Research Note

Isolation and Characterization of a Locus from *Azospirillum brasilense* Sp7 That Complements the Tumorigenic Defect of *Agrobacterium tumefaciens chvB* Mutant

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The chromosomal virulence gene chvB of Agrobacterium tumefaciens is required for pathogenesis. A DNA fragment from the chvB locus can hybridize to DNA from Azospirillum brasilense Sp7. This DNA fragment could restore the tumorigenic activity of the chvB mutant strain A. tumefaciens A1011 towards leaf disks of Nicotiana tabacum. An NH₂-terminal open reading frame, 480 codons long, was most likely responsible for the restoration of the tumorigenic activity. The A. brasilense sequence showed good homology with the NH₂-terminal region of the ndvB gene of Rhizobium meliloti.

Additional keywords: DNA base sequence, lacZ fusion, Rhizobium meliloti ndvB locus.

The diazotroph Azospirillum brasilense can adsorb to root hairs of moncotyledonous plants (Elmerich 1984; Kapulnik et al. 1985; Okon 1985) and also to individual cells (Eyers et al. 1988). Agrobacterium tumefaciens induces tumors on dicotyledonous plants, while Rhizobium meliloti induces nitrogenfixing nodules on the roots of alfalfa plants. The processes of attachment and infection by both bacteria require, in addition to genes encoded by resident plasmids, a related set of chromosomal genes designated chvA and chvB in A. tumefaciens (Douglas et al. 1985) and ndvA and ndvB in R. meliloti (Dylan et al. 1986). DNA fragments containing the ndvA and ndvB genes can complement mutations in chvA and chvB, respectively, in A. tumefaciens (Dylan et al. 1986).

Could there be a genetic locus in A. brasilense that is analogous to chvB and ndvB? From the results of Southern blot hybridization of genomic DNA of several Azospirillum species including Sp7, Altabe et al. (1990) reported that these species did not have chvB homologous sequences. Contrary to this, Waelkens et al. (1987) had reported that genomic DNA from A. brasilense Sp7 hybridized with probes containing the chvB locus of A. tumefaciens.

We report here that A. brasilense Sp7 does have a locus homologous to the chvB locus of A. tumefaciens, and the cloned A. brasilense locus can complement a chvB mutational

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defect in *A. tumefaciens* with respect to tumor formation in leaf disks of *Nicotiana tabacum*. The segment of DNA involved in complementation can encode only a 480 amino acid-long NH₂-terminal polypeptide fragment that has sequence homology with the NH₂-terminal end of NdvB protein of *R. meliloti*.

A sequence homologous to a 1.25-kb *Eco*RI fragment internal to the *chvB* locus (Douglas et al. 1985) was found in the *A. brasilense* Sp7 genome by Southern hybridization. These were a 6.5-kb *Eco*RI fragment, a 5.1-kb *Bam*HI fragment, and a 4.6-kb *Hind*III fragment (data not shown). Restriction analysis revealed that these three were overlapping fragments (Fig. 1).

Complementation studies were done to determine if the chvB-homologous DNA from A. brasilense constituted a locus functionally equivalent to the chvB locus. The recombinant plasmids pSR12 (6.5-kb EcoRI fragment cloned in pRK405) and pSR14 (9.5-kb EcoRI-HindIII fragment cloned in pRK405) were mobilized into A. tumefaciens A1011 (chvB::Tn5) from E. coli S17.1 (Simon et al. 1986) by conjugation. The vector pRK405 is a derivative of pRK404 (Ditta et al. 1985) in which the *Eco*RI site outside the polylinker has been destroyed by filling. The exconjugants were used to infect the Nicotiana tabacum leaf disks. The A. tumefaciens strains A348 (wild type) and A1011 (chvB::Tn5) (Douglas et al. 1985) were used as positive and negative controls, respectively. The defect due to chvB mutation in the strain A1011 could be complemented by both pSR12 and pSR14 plasmids, since virulence was exhibited towards tobacco leaves (Fig. 2). We propose to designate the A. brasilense locus cviB (complements virulence). The sequence on the right side of the broken arrow in the restriction map (Fig. 1) may not be essential for virulence, since pSR12 can also complement the defects of the chvB mutant.

