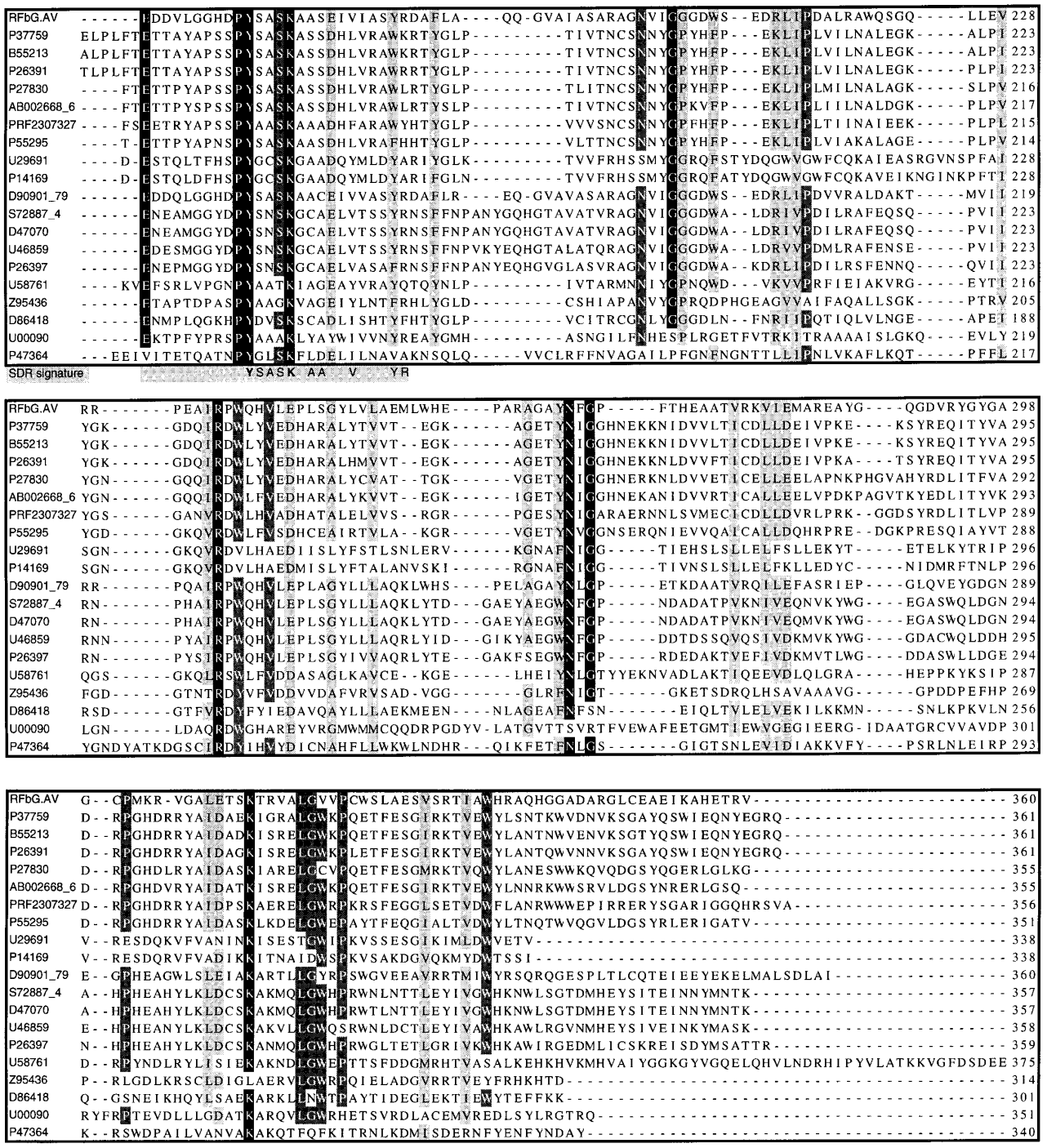

FIG. 2—*Continued*

vinelandii shares a homology of 57% identity with the RfbG of *Synechocystis* and 47% identity with the RfbG of *Yersinia pseudotuberculosis* and the lowest homology of 20% identity with the RfbG of *Mycoplasma genitilium*. Although the extent of overall homology among different *rfb*G homologues vary, there are specific regions of conservation among these peptides (Fig. 2). For example, the short-chain dehydrogenases/reductases family (SDR) signature sequence is conserved in all the sequences compared in Fig. 2.

