

Agrobacterium tumefaciensの染色体上の病原性遺伝子,acv BおよびchvAのtransposn 5挿入非病原性変異菌を用いた機能解析

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Functional Analysis of Two Chromosomal Virulence Genes *chvA* and *acvB* of *Agrobacterium tumefaciens* Using Avirulent Mutants with Transposon 5 Insertion in the Respective Gene*

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Abstract

A mutant (M3 strain) of *Agrobacterium tumefaciens* was isolated by transposon (Tn5) mutagenesis. The M3 strain was avirulent on all host plants tested, *Daucus carota*, *Solanum tuberosum* and *Kalanchoe pinnata*. The avirulent phenotype of M3 strain was due to an insertion of Tn5 into the *chvA* chromosomal virulence gene. Using this M3 strain (*chvA*::Tn5) and two other strains, B119 strain (*acvB*::Tn5) and A208 strain (parent, virulent), the functions of the *chvA* and *acvB* chromosomal virulence genes of *A. tumefaciens* were analyzed. When M3, B119 and A208 strains were co-cultured with tobacco BY2 suspension-culture cells in the presence and absence of acetosyringone (As), efficient T-DNA transfer from an *Agrobacterium* cell to the nucleus of a tobacco cell occurred only with A208 strain in the presence of As. However, the T-strand (an intermediate form of T-DNA for transfer) was produced normally in all three strains in the presence of As. Analyses with tricine-SDS-polyacrylamide gel electrophoresis and electron microscopy demonstrated that pili, which are assumed to mediate T-DNA transfer into the host cell, were not produced on the cell surface of the M3 strain in either media with or without As. On the other hand, perfect pili were produced on the cell surface of the A208 strain in the presence of As. On the cell surface of the B119 strain, "imperfect" pili which were thinner than those on the A208 strain were produced in the presence of As. Taken together, these results indicate that *chvA* and *acvB* likely play roles in virulence by influencing formation of the pili through which the T-strands are thought to be transfer into host cells.

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Key words : *Agrobacterium tumefaciens*, chromosomal virulence gene, functional analysis of gene, transposon tagging, tobacco BY2 cell.

INTRODUCTION

Agrobacterium tumefaciens has the ability to transfer the T-DNA segment from the resident tumor-inducing (Ti) plasmid into plant nuclear genomes, resulting in the crown gall tumor phenotype^{26,28}. For this inter-kingdom DNA transfer process to occur, a set of genes in the virulence region of the Ti-plasmid and some virulence genes on the chromosome are required¹⁴. Upon induction of the *vir* regulon by inducers such as acetosyringone (As) from wounded plants, single-stranded scission occurs on the bottom strand of T-DNA (T-strand) at the 5'-end to which the VirD2 protein binds covalently^{22,23}. Some other proteins may bind to the complex, producing a T-complex. The T-complex is then transferred into

the cytoplasm of the host cell and finally reaches the nucleus of the host cell²⁵. Thus, the outline of the mechanism of crown gall induction by *A. tumefaciens* has been established, although the mechanism of some of the processes have remained elusive.

Previously, we isolated a new chromosomal virulence gene *acvB* from *A. tumefaciens* (A208 strain) harboring a nopaline-type Ti-plasmid using a transposon (Tn5) mutagenesis method²⁷. The B119 strain with a Tn5 insertion in the *acvB* gene shows an avirulent phenotype²⁷. In spite of the avirulent phenotype of B119 strain, the *vir*-genes on Ti-plasmid are induced normally by As, and the T-strands (or T-complexes) are produced normally in the strain¹⁰. The product of the *acvB* gene (AcvB protein, 47 kDa) is localized in the periplasm¹⁰ and is involved in the passage of the T-complex through

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cell walls of the host plant, although its mechanism is still obscure⁹⁾.

In the present work, we isolated an avirulent mutant (M3 strain) of the A208 strain using a transposon (Tn5) mutagenesis method. The M3 strain had a Tn5 insertion in the *chvA* chromosomal virulence gene which had been isolated and characterized earlier²⁾. The gene product (ChvA protein, 64.7 kDa) was reported to be localized in the inner membrane and to participate in the transport of β -1,2-glucans across the inner membrane²⁾. The β -1,2-glucans were once thought to be required for the attachment of *A. tumefaciens* to the host plant cell, an early step in crown gall tumor formation¹⁶⁾. However, β -1,2-glucan is not involved directly in either attachment of *A. tumefaciens* to the host cell or its virulence²⁴⁾.