The DNA sequence of both strands of the segment between the solid vertical arrows (Fig. 1) was determined by the dideoxy chain termination method (Sanger et al. 1977). An open reading frame (ORF) was found, which was preceded by the two terminator codons TAA and TAG (Fig. 3, underlined). The first ATG in this frame (underlined) has been assumed to be the translation initiatior codon. An imperfect Shine and

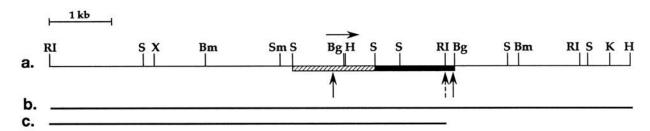


Fig. 1. A, Restriction endonuclease map of the 9.5-kb DNA containing cviB. The hatched and the black rectangles represent the regions homologous to the 1.25-kb EcoRI fragment internal to the chvB locus of Agrobacterium tumefaciens. The SalI fragment (hatched rectangle) was used for constructing the cviB::lacZ promoter fusion. The horizontal arrow represents the direction of transcription. DNA sequence has been determined for the region between the two solid vertical arrows. The region on the right hand side of the broken arrow is not necessry for the tumorigenic activity. B, The 9.5-kb EcoRI/HindIII fragment contained in pSR14. C, The 6.5-kb EcoRI fragment contained in pSR12. Abbreviations: RI, EcoRI; S, SalI; X, XhoI; Bm, BamHI; Bg, BglII; H, HindIII; K, KpnI; Sm, SmaI.

Dalgarno sequence (CGAGC, broken underlined) exists immediately upstream. There is a GTG (underlined) in the same frame, upstream of the putative initiator codon ATG, but there is no semblance of any Shine and Dalgarno sequence preceding it. The ORF extends beyond the sequenced region that ends in a *Bgl*II site (Fig. 3, boxed). The NH₂-terminal segment of the putative CviB polypeptide from *A. brasilense* capable of correcting the *chvB* mutant phenotype with respect to virulence, is only 480 amino acide long.

The direction of transcription was confirmed by constructing a *cviB*::*lacZ* promoter fusion. A 1.4-kb *SaII* fragment (hatched rectangle, Fig. 1) was cloned into the broad host range transcription fusion vector pGD499 (Ditta et al. 1985) in both orientations. The construct with the promoter in the correct orientation, in which the *HindIII* site within the *SaII* fragment was closer to the *lacZ* gene (pSR22.2) and the construct with the promoters in opposite orientation (pSR21.2), were mobilized into *A. brasilense* Sp7 by conjugation. The colonies of exconjugants with pSR22.2 and pSR21.2 elicited β-galactosidase activities of 2,120 and in 180 Miller units (Miller 1972), respectively. Thus, the promoter of *cviB* is likely to be within the 1.4-kb *SaII* fragment, and the direction of transcription is from left to right (horizontal arrow, Fig. 1).

The DNA sequence of the *cviB* locus was found to have considerable homology with the relevant portion of the *ndvB* gene of *R. meliloti* (Ielpi et al. 1990), the gene that could also complement the defect in the *chvB* locus of *A. tumefaciens*. The homology was poor, with bases upstream of the base number 32 (underlined C, Fig. 3), which corresponded to base number 377 designated by Ielpi et al. (1990) in the *R. meliloti ndvB* sequence.

The derived amino acid sequence of CviB had 60% homology with the derived amino acid sequence of NdvB at the level of identical amino acids and 76% homology if similar amino acids were considered. However, the first 38 amino acids at the NH₂-terminal end of NdvB were absent at the NH₂-terminal end of CviB (Fig. 4). The base sequence of the corresponding region has been rechecked by using the oligonucloetide primer, 5'-ATAGGACGCGCGGATCGAATC3' (bases complementary to base numbers 87–67, Fig. 3). The hydropathy plot of the NH₂-terminal region of the putative CviB polypeptide, determined according to Kyte and Doolittle (1982), has revealed the presence of a possible transmembrane segment that corresponds to one of the transmembrane segments of the NdvB polypeptide (box, Fig. 4).

