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Scientific Research

Characterization of Avirulent TnphoA Mutants in *Agrobacterium tumefaciens* to Enhance Transformation Efficiency

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Abstract

Protein fusion with the *Escherichia coli* alkaline phosphatase is used extensively for the analysis of the topology of membrane protein. *Agrobacterium* strain A6007 was mutagenized with *E. coli* strain mm294A plasmid pRK609 having TnphoA to obtain mutants defective in virulence. Because alkaline phosphatase activity is only detected when the PhoA gene product from the transposon is secreted out of the protoplasm, the virulence mutants are located in genes that code for transmembrane or periplasmic proteins. Attempts were made to obtain the sequences adjacent to the TnphoA inserts through several different approaches including Inverse PCR, Cloning, and Tail PCR. Transposon-adjacent sequence was obtained from one membrane anchor subunit in *Bradyrhizobium japonicum i.e.* succinate dehydrogenase which has enhanced transformation efficiency.

Keywords

Transposon, Mutagenesis, *Agrobacterium tumefaciense*, *Bradyrhizobium japonicum*

1. Introduction

Agrobacterium tumefaciens is a Gram-negative, soil-inhabiting, pathogenic bacterium. It causes crown gall diseases in dicotyledonous plants. It harbors a big Ti plasmid, which has vir, con, origin of replication and T-DNA regions. It affects the wounded portion of the plant, which secretes a phenolic substance named as acetosyringone which activates the vir region. It has many vir genes viz vir A, B, C, D, E & G etc. which encode a variety of proteins *i.e.* vir A, B, C, D, E & G etc. Vir A binds with acetosyringone and activates vir genes, and conse-

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quently vir D_2 proteins bind with left and right borders of T-DNA and a single stranded T-DNA comes out which binds with different vir proteins and channelizes through vir B protein made pore and goes to the plant cell and integrates into the plant genome, and consequently the integrated genes express and plant becomes transformed. It was believed that virulence genes were present on Ti plasmid only but it was found that besides Ti plasmid, vir genes were also present on chromosomal DNA viz ChvA, ChvB, and exoC etc. [1] [2]. These chromosomal virulence genes also transfer T-DNA which causes tumor in the plant. On mutation of these, genes cause avirulent strains which do not form tumor. TnphoA construct (**Figure 1**) which is a derivative of Tn5 encoding kanamycin resistance, is successfully used for mutagenesis [3]. This construct generates a hybrid protein which has a version of the alkaline phosphatase (EC 3.1.3.1) gene with its signal sequence and promoter deleted, which will result in a blue colony phenotype on X-phos containing media when secreted into the periplasm, if the insertion occurs in the correct orientation and reading frame. This feature has allowed TnphoA to be used in the analysis of transmembrane protein topology [3] and also to identify chromosomal genes that are secreted into the periplasm [4]. Protein fusions have played a central role in molecular genetics studies of the mechanism of protein export in bacteria [5] [6]. In most of the studies, the hybrid containing amino terminal sequences of exported protein fused with cytoplasmic protein β-galactosidase moiety, which is unable to pass through the cytoplasmic protein. To study the exported protein from transmembrane, TnphoA construction is used to locate the membrane-localized chromosomal genes, where alkaline phosphatase bound protein is secreted from the membrane. If the missing signal peptide is substituted by either own moiety or other exported protein, a TnphoA mutagenesis approach has successfully been used in *Agrobacterium tumefaciens* to identify additional genes critical for virulence. Before the transposon mutagenesis could begin, however, it was first necessary to derive PhoA mutants; this was because *A. tumefaciens* expresses endogenous alkaline phosphatase activity that would completely mask the mutant phenotype [7]. Chemical mutagenesis was used successfully to obtain multiple strains which had stable PhoA minus phenotypes and continued virulence was confirmed to ensure that virulence related genes remained functional (wood, personal communication). TnphoA was introduced into the PhoA minus, virulent strain A6007 on a plasmid (pRK 609 from *E. coli* strain MM294A) which was not maintained in *A. tumefaciens* (wood, personal communication) identified approximately 37 avirulent or weakly virulent mutants which expressed alkaline phosphatase activity (wood, personal communication).

Various approaches were used in an attempt to obtain the sequences adjacent to the transposon insertion. This would allow for the determination of whether the avirulent phenotype was due to mutating a previously identified gene or an unknown gene; these approaches included Inverse PCR, Cloning (including southern analysis) and Tail PCR. Additionally, the mutants were screened again for both virulence and alkaline phosphatase activity.

A chromosomal virulence locus, ChvE, codes for a glucose-galactose-binding protein that interacts with the periplasmic domain of the sensory protein vir A and is involved in a synergistic induction of vir genes by plant phenolic compounds and sugars [8]-[10]. It has been reported that a succinate dehydrogenase mutant strain of *Rhizobium meliloti* showed delayed nodulation of Lucerne plants, and the nodules were white and ineffective [11]. It is also reported that the succinate dehydrogenase mutant has 3.5 lower rate of consumption of oxygen than the wild type [12].