The objective of this study is to elucidate the function of the *chvA* gene as well as the *acvB* gene by using M3 (*chvA*::Tn5), B119 (*acvB*::Tn5) and A208 (parent, virulent) strains.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions *Agrobacterium tumefaciens* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. *A. tumefaciens* strains were grown in/on either AB minimal³⁾ or LB medium¹⁷⁾ at 28°C. *E. coli* strains were grown in/on LB medium at 37°C. The concentrations (μ g/ml) of antibiotics used in media were as follows: kanamycin, 100; gentamycin, 30; ampicillin 50; tetracycline 10; hygromycin, 50. Introduction of plas-

mids into *Agrobacterium* was performed by transformation⁶⁾. DNA manipulation was carried out by the methods of Sambrook *et al.*¹⁷⁾, unless otherwise noted.

Transposon (Tn5) mutagenesis and isolation of a mutant Tn5 was introduced into *A. tumefaciens* (A208) by conjugation with *E. coli* harboring a conjugative plasmid, pJB4JI, with Tn5 as reported previously²⁷⁾. Transconjugants were first selected on AB minimal medium containing kanamycin. The kanamycin-resistant colonies were then transferred with tooth picks onto plates of LB agar with and without gentamycin to examine the presence of the pJB4JI plasmid. The kanamycin-resistant and gentamycin-sensitive transconjugants were assayed for virulence on root disks of *Daucus carota*. Total DNAs containing both the Ti-plasmid and chromosomal DNA were isolated by the method of Kado and Liu⁹⁾ from transconjugants with the avirulent phenotype. The DNAs were subjected to Southern blot analysis using the probe (probe 1) which was prepared by labeling the IS50L segment in Tn5 with [³²P] dCTP (Amersham Pharmacia Biotech, Takara Random Primer DNA Labeling Kit, Ver. 2) (Fig. 3). Thus, one avirulent mutant with a Tn5 insertion in its chromosome was isolated and named M3 strain.

Cloning of DNA segment with Tn5 insertion in chromosome of M3 strain Chromosomal DNA of the M3 strain was digested with *Eco*R1 which has no cleavage site within Tn5¹⁹⁾. The digested DNA was electrophoresed on an agarose gel, blotted onto a nylon membrane and hybridized with probe 1. A hybridized

Table 1. Bacterial strains and plasmids

Strain or plasmid	Characterization(s) ^{a)}	Reference or source
<i>A. tumefaciens</i>		
A208	Wild type, nopaline-type pTi, tumorigenic	13
B119	Tn5 insertion in <i>acvB</i> gene on chromosome of A208, avirulent, Km ^r	27
M3	Tn5 insertion in <i>chvA</i> gene on chromosome of A208, avirulent, Km ^r	This study
<i>E. coli</i>		
JM109	Host of pUC119, <i>recA1</i> , <i>supE44</i> , <i>hsdR17</i> , <i>gryA96</i>	Commercial source
DH1	Host of charomid 9-36, cosmid vector, <i>supE44</i> , <i>hsdR17</i> , <i>recA13</i> , <i>rpsL20</i>	Commercial source
Plasmids		
pJB4JI	Inc P, Km ^r , Gent ^r , Str ^r , Mu::Tn5	8
pBR333::IS50	IS50 associated pBR333	18
Charomid 9-36	Cosmid vector, Amp ^r	Commercial source
pUC119	Cloning and sequence vector, Amp ^r	Commercial source
pUCD2	Broad host range plasmid vector, Tc ^r , Amp ^r , Km ^r , Sp ^r	4
pCM3	Charomid 9-36 containing Tn5 and flanking segment 3.3 kb from M3 strain, Km ^r	This study
pUCM3	pUC119 containing the 3.3 kb <i>Sac</i> I- <i>Sal</i> I segment from pCM3	This study
pUC9CA	pUC119 containing <i>chvA</i>	This study
pUCD2CA	pUCD2 containing <i>chvA</i>	This study
pIG121-Hm	Binary vector, derivative of pBI121, an intron in β -glucuronidase gene, Km ^r , Hyg ^r	15

a) Abbreviations: Km^r, Amp^r, Gent^r, Str^r, Tc^r, Sp^r, Hyg^r, are kanamycin, ampicillin, gentamycin, streptomycin, tetracycline, spectinomycin, hygromycin resistance, respectively.

band DNA (9.6 kb) was recovered from agarose gel and ligated into a cosmid vector, Charomid 9-36, packaged *in vitro* and introduced into *E. coli* (DH1). The ampicillin- and kanamycin-resistant colonies were selected. The cloned DNA in these colonies was analyzed by Southern hybridization analysis with probe 1. The cosmid vector with the inserted Tn5-containing DNA fragment (9.6 kb) was thus selected and named pCM3.

Sequencing of the Tn5-containing DNA fragment (3.3 kb) from M3 strain The DNA fragment containing Tn5 and the flanking DNA was recloned in the *Sac*I/*Sal*I sites of the pUC119 plasmid and sequenced using the dye primer (21 M13) and sequencer (model 370A/373A) of Applied Biosystems. The determined sequence was analyzed with GENETYX software.