Since A. brasilense is not known to produce tumors or

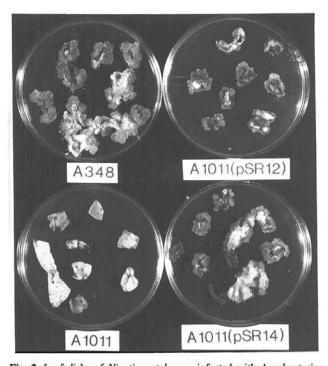


Fig. 2. Leaf disks of Nicotiana tabacum infected with Agrobacterium tumefaciens A348, wild type; A. tumefaciens A1011, chvB::Tn5; A. tumefaciens A1011/pSR12; and A. tumefaciens A1011/pSR14. The photograph was taken after incubation for a period of 20 days at 25° C. The leaf pieces were incubated on Murashige and Skoog (MS) basal medium (Himedia Laboratories, India) in a petri plate at 25°C in the dark for 24 hr. Cultures of different strains of A. tumefaciens grown overnight were centrifuged, and the cells were washed and resuspended in sterile water. The explants (six to eight for each strain) were submerged in the bacterial suspension for 10 to 15 min, soak dried on sterile blotting paper, placed back on the MS basal medium, and incubated at 25°C in the dark for 48 h. The explants were then removed from the plate and washed with autoclaved water, soak dried on a blotting paper, and placed back on MS basal medium containing the necessary antibiotics. Explants infected with the strains A348, A1011, A1011/pSR12, and A1011/pSR14 were placed on MS basal medium containing Cb (carbenicillin, 100 μg/ml); Cb and Km (kanamycin, 100 μg/ml); and Cb, Km, and Tet (tetracycline, 10 µg/ml); and Cb, Km, and Tet, respectively. The plates were then incubated in diffused light at 25°C for 20 days. After 10 to 12 days swellings from the edges were seen which grew into crown gall tumors.

nodules in plants, but adsorbs to the roots, we speculate one of the functions of this locus is to participate in the adsorption.