2. Materials and Methods

Agrobacterium tumefaciens wild strain A6007 was mutated by mixing with *E. coli* strain mm294A having TnphoA transposited plasmid on nitrocellulose membrane and incubated for 6 - 8 hrs on MG/L medium. Blue

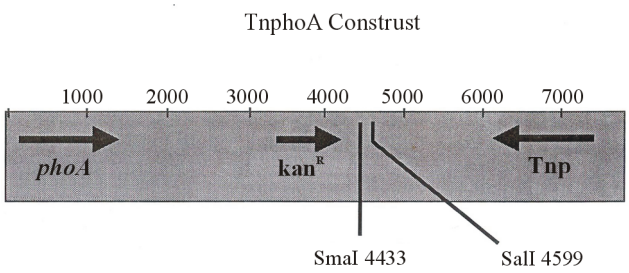


Figure 1. TnphoA construct.

colonies were selected on MG/L medium supplemented with Kanamycin (950 μg/ml), Nalidixic acid (50 μg/ml), Streptomycin (100 μg/ml) and X-phos (5-bromo-4-chloro-3-indolyl phosphate toluidine salt) and screened for virulence on Kalanchoe leaves (**Figure 2**). Mutant strains were rescreened by streaking out from glycerol onto MG/L medium having Km/Sm/Nal/X-phos in the same concentration as stated above and each strain was tested for virulence on Kalanchoe leaves with A6007 strain as a positive control on each leaf. Chromosomal DNA was isolated by the CTAB method from 17 of the 37 mutants, including all of those which were totally avirulent on plants and expressed alkaline phosphatase on X-phos containing media. These approaches were attempted to characterize the TnphoA adjacent sequences:

1) The first approach used in the attempt to characterize TnphoA adjacent sequences was Inverse PCR. Chromosomal DNA of all 17 mutants including 45-B and A6007 were digested with SalI enzyme from BRL, which cuts at only one place in the transposon. Followed by SalI digestion an overnight self ligation was carried out at 16˚C with T_4DNA ligase enzyme from BRL. Inverse PCR was then performed using two outward pointing primers (Jon 1: 5' GCAGTAATATCGCCCTGAGCAGCC 3', TnphoA 2: 5' CCAGGAAACCAGCAGCGGCTATCC 3') which amplified the intervening sequence between them containing TnphoA adjacent sequence up to the next SalI site and visible as clear bands (**Figure 3**). These bands were sliced from the gel and extracted the DNA through QIAgen extraction kit and re-amplified through PCR using both above mentioned primers. The amplified DNAs were PCR sequenced and sent for sequencing.

2) The second approach attempted was cloning scheme. It was done by two methods. (a) Selection by Kanamycin resistance—genomic DNAs of all mutants including 45-B and pBluescript II vector were digested with SalI enzyme from BRL. All mutants DNAs were digested and run on gel (2%) and approximately above than 4.0 kb DNA was sliced from each lane of gel mutant DNA and DNAs were extracted through QIAgen extraction kit. These extracted DNAs were treated as inserts. Both vector and inserts were ligated together in the ratio of 1:4 with T_4DNA ligase from BRL. Transformation was performed both by electroporation using protocol of [7] as well as $CaCl_2$ using protocol of [13] and plated onto MG/L Kan (50 μg/ml) plates. If fragments from the SalI digested containing the kanamycin resistance gene of TnphoA were ligated into the vector, colonies should grow up but none appeared on these plates (**Figure 4**). So second method was used to increase the efficiency of cloning. (b) Southern analysis—in this method six different digests and double digested were designed using SalI and BamHi (which cut only once within the transposon) and EcoRV and XbaI (which do not cut within the transposon) in combination with the single cutters. This should result in chromosomal fragments containing the TnphoA Kanamycin resistance gene and extending varying lengths beyond the transposon into the adjacent sequence depending on whether the restriction sites lie in that area of the genome. After these digestions were performed, they were run out on a 0.8% agarose gel for 2 hr at 80 V. Followed by they were transferred to a

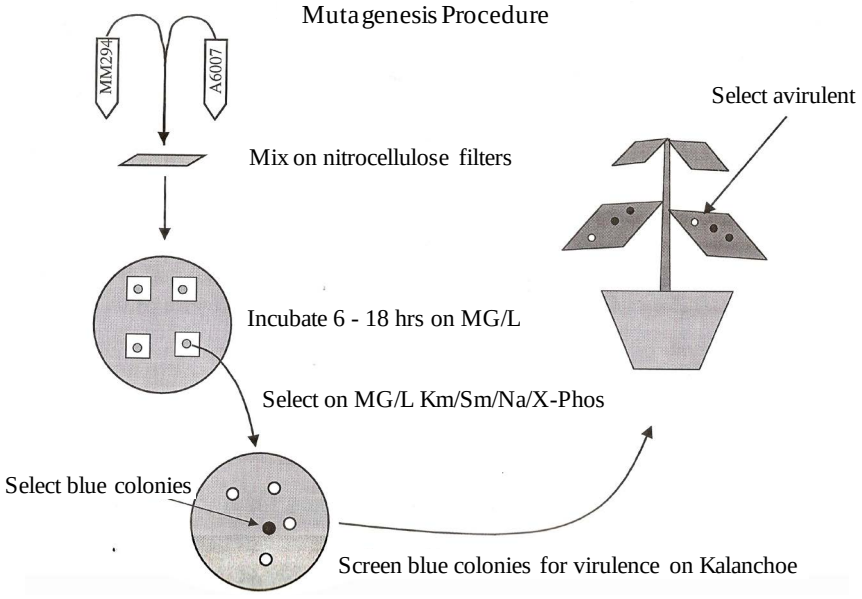


Figure 2. Mutagenesis procedure & virulence on Kalanchoe leaves.

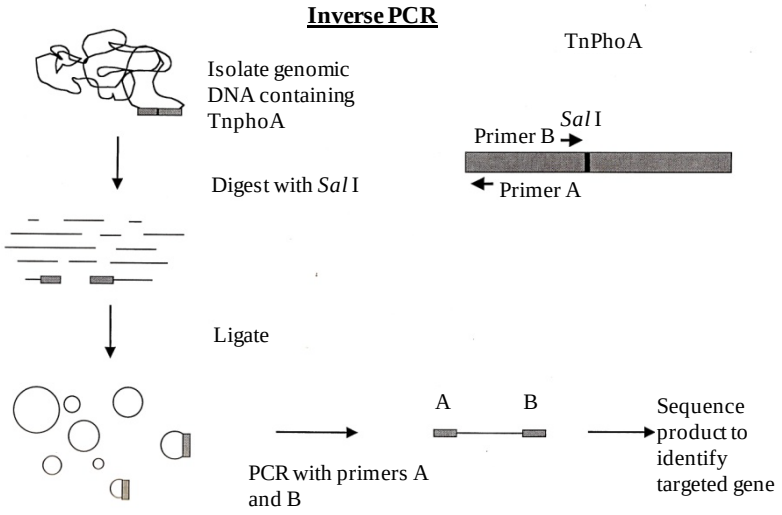


Figure 3. Invese PCR.