Cloning of *chvA* gene The entire *chvA* gene was cloned by PCR reaction using chromosomal DNA of A208 strain as a template and primers which were synthesized based on the reported sequence of *chvA*²⁾: 5'-terminal primer, 5'-CGGGATCCGCCAATTTGTTA-AGCCGCCATTTCAGACAG-3'; 3'-terminal primer, 5'-TTACTGCAGAAAGAAAAAGGGCGGACAATGCTG-3'. The restriction sites of *Bam*HI and *Pst*I, which are indicated with underlines, were included in the 5'- and 3'-terminal primers, respectively. The PCR reaction was carried out at 94°C for 30 sec, 57°C for 1 min, 72°C for 1 min for the first 30 cycles, then 72°C for 10 min with the PCR Amplification kit of Takara. After PCR, the product was digested with *Bam*HI and *Pst*I and applied to agarose gel electrophoresis. The amplified DNA band (1.9 kb) was recovered from agarose gel, inserted in *Bam*HI/*Pst*I sites of pUC119 (pUC9CA) and transformed to *E. coli* (JM109).

Complementation of avirulent phenotype of M3 strain with *chvA* gene The *chvA* gene was prepared from the pUC9CA plasmid and inserted into the *Bam*HI/*Pst*I sites of pUCD2 (pUCD2CA). The pUCD2CA plasmid was introduced into the M3 strain by transformation. The pUCD2 plasmid without the *chvA* gene was also introduced into the M3 strain to use as a control in the complementation assay. To confirm the presence of the plasmid (pUCD2CA or pUCD2), plasmid DNA was isolated from the transformed M3 strain and analyzed by agarose gel electrophoresis after being digested with *Bam*HI and *Pst*I. The A208, M3, M3 strain harboring pUCD2CA and the M3 strain harboring pUCD2 were used to inoculate stems of *Kalanchoe pinnatas* and gall induction was examined 6 weeks after inoculation.

Assay of T-DNA transfer from *Agrobacterium* cells to tobacco BY2 cells A binary vector pIG121-Hm¹⁵⁾, in which the T-DNA region contains a β -glucuronidase (GUS) reporter gene with an intron within the coding sequence downstream of the cauliflower mosaic virus 35S promoter, was introduced into the M3, B119 and A208 strains. The *A. tumefaciens* strains

harboring the binary vector were co-cultured with tobacco BY2 cells, and T-DNA transfer from *A. tumefaciens* to tobacco cells was monitored by transient expression of the GUS gene as reported⁶⁾. Since the binary vector (pIG121-Hm) contains an intron sequence in β -glucuronidase (GUS) gene in the T-DNA region, the GUS enzyme protein can be synthesized in plant cells only after being transferred into plant nuclei¹⁵⁾. One unit of GUS activity was defined as the activity producing one pmol of 4 methylumbelliferon/min/mg protein. The media for induction of *A. tumefaciens* and co-culture included 200 μ M As, unless otherwise stated.

Detection of T-strand formation in M3 and A208 strains The two strains were cultured at 28°C for 18 hr in the conditioned LS media¹²⁾ with and without As. The conditioned LS medium was used to obtain good growth of *A. tumefaciens*. The supernatant of the 3-day-old tobacco BY2 culture (LS-medium, pH 5.5) was sedimented, then used as the conditioned LS medium. Total DNAs were prepared from 7.8×10^{10} cells of the two strains and dissolved in 200 μ l of water. The DNA (20 μ l) was digested with 60 units of S1 nuclease at 23°C for 20 min. The digested and non-digested DNA were separated on agarose gel and blotted on nylon membrane (Hybond N⁺, Amersham Pharmacia Biotech.) under non-denaturing conditions²³⁾. The blotted membrane was hybridized with a ³²P-GUS DNA probe.

Preparation of outer membranes, periplasmic soluble proteins and spheroplasts from *A. tumefaciens* cells The three strains were cultured at 28°C for 3 days in media (200 ml) composed of fresh LS medium (50 ml) and the conditioned LS medium (150 ml) which contained either As or no As. The cells were harvested by centrifugation at $6000 \times g$ for 10 min and washed twice with distilled water. Subsequently, *Agrobacterium* cells were fractionated by the reported method²¹⁾ with some modifications. The washed cells were suspended in 17.5 ml of ice cold distilled water. The following chilled solutions were added gradually over the indicated time with gentle shaking: 10 ml of 0.1 M Tris-HCl, pH 8.3 (5 min); 9 ml of 2 M sucrose (5 min); 1.75 ml of 1% Na₂-EDTA, pH 7.0 (3 min) and 1.75 ml of 3% lysozyme (3 min). The mixture was incubated at 37°C for 15 hr and then centrifuged at $20,000 \times g$ at 4°C for 15 min to collect spheroplasts. Thereafter, the resultant supernatant was centrifuged at $95,000 \times g$ at 4°C for 45 min to collect the outer membrane fraction. The final supernatant contained the periplasmic soluble protein fraction.