GTCCCGAGTTTCGCCGTTTTTTACGGTGCTTTTTCAAAATGACATAGCAGATATGCATGGGCATGTAAGGGGCGCCACA GGCACTCAGACGGCCTGAAATTCAGCGCTTGCATCGGGTCGCGACAGGTGGCATGGAACTATTGATGTCCTGGGGCATT AGCGTCTTTCGGATGTAGCGCGAACGTTCCGGTTTCGTGTTGCCCTTCAGGTTATCAACGGGGGACCATCGGGCGTTGC ${\tt GCGCACAAATTTCGCCGTCGGGGAGGGTGCGAAATCCGGTCGGAACCGTTGCCGCGCCGCAGGATC{\tt AGATCT}$ ${\tt TCTGTC}$ ACCGGAAGCTTCCTGCACGCGCTGATCTCAAGCCCCGCAAACAGATTGCAAACATTGTGCAGCAGTATGCGGCGCCTTC $\tt CTGCCGGACGGTCCTCTCAGCCGGGTGTCCGGTTCAGCGAAGGTTCAGGATAAGTCA\underline{TAA}ATA\underline{TAG}CGTGGCCG$ ATTTTCGTGTGTGTTCCCGCCACGGACGAAATGCCGGTCAAGTCGAACAAGTCTCCATAAAGTTCAGAATACGAGCCCG ATG TCA TIT CAT ATC ACC CCG ACC GCC GCC GCC CGT GAT TCC GAG ACG AAG CAG ATC GAT 60 20 E T K T A A R D S H T A I CAT AAT GAT TCG ATC CGC GCG TCC TAT, ATG ACG GTC GAG GAA CTG CAT GAT GCG GGC GCC 40 E H I R A S Y M T V E GCG CTT TCC CGC GAT GGC GCC GAC AGC CTT CCC GGC TTC ATG GAG TTC GAT TTC TTC GAG 180 F D S G M E F D L CGT CAT CGC GAG AAC GAA AAG GAA ATC CTC AGG GTC TAT CGC ACA ACG GCG GTC GAC GCG 240 80 R T N E K I L GAA AAT GGC GCG ACG ATA ACG CCG GCG GCA GAA TGG CTT CTC GAC AAC CAC TAC GTC ATC 300 D V 100 W L T P A E L T I A GAA GAG GCG ATC CAG GAA GTC CGC CGT GAT TTT CCG CGC AAG TTC TAT CGC CAG CTG CCG 360 F Y R Q I. 120 K E V R R D F P R 0 ACG ATG ACG GTG GGC GTG ACG ATC CGG CGG GTC ATG GCG CTC GGC TGG CTT TAT GAC 420 140 V L G W Y G G V T I R R M A GCC CAC ACC CAC AGC ACG GTA TCG CGC GAA AAC ATG ACG GCG CTG GTG GAT GGC TAC CAG 160 Т L v E H S T S R N M A 540 ACC TCG AAG ACG GTG CAG ATC GGT GAA TTG TGG GCG CTG CCA TGG ATC ATC CGT TTT GTC 180 R F P W I I Ι G E L W A L CTC ATC GAA AAT CTT CGC CGC ATC TCC ATC CGT GTG GAG CGT TCG CGC CGC ATG CGC CAG 600 200 R S R R M N L R R I S I R V E AAG GCG AAT CAG GTG GTC GAC GAG ATT ATC CGC CTG AAC GAT GCG GAA GCG TCG GCA ACA 660 220 E S V I R L N D N 0 D E I CTT CTC AAG CAG GTC GAT TCG CTG GTC GAT GAC CCG ACT TTC GCG ACG CAT GTC CTT TAT 720 F T H 240 S V D D P T A V D L CGC CTC CGA AAC GGC TCG CAG ACA TCG GGT TTT GCC GTT GCA TGG CTC GAA GAG CGT CTT 780 260 S G V W L E G S F A CAC GCT GCC GGC ACC GAC GCT GAA AAC GTG ATG ATG TCG GAA CAT AAT CGC CTG GCA TCC 840 280 N V E H T D A E M M GGC AAC GTG ACG ATG GGC AAC ATC GTC AAG AGC CTG CGC GAG ATC GAT GAT ACC GAA TGG 900 300 E G N I V K S L R E T D D T TCG GTG TGG TTC GAG GAA GTC AGC CAT ATC GAC AAG GTT CTG CGC GAA GAA ACC GAT TAC 960 320 E D V R E E GAA ACG CTC GAT TTC GGC TCC CGG AAC ACC TAC CGC AAT ACC ATC GAA CTT CTG GCG CGT 1020 E L 340 D F G S R N Y R т CGT TCT CCC AAG ACC GAA GTT GAG GTC GCC CGT GCC GCT GTC GAA ATG GCG CGT ACC GAT 1080 V V E M A R 360 V E A R A A K T E ATG CCC GCA GAG GCG GAC GAG ACG CAT CCC GTC AAT GTC GGC TCC GTG CTG GGT CAG 1140 380 V G A E A D E T H P V N V G S L V CGT CGT TTC GAG CTT GAA AAG GCG CTG GGA TAC CGG CCC CTA GTA TCG CAG CGT ATC GTC K G Y R P L V 400 E L Α CGG GCG ATG CGG AAG TTC AAC TGG CTG GCA ATT GCA GCG CCG GTG CTT CTC ATC ACC GCG 1260 v L T 420 N W L A I A A P Ι. T GTC GCC ATG CTG GCG GTC GGG TGG TTC CTC GCC GAA GCG GGC ATG CCC TGG TAT GTT GTC 1320 440 W F E A G M P V G A ACC GCT TTC CTG CTG ATG TTC GCT CTG CCC GCC TCG GAG GGC GCG ACC GGC CTC TTC AAC 1380 P S G G 460 E A F L A M Α ACC CTT GTC ACC TTC TTC GTG AAA CCG TTC CGG CTG GTC GGA ATC GAG TTC AAG AAT CCA 1440 V G I E F K 480 V K P F R L v T F ATT CCC GAG GAC GCG CGA TCG TTG GTC GCC GTG CCC GTC ATG CTG ACC AGT CGC GAC AGC 1500 T D 500 V V P V M L S R E D R S L A GTT GAC GAG ATG ATG CGC AAT ATC GAG GTG CAT TAT CTC GCC AAT CCG CAT GGT GAG ATC 1560 P H E 520 N I E H Y A N E M M R