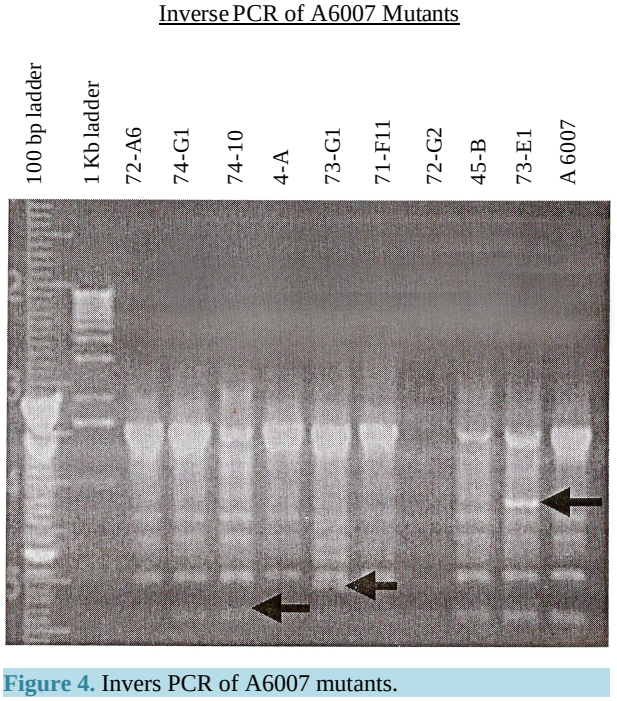


Figure 4. Invers PCR of A6007 mutants.

nylon (Hybond, Amersham) membrane and PhoA gene with primers TnphoA 5': 5' CCGCTCGAGGATCCTG-TTCTGGAAAACC 3' and TnphoA 3': 5' GGCTCTAGATTATTTCAGCCCCAGAGC 3' (from Lishan Chen). This probe was gel extracted with QIAgen kit and labeled with a kit from Amersham. Wash buffers and hybridization buffers were also made according to the Amersham kits protocol. Hybridization was carried out at 55˚C overnight with four washes the following day. ECF (Electro Chemical Front) substrate was applied to blots which were then incubated and scanned (**Figure 5** and **Figure 6**). Genomic DNAs were partially digested with various restriction enzymes though some of them were Cs (Cesium chloride) purified was big hurdle to take Southern results of all mutants, so third approach Tail PCR was used.

3) The third approach was Tail PCR: Tail (Thermal asymmetric interlaced) PCR was used with some modification of protocol of [14]. In this method four primers were used (**Figure 7**). Primer 1 (TnphoA II: 5' GTGC-AGTAATATCGCCCTGAGCA 3') anneals to the 5' end of TnphoA and is used in combination with the partially degenerate primers (a), (b), or (c) that all contain the following sequence: 5' GGCCACGCGTCGTC

GACTAGTACNNNNNNNNNN 3' followed by AGAG (a), ACGCC (b), or GATAT (c), for two rounds of PCR at very low annealing temperatures (30˚C and 43˚C sequentially). A 1:1 dilution of this PCR product is made and 1 µl of this dilution is used to do another PCR reaction for the normal 30 cycles with a 60˚C annealing temperature. This second PCR reaction uses primer 2 (Hah-1: 5' GTTTTCCAGAACCAGGGCAAAACGG 3') which anneals to the complement of the tail end of the TnphoA sequence and primer D (CEKG4: 5' GGCCACGCGTCGACTAGTAC 3') which anneals to the complement of the tail end of primer (a), (b), (c). In this way unknown sequences adjacent to a known sequence can be amplified by PCR which can then be followed by a sequencing PCR reaction [14]. Resulting bands (**Figure 7** and **Figure 8**) were gel extracted, re-amplified with another round of PCR, and sequenced.

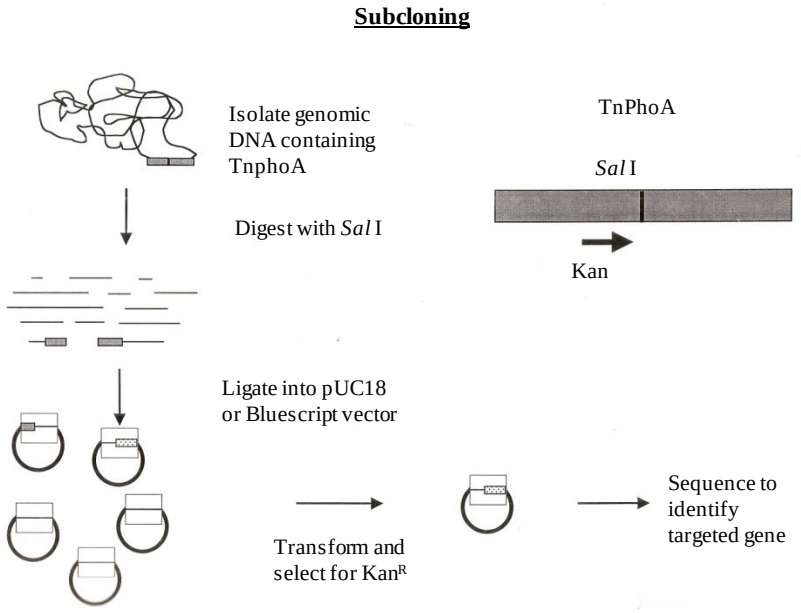


Figure 5. Cloning & subcloning of DNA containing TnphoA.