Tricine SDS-polyacrylamide gel electrophoresis (tricine-SDS-PAGE) The protein contents of the sample solutions were determined with a protein assay kit from BIO-RAD. The sample solutions containing the same amount of proteins were boiled in 10 mM Tris-HCl, pH 6.8, including 1% SDS, 1% 2-mercaptoethanol and 20% glycerol for 3 min and then subjected to tricine-SDS-PAGE (16.5%) as described by H. Schägger and G. V. Jagow²⁰⁾. After electrophoresis, proteins were stained

with silver stain II kit (Wako).

Electron microscopy *A. tumefaciens* cells and the outer membrane fractions of the *A. tumefaciens* cells were suspended in 50 mM MgCl₂ solution. Both suspensions were mixed with an equal volume of 2% phosphotungstic acid solution (pH 7.4) and deposited on Formvar films on 200-mesh copper grids (VECO GRID). Usually about 10 µl of the mixture was placed on each grid for 5 min, and then excess fluid was removed with filter paper. Subsequently, the grids were air-dried for 2 min. The samples were observed in an electron microscope (model JEM 1200, JEOL) at 80 kV.

RESULTS

Isolation of an avirulent mutant of *A. tumefaciens* with Tn5 insertion in its chromosome

Tn5 was introduced into A208 strain by using a conjugative plasmid, pJB4JI (kanamycin-resistant (from Tn5) and gentamycin-resistant). Then the kanamycin-resistant and gentamycin sensitive transconjugants were selected and assayed for virulence on carrot root disks. Total DNAs were isolated from avirulent transconjugants and subjected to Southern blot analysis using probe 1 (IS50L in Tn5) to determine whether Tn5 was inserted into the Ti-plasmid or the chromosome. Finally, one avirulent mutant with a Tn5 insertion in its chromosome was isolated and named the M3 strain (Fig. 1).

Characterization of M3 mutant strain

The growth rate of the M3 strain on AB minimal and

LB media was similar to that of A208 strain (parent strain) and the morphological features such as shape and color of the colonies of both strains were identical (data not shown). The M3 strain was avirulent on not only on carrot root disks (*Daucus carota*) but also on potato tubers (*Solanum tuberosum*) and stems of Kalanchoe (*Kalanchoe pinnata*) (data not shown). To determine the number of Tn5 insertions in the chromosome, the chromosomal DNA of M3 was digested by either *Eco*R1 (has no cleavage site within Tn5) or *Bam*H1 (has one cleavage site)¹⁹. The digested DNA samples were analyzed by Southern hybridization using probe 1. Only one band (9.6 kb) was detected in the sample digested with *Eco*R1, while two bands (15.0 and 8.5 kb) were detected in the sample digested with *Bam*H1 (Fig. 2). The results indicated that one Tn5 is inserted in the chromosome of M3 strain. The DNA segment (9.6 kb) with a Tn5 insertion in the chromosome of M3 was cloned (Fig. 3), and the DNA segment flanking IS50R of Tn5 in Fig. 3 was sequenced (data not shown). The determined sequence was subjected to a homology search, which indicated that Tn5 was inserted at base1224 from the 5'-end of the *chvA* gene for which the sequence had already been reported².

Cloning of the *chvA* gene and complementation of M3 strain with the *chvA* gene

The *chvA* gene was amplified using PCR, the chromosomal DNA of the A208 strain was used as a template, and the primers were synthesized based on the reported sequence of *chvA*². The amplified DNA (1.9 kb) was inserted into *Bam*H1/*Pst*I sites of pUCD2 (broad-

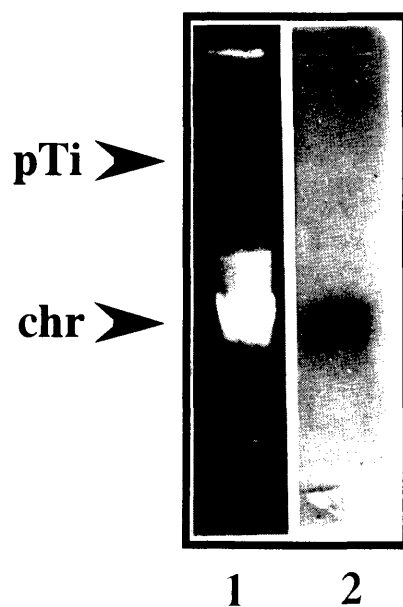


Fig. 1. Cellular location of the Tn5 insertion in avirulent mutant M3 strain. Total DNA from M3 strain was electrophoresed on agarose gel. The gel was stained with ethidium bromide (lane 1) and blotted onto a nylon membrane, which was hybridized with probe 1 (lane 2). pTi, Ti plasmid; chr, chromosomal DNA.