Characterization of the Functional Properties of the rfbG Gene of A. vinelandii

To determine whether we have cloned the functional copy of the *rfbG* gene from *A. vinelandii*, we examined

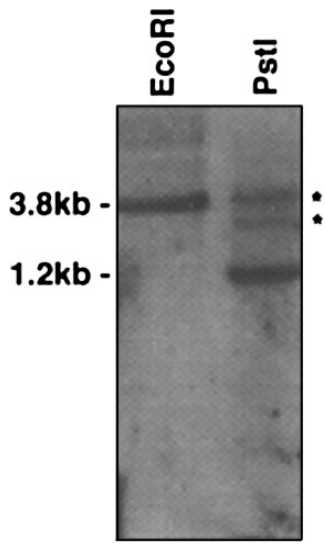

FIG. 3. Autoradiogram showing the Southern blot analysis of chromosomal DNA isolated from *A. vinelandii*. The chromosomal DNA was cleaved either with *Eco*RI or with *Pst*I and the resulting fragments were separated on 0.8% agarose gel. DNA was transferred and fixed to the MagnaGraph Nylon transfer membrane (Micron Separations Inc.), as described in the "Genius System user's guide for membrane hybridization" obtained from Boehringer Mannheim Corporation, Indianapolis, IN. Hybridization was performed using digoxigen-labeled 1.1kb *Pst*I DNA (corresponding to *rfbG*) probe which was labeled with digoxigenin-11-UTP using random primed method. * Indicates partial cleavage products in the *Pst*I digested chromosome.

the number of copies of *rfbG* gene per chromosome of *A. vinelandii*. To do this we employed Southern blot analysis technique. Initially, we purified the total chromosomal DNA of *A. vinelandii*. This DNA was digested with *Eco*RI and *Pst*I separately and the fragments were separated on 0.8% agarose gels. The fragments were transferred to MagnaGraph nylon transfer membrane by Southern blotting and the blot was probed with the 1.1kb *Pst*I fragment that was previously labeled with digoxigenin-11-UTP. This analysis showed that the chromosome of *A. vinelandii* harbors only one copy of the *rfbG* gene (Fig. 3). It was observed in other bacteria that mutations in the *rfb* genes had caused the mutants to exhibit agglutination during their growth in liquid medium, giving the culture a gummy appearance (33). Therefore, we decided to test whether the *rfbG* gene of *A. vinelandii* is functional by mutating this gene and analyzing how the disruption of this gene could affect the growth characteristics of *A. vinelandii*.

To isolate an *A. vinelandii* strain defective in *rfbG* function, initially we constructed a plasmid that carried the *rfbG* disrupted by inserting a kanamycin resistance gene in the 3.8kb *Eco*RI fragment (Fig. 1). This was constructed as follows. The 3.8kb *Eco*RI fragment has a unique *Hind*III site (Fig. 1). Since the fragment was cloned in the *Eco*RI site of pUC18, cleaving with the restriction enzyme *Hind*III resulted in removing a portion of 3.8kb *Hind*III fragment and all the polycloning sites of pUC18. The plasmid was subjected to religation and the *E. coli* strain TG1 was transformed with the ligation mixture. DNA was isolated from transformants and restriction digestion with *Hind*III was used to identify the plasmids that lost the portion of 3.8kb *Eco*RI fragment and all the polycloning sites of the pUC18. This plasmid was designated pBG224. This plasmid contained two *Pst*I sites located in the 3.8kb *Eco*RI fragment and upon digestion with *Pst*I generated two fragments, 1.1kb and 4.6kb in size. To construct pBG225 (Fig. 4), we gel purified the 4.6kb *Pst*I fragment of pBG224 and ligated it with a *Pst*I fragment carrying the kanamycin resistance marker. *E. coli* strain TG1 was transformed with this ligation mixture and transformants were selected on 2YT agar plates supplemented with ampicillin and kanamycin. The plasmid DNA was isolated from the transformants and subjected to restriction enzyme analysis to confirm