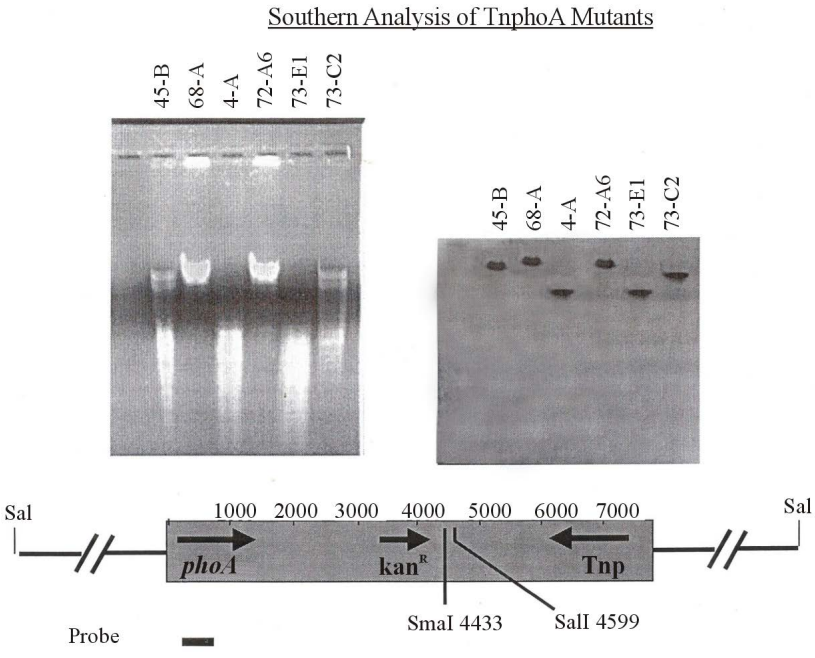


Figure 6. Southern analysis of TnphoA mutants.

Thermal asymmetric interlaced PCR (TAIL)

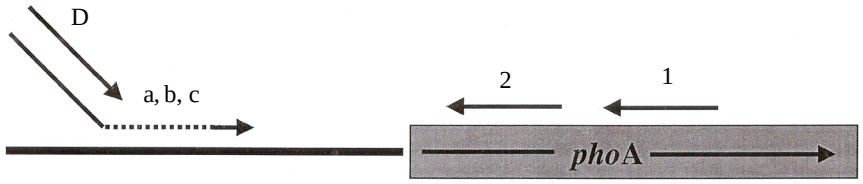


Figure 7. Tail PCR.

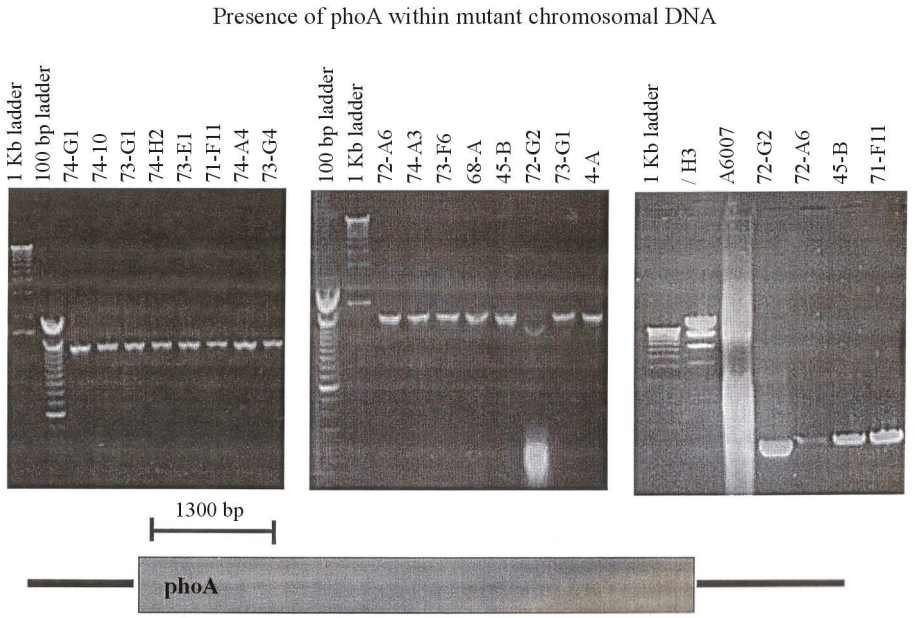


Figure 8. Presence of PhoA within mutant chromosomal DNA.

3. Results

The results of the alkaline phosphatase activity assayed of all mutants were shown in **Table 1**, **Table 2**, **Table 3**, **Table 4** and **Table 5** along with the results of re-screening the mutants for virulence on Kalanchoe leaves. In Inverse PCR the shelf ligated DNAs were not amplified with the use of Taq DNA polymerase so that no bands were visible but with the use of Pfu enzyme which is a proof reading DNA polymerase and high fidelity in DNA synthesis, bands were appeared (**Figure 4**). In cloning approach followed by ligation and transformation, no colonies were grown up on MG/L Kan (50 µl/ml) selection plates but in southern analysis discreet bands of TnphoA adjacent sequences of different mutants digested with SalI enzyme, were visible (**Figure 5** and **Figure 6**). In Tail PCR the presence of PhoA was screened by using primers TnphoA 5' and TnphoA 3' with the results being positive in each mutant except 74-G2 showed somewhat ambiguous. In all mutants one TnphoA band was seen in comparison to A6007 (control, no TnphoA band) (**Figure 7** and **Figure 8**). In the Tail and Inverse PCR many bands were sequenced but except 45-B mutant band sequence (**Figure 9**) no other mutant's band sequence gave any promising result. Its (45-B) band sequence showed a high degree of homology 984% similar to a succinate dehydrogenase membrane anchor subunit in *Bradyrhizobium japonicum* (**Figure 9(a)** and **Figure 9(b)**). Two distinct regions of homology were present, the first being 26 amino acids in length and the second being 13 amino acids in length. The E-value for this homology was 5×10^{-11}, showing that the protein coded by the 45-B gene and the polypeptide in *B. japonicum* are almost certainly homologues (**Figure 10** and **Figure 11**) and confirming that the succinate dehydrogenase gene is adjacent to the transposon (**Figures 11(a)-(d)**).