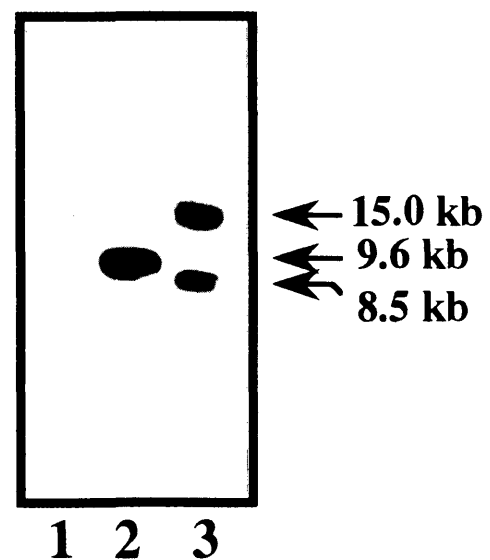


Fig. 2. Determination of number of Tn5 insertions in chromosome of M3 strain. The chromosomal DNA was isolated from A208 and M3 strains and digested with either *Eco*R1 or *Bam*H1. The digested DNA was subjected to Southern blot analysis using probe 1. Lane 1, A208 strain, *Eco*R1-digested; lane 2, M3 strain, *Eco*R1-digested; lane 3, M3 strain, *Bam*H1-digested.

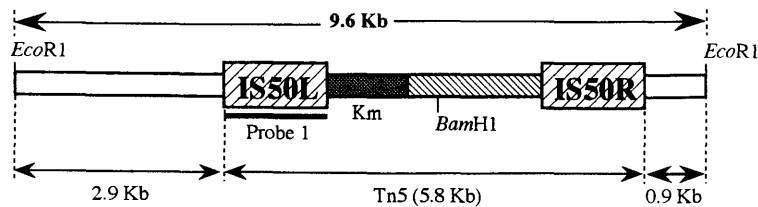


Fig. 3. The structure of the Tn5-containing DNA fragment (9.6 kb) which was cloned from M3 avirulent mutant. "Probe 1" shows the sequence used for preparation of probe 1 which was used in the experiments of Fig. 1 and 2. K_m shows the segment of kanamycin-resistance gene. *EcoRI* and *BamHI* indicate the restriction sites of the respective enzymes. The open bars indicate the DNA segments derived from the chromosome of *A. tumefaciens*.

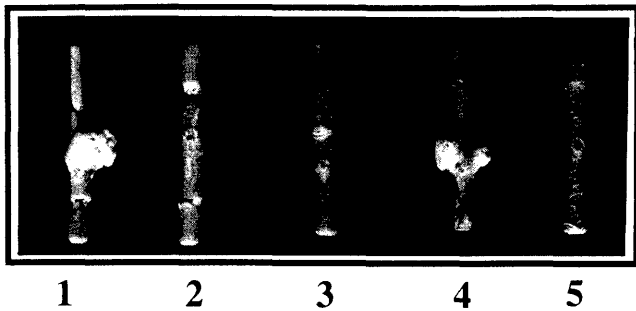


Fig. 4. Complementation of M3 strain (avirulent) by the cloned *chvA* gene. The cloned *chvA* gene was inserted into pUCD2 plasmid (pUCD2CA) and introduced into the M3 strain. Various strains of *A. tumefaciens* were inoculated on the stems of *Kalanchoe pinnata*. Photographs were taken 6 weeks after inoculation. 1, A208 strain (parent, virulent); 2, water-inoculated; 3, M3 strain; 4, M3 strain harboring the *chvA*-inserted pUCD2 (pUCD2CA); 5, M3 strain harboring pUCD2 vector without *chvA*.

host-range plasmid vector), producing pUCD2CA. The pUCD2CA plasmid was introduced into M3, and the resulting M3 strain harboring pUCD2CA was assayed for virulence on stems of *Kalanchoe pinnata* (Fig. 4). The M3 strain harboring pUCD2CA induced big galls, while M3 strain harboring the pUCD2 vector without *chvA* gene did not. The result confirmed that the avirulent phenotype of M3 strain was due to a Tn5 insertion in the *chvA* gene on its chromosome.