Fig. 3. Partial nucleotide sequence of the Azospirillum brasilense cviB locus. The deduced amino acid sequence is shown below the nucleotide sequence. The presumed initiator ATG is underlined as also the termination codons TAA and TAG upstream of initiator ATG. The imperfect Shine and Delgarno sequence is represented by the broken line under the sequence. The restriction endonuclease sites, BgIII (AGATCT), HindIII (AAGCTT), SaII (GTCGAC), and EcoRI (GAATTC) are boxed. Significant homology with the ndvB gene of R. meliloti begins at the base C at position 32 (underlined).

CviB	1	MSFHITPTAAARDSETKQIDHNDSIRASYMTVEELHDAGAALSRDGADSL 	50
NdvB	39	MLQNTTQSNLPREPEAKQIDYNDSIRSTYFSIDDLRACGASLAEKGASAL	88
CviB	51	PGFMEFDFFERHRENEKEILRVYRTTAVDAENGATITPAAEWLLDNHYVI	100
NdvB	89	+	138
CviB	101	EEAIQEVRRDFPRKFYRQLPTMTVGGVTIRRVMALGWLYDAHTHSTVSRE	150
NdvB	139		188
CviB	151	NMTALVDGYQTSKTVQIGELWALPWIIRFVLIENLRRISIRVERSRRMRQ	200
NdvB	189	++ + + + ++ + +	238
CviB	201	KANQVVDEIIRLNDAEASATLLKQVDSLVDDPTFATHVLYRLRNGSQTSG	250
NdvB	239	$ \cdot \cdot$	288
CviB	251	FAVAWLEERLHAAGTDAENVMMSEHNRLASGNVTMGNIVKSLREIDDTEW + + +++ + ++ +	300
NdvB	289	AVIAWIEERLERRGTDVEEALVAEQNRLSSGNATMSNIIRSLREIDDTDW	338
CviB	301	SVWFEEVSHIDKVLREETDYETLDFGSRNTYRNTIELLARRSPKTEVEVA +	350
NdvB	339	AVWFESVSKIDATLREGSDYAALDFGSRNTYRDTIEKLARRSGHSEHEVT	388
CviB	351	RAAVEMARTDMPAEADETHPVNVGSVLVGQRRFELEKALGYRPLVSQR	398
NdvB	389	EIAIEMVEEAKAAAAVEAPLQEPNVGSFLVGKQRLALEKRIGYSPSIFQH	438
CviB	399	IVRAMRKFNWLAIAAPVLLITAVAMLAVGWFLAEAGMPWYVVTAFLLMFA ++	448
NdvB	439	LIRSVRKLDWFAIAGPNILLTILAMIVVYAFYSPMDIPSGAKLIMLLLFA	488
CviB	449	LPASEGATGLFNTLVTFFVKPFRLVGIEFKNGIPEDARSLVAVPVMLTSR	498
NdvB	389	+	538
CviB	499	DSVDEMMRNIEVHYLANPHGEI 520	
NdvB	539	DHVDELVRNLEVHYLANPRGEI 560	

Fig. 4. Amino acid sequence comparison of NH₂-terminal regions of CviB and NdvB proteins. Identical amino acids are represented by vertical bars and the similar amino acids are represented by plus signs. The potential transmembrane domains deduced from the hydropathy plots of the two polypeptides are boxed.

ACKNOWLEDGMENTS

We thank J. Dobereiner, G. Ditta, and E. W. Nester for providing bacterial strains and plasmids, U. K. Bageshwar, and K. Latha for help in the experiments and D. K. Chattoraj for critical reading of the manuscript. This work was supported by the Indian Council of Agricultural Research and the Department of Biotechnology, Government of India.

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