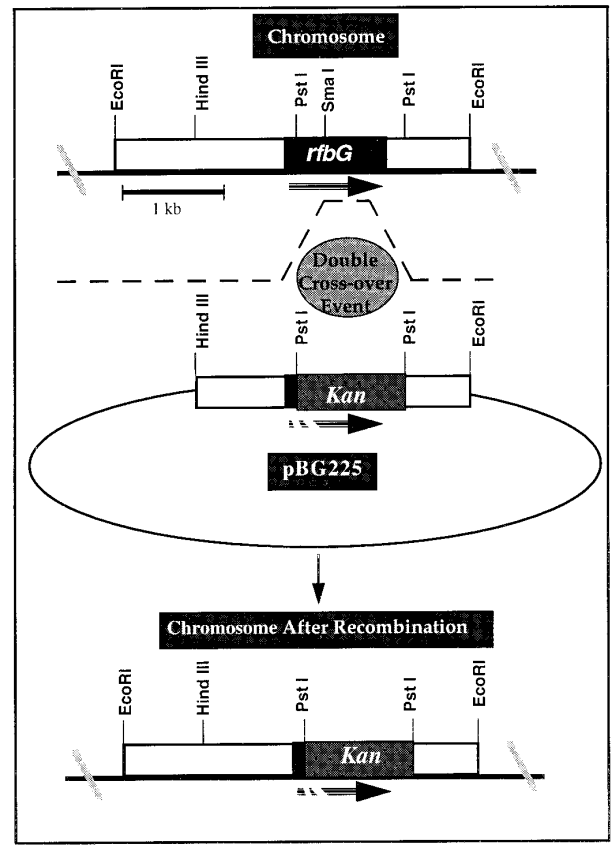

FIG. 4. Strategy for the construction of *rfbG*$^-$ *A. vinelandii* strain. Organization of the *rfbG* on the chromosome before and after the double cross-over event with the mutated *rfbG* and its neighboring sequences present on the plasmid pBG225 (pUC18 derivative) is shown. The plasmid pBG225 contains an *rfbG* disrupted by the insertion of a kanamycin resistance marker. This kanamycin resistance marker had replaced the sequences located between the two *Pst*I sites.