Table 1. Expression of TnphoA mutant's alkaline phosphatase activity on X-phos & virulence on Kalanchoe leaves.

Mutant ID	Virulent?	Overall virulence	PhoA expressed? (blue on X-phos)	PhoA present (from PCR)	Genomic DNA isolated?
68-A	no	0	yes	yes	yes
45-B	no	0	yes	yes	yes
45-A	no	0	no		
4-A	no	0	yes	yes	yes
74-A3	no	0	yes	yes	yes
74-A4	no	0	yes	yes	yes
71-F11	no	0	yes	yes	yes
73-C2	no	0	yes	yes	yes
73-E1	no	0	yes	yes	yes
73-F6	no	0	yes	yes	yes
72-G2	no	0	yes	yes	yes
74-G1	no	0	yes	yes	yes
74-H2	no	0	yes	yes	yes
74-10	no	0	yes	yes	yes
73-G4	no	0	yes	yes	yes
73-G1	no	0	yes	yes	yes
71-C11	attenuated	0.5	yes		
73-D1	attenuated	0.5	yes		
72-A6	attenuated	0.5	no	yes	yes
74-A12	attenuated	0.5	yes		
74-9	attenuated	0.5	yes		
73-G6	attenuated	0.5	yes		
72-G4	attenuated	0.5	yes		
72-G8	attenuated	0.5	yes		
74-B2	attenuated	1	yes		
74-E11	attenuated	1	yes		
133-A	attenuated	1	yes		
74-F7	yes	2	yes		
74-B6	yes	2	yes		
74-C7	yes	2	yes		
72-A9	yes	2	yes		
73-C8	yes	2	yes		
73-F9	yes	2	yes		
51-A	yes	3	yes		
144-A	yes	3	no		
16-B	yes	4	Yes		
100-A	yes	4	yes		

4. Discussion

Attempts were made to characterize transposon adjacent sequences in *Agrobacterium tumefaciens* mutants defective in tumor-forming ability using a variety of approaches. One likely reason for the lack of success with the Inverse PCR approach was possibility that the size of DNA that was to be amplified was too large. Taq polymerase can't reliably amplify fragments larger than 3 kb and if the closest SalI or BamHI site was farther away than that from the 5'-end of TnphoA, then successful Inverse PCR would have been difficult. Similarly, size may have also been a factor in the attempt to clone part of the adjacent gene, since the transposon fragment remaining after digestion with SalI or BamHI would have been at least 4 kb. It is right because large fragments can be cloned and make many copies in the vector like the PCR; if the next SalI or BamHI occurred at a great distance from the 5' end of the transposon, size would have likely been prohibitive.

Table 2. Expression of TnphoA mutant's alkaline phosphatase activity on X-phos.

Serial number	Mutants	Colonies grown on Kanamycin	PhoA expressed (blue on X-phos)
1	A6076	yes	yes
2	A6075	yes	yes
3	A6067	yes	yes
4	A6055	yes	yes
5	A6053	yes	yes
6	A6016	yes	yes
7	A6012	yes	yes
8	A6096	yes	yes
9	A6093	yes	yes
10	A6092	yes	yes
11	A6084	yes	yes
12	A6083	yes	yes
13	A6080	yes	yes
14	A6077	yes	yes
15	A6221	yes	yes
16	A6220	yes	yes
17	A6216	yes	yes
18	A6145	yes	yes
19	A6136	yes	yes
20	A6132	yes	yes
21	A6107	yes	yes
22	A6312	yes	yes
23	A6310	yes	yes
24	A6296	yes	yes
25	A6290	yes	yes
26	A6251	yes	yes
27	A6250	yes	yes
28	A6226	yes	yes
29	A6366	yes	yes
30	A6352	yes	No
31	A6348	yes	yes
32	A6343	yes	yes
33	A6341	yes	yes
34	A6340	No	yes
35	A6327	yes	yes
36	A6442	yes	yes
37	A6436	yes	yes
38	A6433	yes	yes
39	A6405	yes	yes
40	A6402	yes	yes
41	A6397	yes	yes
42	A6384	yes	yes
43	A6556	no	no

Table 3. Expression of TnphoA mutant's alkaline phosphatase activity on X-phos.

Serial number	Mutants	Colonies grown on Kanamycin	PhoA expressed (blue on X-phos)
44	A6535	yes	yes
45	A6498	yes	yes
46	A6497	yes	yes
47	A6496	yes	yes
48	A6457	no	No
49	A6450	yes	yes
50	A6595	yes	yes
51	A6593	yes	yes
52	A6587	yes	yes
53	A6586	yes	yes
54	A6578	yes	yes
55	A7019	yes	yes
56	A6990	yes	yes
57	A6961	No	No
58	A6909	yes	yes
59	A6879	yes	yes
60	A6822	yes	yes
61	A6758	yes	yes
62	A7016	yes	yes
63	A6983	yes	yes
64	A6958	yes	yes
65	A6906	yes	yes
66	A6864	yes	yes
67	A6791	yes	yes
68	A6748	yes	yes
69	A7015	yes	no
70	A6982	yes	no
71	A6957	yes	no
72	A6899	yes	no
73	A6859	yes	no
74	A6776	yes	no
75	A6747	yes	no
76	A7011	yes	no
77	A6981	yes	no
78	A6955	yes	no
79	A6898	yes	no
80	A6853	yes	no
81	A6769	yes	yes
82	A6746	yes	no
83	A6999	yes	no
84	A6980	yes	no
85	A6942	yes	no
86	A6889	yes	no

Table 4. Expression of TnphoA mutant's alkaline phosphatase activity on X-phos.