T-DNA transfer to host plant cell in media with and without As

The M3, B119 and A208 strains harboring a binary vector pIG121-Hm were co-cultured with tobacco BY2 cells in LS media (pH 5.5) with or without As, and T-DNA transfer to nuclei of host cells were monitored by transient GUS activity (Fig. 5). In the media with As, high GUS activity (22 units at the maximum) was detected with A208 strain, while negligible activity was detected with M3 and B119. On the other hand, reduced GUS activity (8 units at the maximum) was detected with A208 strain, and very low activity (1-4 units at the maximum) was detected with M3 and B119 strains in the media without As. The results showed that the addition of As was required for efficient T-DNA transfer from *A.*

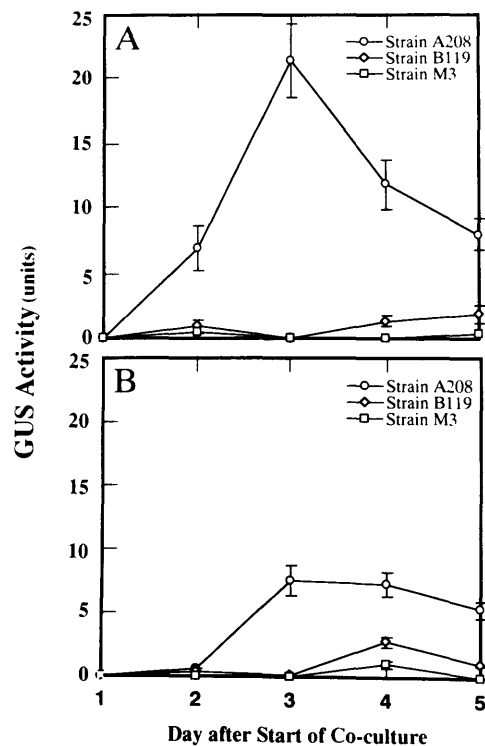


Fig. 5. T-DNA transfer from M3, B119 and A208 strains to nuclei of tobacco BY2 cells in media with and without As. The three strains harboring a binary vector, pIG121-Hm, were co-cultured with tobacco BY2 cells in LS media (pH 5.5) with or without As (100 μ M), and T-DNA transfer to nuclei of host cell was monitored by a transient GUS activity. Panel A, in the medium with As; panel B, in the medium without As. Experiments were repeated three times. The bar at each point represents the standard deviation.

tumefaciens cell to host cell. The optimal concentration of As changed with cultures; either 100 μ M or 200 μ M of As was used in this study.

T-strand formation in M3 strain

As mentioned in the INTRODUCTION, the T-strand is formed at an early stage in crown gall tumor induction by single-stranded scission of T-DNA on the Ti-plasmid, and the resultant T-DNA is transferred to host plant cells. Previously, the T-strand was produced nor-

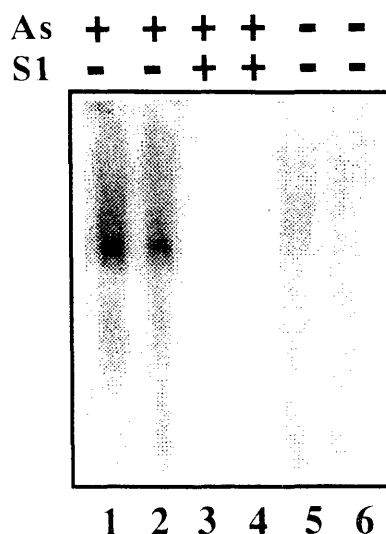


Fig. 6. Detection of T-strand formation in M3 and A208 strains by Southern blot analysis. The two strains harboring a binary vector, pIG121-Hm, were cultured in LS media (pH 5.5) with or without As. Total DNA was isolated from the cultured cells and applied to agarose gel electrophoresis after either digestion with S1 nuclease or no digestion. Then the gel was blotted under non-denaturing conditions and hybridized with a probe of ^{32}P -GUS DNA. As +, cultured in media with As; As -, cultured in media without As; S1 +, digested with S1 nuclease; S1 -, non-digested with S1 nuclease. Lanes 1, 3 and 5, A208 strain; lanes 2, 4 and 6, M3 strain.

mally in B119 strain as it is in A208 strain, when cultured in media containing As¹⁰). Thus, the experiment was performed to examine whether or not T-strand was formed in the M3 strain cultured in the medium with As (Fig. 6). Total DNAs were prepared from the M3 and A208 strains harboring a binary vector, pIG121-Hm, which were cultured in media with or without As. The DNA samples were separated on agarose gel, blotted onto a nylon membrane under non-denaturing conditions²³) and hybridized with ^{32}P -probe of GUS DNA. Positive bands were detected with both strains cultured in media containing As, but not with both the strains cultured in media containing no As. The bands disappeared when DNAs were analyzed after treatment with S1 nuclease, an enzyme that digests single-stranded DNA but not double-stranded DNA. These results indicated that the T-strand was normally produced in M3 strain as well as in A208 strain when cultured in media with As.