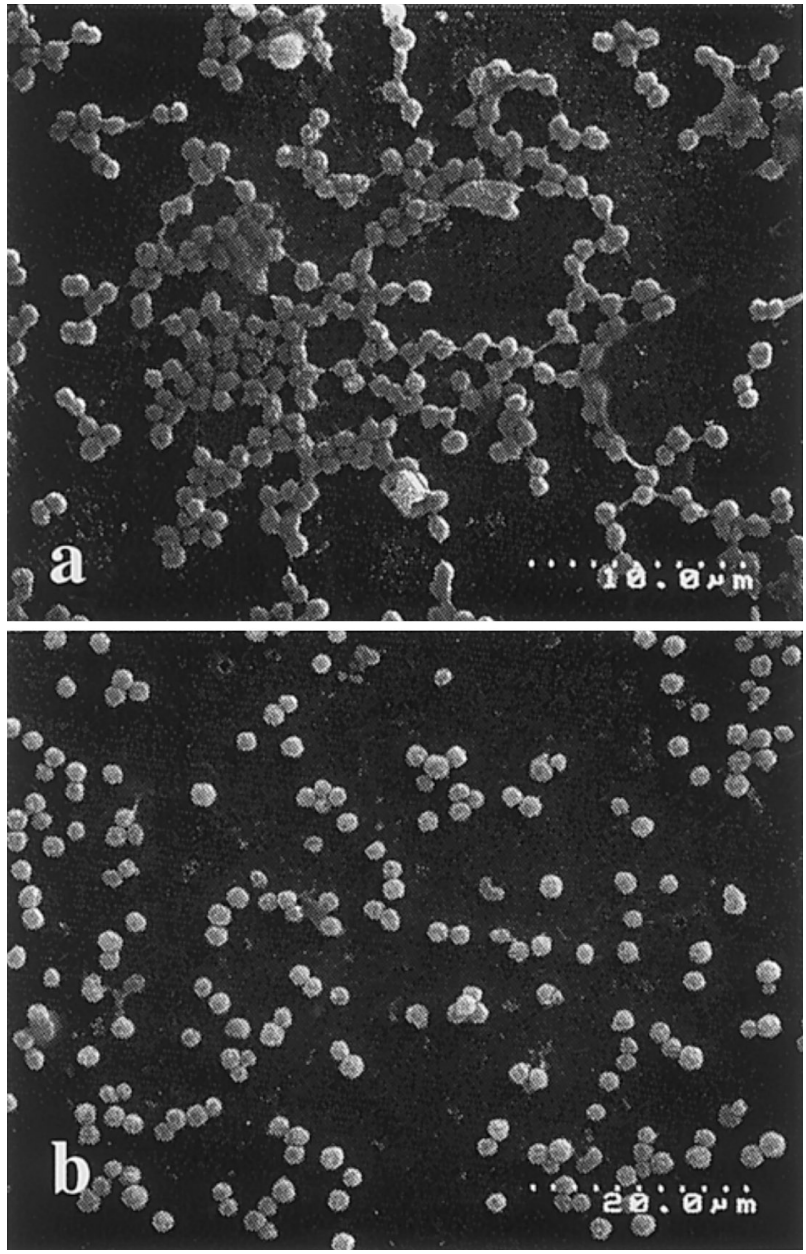

FIG. 5. Scanning electron micrographs of *A. vinelandii* strains harboring disrupted *rfbG* (a) and intact *rfbG* (b). Cells were visualized by using the methods described previously (3). The disruption of *rfbG* had resulted in the agglutination of the cells and decrease in cell volume.

that the *rfbG* gene on the plasmid was disrupted by kanamycin resistance marker. The *A. vinelandii* strain DJ54 was transformed with this plasmid and transformants were selected on BN^+ agar plates supplemented with kanamycin. Since these plasmids can not replicate in *A. vinelandii,* kanamycin resistant transformants represent colonies in which the kanamycin resistance marker has been rescued onto the chromosome by homologous recombination via DNA corresponding to *rfbG* and its neighboring sequences present on the plasmid and on the chromosome (Fig. 4). The growth characteristics of these kanamycin resistant *A. vinelandii* colonies were examined by growing in BN^+ liquid medium. It was observed that the kanamycin resistant *A. vinelandii* cells took longer to grow when compared to the growth of the parental strain DJ54. In liquid medium, agglutination of the cells was evident and this was further examined by scanning electron microscopic analysis. Fig. 5 shows that the *A. vinelandii* cells in which the chromosomal *rfbG* was disrupted by the insertion of kanamycin resistance marker agglutinate in liquid medium. This observation is consistent

with previous studies on *rfb* mutants of the members of Enterobacteriaceae.

In summary, we have identified a functional homologue of *rfbG* in the free-living soil bacterium *A. vinelandii*. Since the *rfb* genes specify O-antigen biosynthesis and bacteria with O-antigens are better adapted for symbiotic or parasitic association with eukaryotes, it will be interesting to see what role *rfb* genes play in a free-living soil bacterium like *Azotobacter*.

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