Serial number	Mutants	Colonies grown on Kanamycin	PhoA expressed (blue on X-phos)
87	A6851	yes	no
88	A6968	yes	yes
89	A6679	yes	yes
90	A6997	yes	no
91	A6977	yes	no
92	A6940	no	no
93	A6888	yes	no
94	A6850	yes	no
95	A6762	yes	yes
96	A6703	yes	yes
97	A6992	yes	yes
98	A6970	yes	no
99	A6930	yes	no
100	A6880	yes	no
101	A6825	yes	no
102	A6759	yes	yes
103	A6623	no	no
104	A7092	yes	no
105	A7067	yes	yes
106	A7081	yes	no
107	A7051	yes	no
108	A7044	yes	no
109	A7041	yes	no
110	A7035	no	no
111	A7302	yes	no
112	A7212	yes	yes
113	A7384	yes	no
114	A7211	yes	yes
115	A7134	yes	no
116	A7106	yes	no
117	A7098	yes	yes
118	A7379	yes	no
119	A7365	yes	yes
120	A7340	no	no
121	A7326	yes	no
122	A7313	yes	no
123	A7305	yes	no
124	A7516	yes	no
125	A7479	yes	no
126	A7474	yes	no
127	A7443	yes	no
128	A7431	no	no
129	A7423	no	no

Table 5. Expression of TnphoA mutant's alkaline phosphatase activity on X-phos.

Serial number	Mutants	Colonies grown on Kanamycin	PhoA expressed (blue on X-phos)
130	A7419	yes	no
131	A7620	yes	no
132	A7616	yes	no
133	A7601	yes	no
134	A7551	yes	no
135	A7531	yes	no
136	A7522	yes	no
137	A7751	yes	no
138	A7695	yes	no
139	A7694	yes	no
140	A7692	yes	no
141	A7678	yes	no
142	A7672	yes	yes
143	A7654	yes	yes
144	A7375	yes	no
145	A7365	yes	no
146	A6970	yes	no
147	A6067	yes	no
148	A6340	yes	no
149	A6880	yes	no
150	A7672	yes	no
151	A7642	yes	yes
152	A6007 (control, wild type)		

From the southern analysis which was intended to determine the size of the fragment, it was seen that all mutants have a single TnphoA insert and its size is about 23 kb; we were unable to get the size standard included in the Amersham kit to light up on the blot so we had to develop our own ladder to know the size of fragment. Southern results couldn't get with all mutants due to incomplete digestion of chromosomal DNA though we used Cs (Cesium chloride) purified DNA and used highly concentrated BamHi. Rather than continue working to figure out and solve this problem, we chose to attempt the Tail PCR approach.

The Tail PCR approach has been used successfully in a number of bacterial species by Manoil (wood, personal communication) and although our attempts have not yielded instant and easy success in the case of each mutant, the results we have obtained from 45-B are very promising; we cannot be sure that whether the succinate dehydrogenase homologue in *Agrobacterium tumefaciens* is critical for virulence at this time. It has been seen that succinate dehydrogenase mutant strain of *Rhizobium meliloti* showed delayed nodulation of Lucerne plants and the formed nodules were white and ineffective.

Revertant strain induced red and effective nodules. Succinate dehydrogense mutant rate of oxygen consumption also goes down due to choking of respiratory pathway for the liberation of kinetic energy. Sugar and plant phenolic compound-acetosyringone activates the vir A protein which induces vir genes and consequently transfers the T-DNA from agrobacterium to the plant. Succinate dehydrogenase which catalyses the conversion of

http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast BLAST Search Results Monday, June 7, 1999

NCBI **BLAST Search Results** BLAST Entrez ?

Commencing search, please wait for results.

BLASTX 2.0.8 [Jan-05-1999] (w/ SH-4, 45-B lower band sequence)

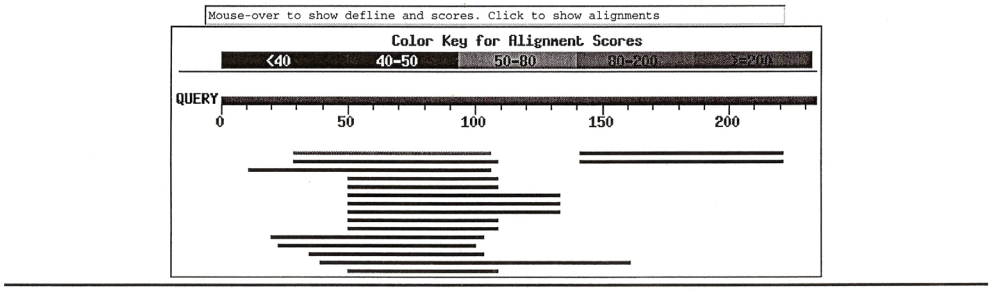
Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs", Nucleic Acids Res. 25:3389-3402.