Tricine SDS-PAGE of outer membrane proteins, periplasmic soluble proteins and spheroplast proteins of the three strains of *A. tumefaciens* cultured in media with or without As

The M3, B119 and A208 strains were cultured in media with or without As and then fractionated into outer membrane, periplasmic soluble protein and spheroplast fractions. The protein compositions of the three

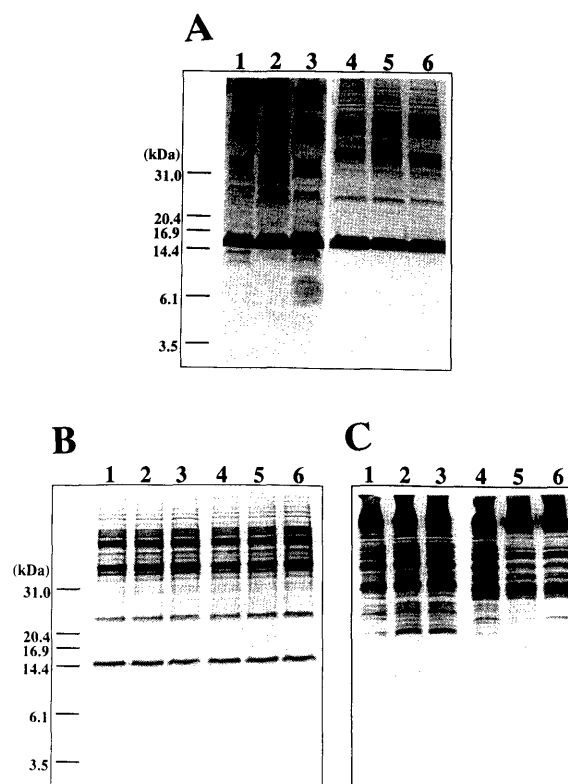


Fig. 7. Tricine-SDS-polyacrylamide gel electrophoresis of various subcellular fractions from M3, B119 and A208 strains which were cultured in media with and without As. The outer membrane proteins (panel A), periplasmic soluble proteins (panel B) and spheroplast proteins (panel C) were prepared from the cells of the three strains which were cultured in media with and without As and then applied to electrophoresis. In all panels, lanes 1-3 are samples from cells cultured in media with As. Lanes 4-6 are samples from cells cultured in media without As. Lanes 1 and 4, B119 strain; lanes 2 and 5, M3 strain; lanes 3 and 6, A208 strain.

fractions were analyzed with tricine-SDS-PAGE (Fig. 7). Tricine as a trailing ion provides sharper resolution of small proteins than when glycine is used. As can be seen from a comparison of the panels A, B & C in Fig. 7, the protein composition clearly differed among the three fractions, indicating that the three fractions had been well-separated.

The protein band patterns of the outer membrane fraction of the three strains cultured in media containing As (lanes 1-3, panel A, Fig. 7) were different from those of the three strains cultured in media containing no As (lanes 4-6, Fig. 7). This result showed that As induced changes in cell surface structure of *A. tumefaciens*. In addition, when the three strains were cultured in media with As, the protein bands differed in the range between 15 and 5 kDa of the outer membrane fractions (lanes 1-3, panel A, Fig. 7); 7-8 bands were detected with A208 strain (lane 3, panel A, Fig. 7). Two clear bands were observed with B119 (lane 1, panel A, Fig. 7) and two faint

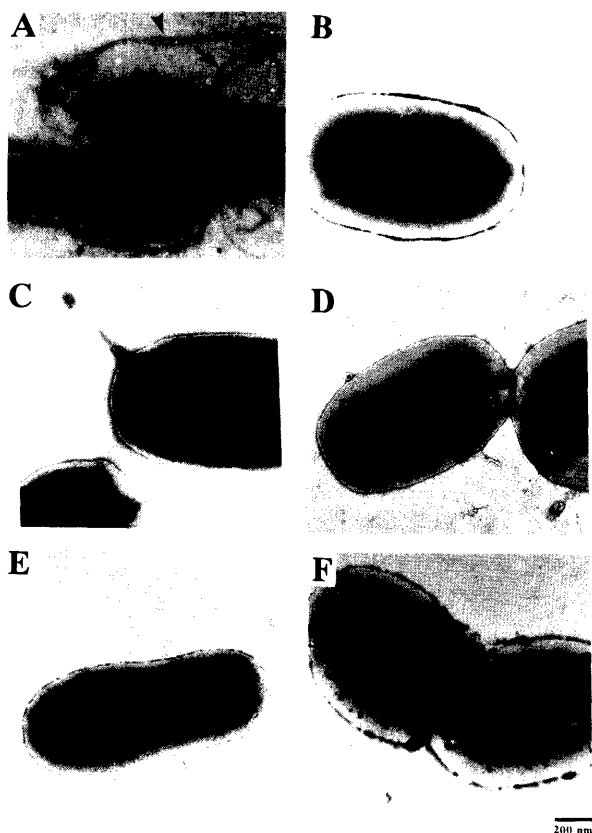


Fig. 8. Electron micrographs of cells of M3, B119 and A208 strains cultured in media with and without As. The cultured cells were examined with an electron microscope at a magnification of 30 K after being stained with phosphotungstic acid. Panels A, A208 strain cultured with As; B, A208 strain cultured without As; C, B119 strain cultured with As; D, B119 strain cultured without As; E, M3 strain cultured with As; and F, M3 strain cultured without As. An arrow in panel A points to a flagellum with diameter of about 13 nm and an arrowhead points to pili.

bands were seen with M3 (lane 2, panel A, Fig. 7). This result demonstrated that the M3 and B119 strains were differently impaired by the change in cell surface structure induced by As.