Query=
(234 letters)

Searching.....done

If you have any problems or questions with the results of this search
please refer to the BLAST FAQs

Distribution of 17 Blast Hits on the Query Sequence



Sequences producing significant alignments:	Score (bits)	E Value

Page 1

(a)

http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast BLAST Search Results Monday, June 7, 1999

gi|3169722	(AF007569) succinate dehydrogenase membrane anchor s...	54	2e-07
sp|O59659|DHSC_PARDE	SUCCINATE DEHYDROGENASE CYTOCHROME B-556 S...	38	0.012
sp|P41085|DHSC_RICPR	SUCCINATE DEHYDROGENASE CYTOCHROME B-556 S...	37	0.020
gi|15518874	(U31241) integral membrane protein CII-3 [Cricetulus...	34	0.18
pir||T48085	integral membrane protein CII-3 - Chinese hamster (...	34	0.18
bbs|161801	succinate-ubiquinone reductase QPs1 subunit [cattle,...	34	0.23
pir||A45159	succinate-ubiquinone reductase QPs1 - bovine >gi|16...	34	0.23
sp|P35720|C560_BOVIN	SUCCINATE DEHYDROGENASE CYTOCHROME B560 SU...	34	0.23
ref|NP_002992.1|PSDHC1	succinate dehydrogenase complex, subunit...	33	0.30
gi|3420826	(AF081495) integral membrane protein CII-3b [Homo sa...	33	0.30
dbj|BAA31213|	(AB015757) fumarate reductase cytochrome b large ...	33	0.40
sp|P35721|C560_MARPO	SUCCINATE DEHYDROGENASE CYTOCHROME B560 SU...	32	0.52
emb|CAA74085.1|	(Y13760) SdhC protein [Shewanella frigidimarina]	31	1.2
emb|CAB03797|	(Z81453) B0250.9 [Caenorhabditis elegans]	31	1.5
sp|P80481|C560_RECAM	SUCCINATE DEHYDROGENASE CYTOCHROME B560 SU...	30	3.5
emb|CAB41546.1|	(AL022316) bK126B4.3 (novel protein) [Homo sapi...	29	7.8
gb|AAD34091.1|AF151854_1	(AF151854) CGI-96 protein [Homo sapiens]	29	7.8

gi|3169722 (AF007569) succinate dehydrogenase membrane anchor subunit
[Bradyrhizobium japonicum]
Length = 132

Score = 53.9 bits (127), Expect = 2e-07
Identities = 19/26 (73%), Positives = 24/26 (92%)
Frame = +3

Query: 30 LIGYTWALVHHMLGGLRHFMWDLGHG 107
+ GYTWAL+HHML G+RHF+WDLG+G
Sbjct: 72 VFGYTWALMHHMLSGIRHFVWDLGYG 97

sp|O59659|DHSC_PARDE SUCCINATE DEHYDROGENASE CYTOCHROME B-556 SUBUNIT >gi|975316
(U31902) succinate dehydrogenase b-type cytochrome
subunit [Paracoccus denitrificans]
Length = 130

Score = 37.9 bits (86), Expect = 0.012
Identities = 15/27 (55%), Positives = 19/27 (69%)
Frame = +3

Query: 30 LIGYTWALVHHMLGGLRHFMWDLGHGL 110
L G WAL +H+L GLRH +D G+GL
Sbjct: 74 LTGSMWALWYHLLAGLRHLFYDAGYGL 100

sp|P41085|DHSC_RICPR SUCCINATE DEHYDROGENASE CYTOCHROME B-556 SUBUNIT >gi|409934
(U02603) putative hydrophobic subunit of succinate
dehydrogenase [Rickettsia prowazekii]
>gi|3860694|emb|CAA14595| (AJ235270) SUCCINATE
DEHYDROGENASE CYTOCHROME B560 SUBUNIT (sdhC) [Rickettsia
prowazekii]
Length = 124

Score = 37.1 bits (84), Expect = 0.020

Page 2

(b)

Figure 9. (a) Blast search of 45-B lower band sequence producing significant alignment: score and E-value; (b) Succinate dehydrogenase membrane anchor subunit.

LAST Search Result Page 1 of 8

Commencing Search. Please wait for results.

BLASTX 2.0.8 [jan 05 1999]

Reference :

Altchul, Stephen F. Thomas L. Madden, Alenjandro A Schäffer, Uinghul Zhang, Zheng Zhang, Webb Miller , and David J. Lipman (1997) "Gapped BLAST and PSI- BLAST : a new generation of protein database search programs", Nuscleic Acids Res. 25:3389-3402.

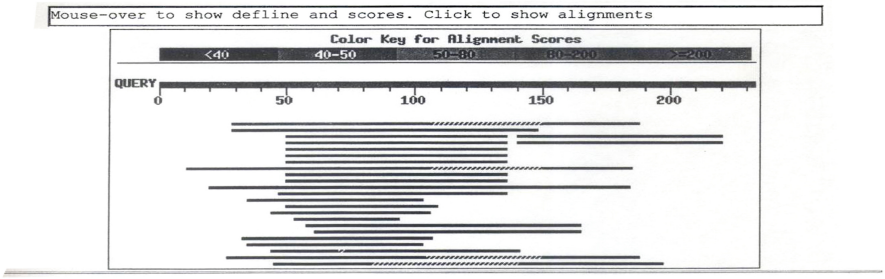
query =

(233 Letters)

searching.....done

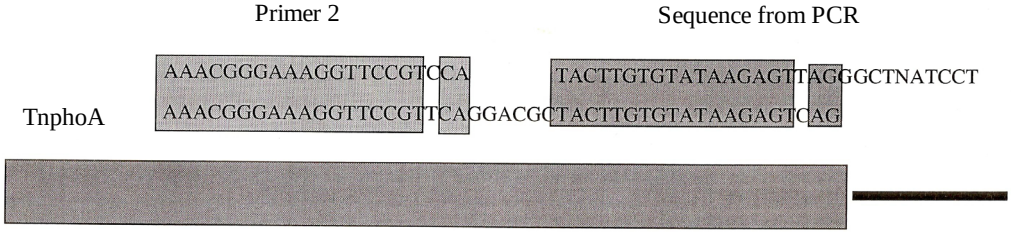
If you have any problems or questions with the results of search please refer to the BLAST FAQs

Distribution of 25 Blast hits on the Query sequence



Sequences producing significant alignments:	Score bits	E Value	N
si | 3169722 (AF 007569) succinate dehydrogenase membrane anch.....	61	5e-11	2
sp |P41085 |DHSC_RICPR SUCCINATE DEHYDROGENASE CYTOCHROME B-5.....	41	2e-05	2
sp |Q5965 |DHSC_PARDE SUCCINATE DEHYDROGENASE CYTOCHROME B-5.....	46	6e-05	1
sbs|161801 succinate - ubiquinone reductase QPs1 subunit [cat.....	44	4e-04	1
pir| |A45159 succinate - ubiquinone reductase QPs1 - bovine >g	44	4e-04	1
sp| P35720 C560_BOVIN SUCCINATE DEHYDROGENASE CYTOCHROME B56.....	44	4e-04	1
pir| |A48085 integral membrane protein CII-3 Chinese - hamst.....	42	0.001	1
si | 1518874 (U31241 integral membrane protein CII-3 - [Cricet.....	42	0.001	1
qi | 3420826 (AF081495) integral membrane protein CII-3b [Hom...	40	0.007	1
sef | NP_002992.1 PSDHC succinate dehydrogenase complex, sub ...	40	0.007	1
dbj| BAA31213| (AB015757) fumarate reductase cytochrome b la.....	37	0.047	1
sp | P35721| C560_MARPO SUCCINATE DEHYDROGENASE CYTOCHROME B56.....	36	0.088	1
emb| CAA74085.1| (413760) SdhC protein [Shewanella frigidima	35	0.23	1
sp| P80481 C560_RECAM SUCCINATE DEHYDROGENASE CYTOCHROME B56.....	33	0.81	1
gb|AA D34091.1 AF 151854_1(AF151854) CGI-96 protein [Homo sa	31	2.1	1

Figure 10. Blast search of 45-B lower band sequence.



(a)

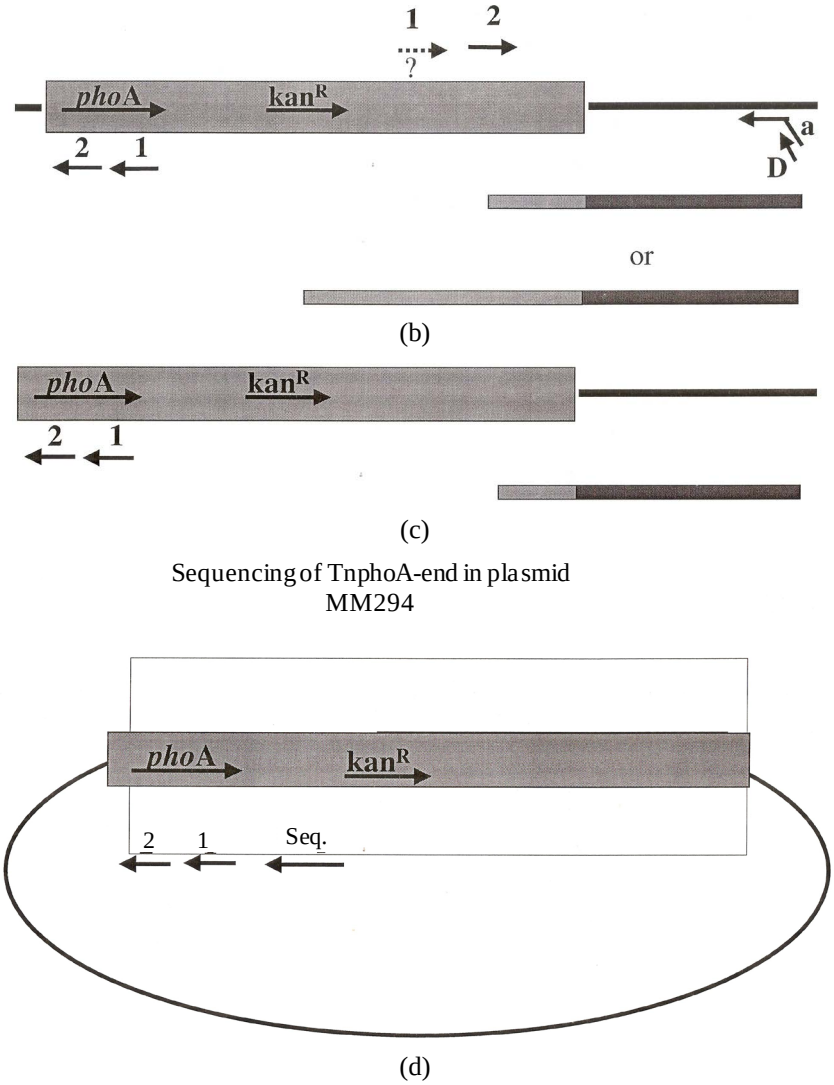


Figure 11. (a) Binding of primer & with TnphoA and adjaunt sequence; (b) Tail PCR shows the action of primer and (a) (b) or (c) & (d) on right side of TnphiA adjacent sequence; (c) TnphoA adjacent sequence of Tail PCR; (d) Sequencing of TnphoA-end in plasmid MM294.

succinate to fumarate and goes in further step of the production of energy, to convert fumarate to phosphoenolpyruvate which later on by the reversal of glycolysis changes into sugar which activates vir genes. It seems that succinate dehydrogenase may be involved in virulence and T-DNA transfer from agrobacterium to the plant with high efficiency for transformation.

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