In contrast, As (acetosyringone) did not affect the protein composition of the periplasmic soluble protein and spheroplast fractions (panel B and C, Fig. 7). Moreover, the protein composition of those two fractions was almost the same among the three strains when they were cultured in both media with and without As (panels B and C, Fig. 7).

Electron microscopic observation of the three strains cultured in media with or without As

The M3, B119 and A208 strains were cultured in media with and without As and then observed with an electron microscope after staining with phosphotungstic acid (Fig. 8). Pili were seen with the A208 strain when cultured in media with As (panel A, Fig. 8), while pili were not seen in culture media without As (panel B, Fig.

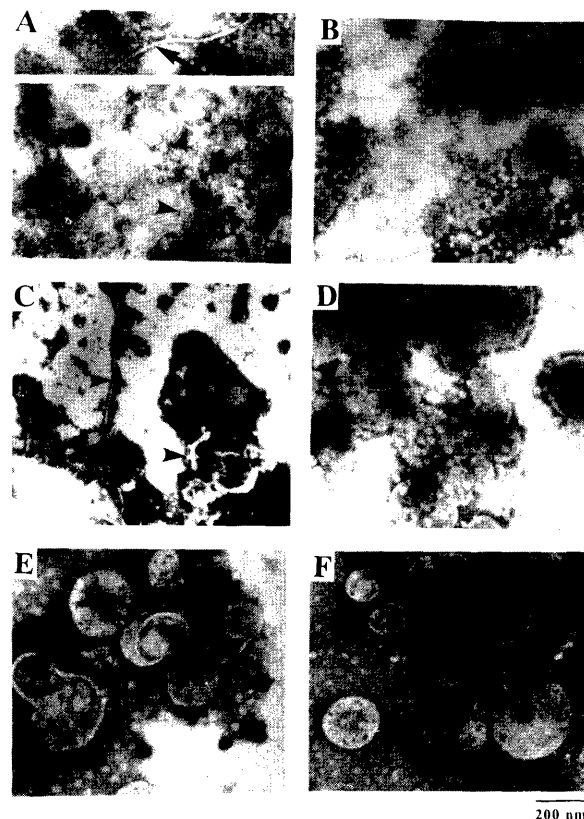


Fig. 9. Electron micrographs of outer membranes from cells of M3, B119 and A208 strains which were cultured in media with and without As. Outer membranes were stained with phosphotungstic acid and then observed at a magnification of 40 K. Panels A, A208 strain cultured with As; B, A208 strain cultured without As; C, B119 strain cultured with As; D, B119 strain cultured without As; E, M3 strain cultured with As; and F, M3 strain cultured without As. Arrow and arrowheads in panel A shows a flagellum (13 nm in diameter) and perfect pili (4 nm in diameter), respectively. The arrowheads in panel C showed "imperfect" pili which are a little thinner (2-3 nm) than "perfect pili" of A208 (parent) strain in panel A.

8). Some pilus-like structures were observed on the cell surface of B119 cultured in medium with As (panel C, Fig. 8), whereas no such fibrous structure was seen in media without As (panel D, Fig. 8). With M3, no fibrous structure was observed whether the cells were cultured in media with or without As (panel E and F, Fig. 8).

Electron microscopic observation of outer membranes from the three strains cultured in media with and without As

Many fibrous structures about 4 nm in diameter could be seen in the outer membrane fraction from the A208 strain cultured in medium with As (panel A, Fig. 9). Those thin fibrous structures were regarded as pili since the diameter of these structures was smaller than that (about 13 nm) of a flagellum (arrow in panel A) and coincided with the reported diameter (3.8 nm) of pili⁷⁾.

These structures were not seen with A208 strain when cultured in medium without As (panel B, Fig. 9). In the outer membranes from B119 strain cultured in medium with As, there were fewer fibrous structures which were thinner (2–3 nm in diameter) than that of A208 (panels A and C, Fig. 9), whereas no pilus-like structure was found in B119 cultures in medium without As (panel D, Fig. 9). Pilus-like structures were not seen in outer membranes from M3 cultured in media with and without As (panels E and F, Fig. 9). In the panels of Fig. 8 in which *A. tumefaciens* cells were observed, flagella were seen only on the A208 strain cultured in medium with As. However, in the samples exemplified in Fig. 9 in which outer membranes from a large number of cells were observed, all samples included a limited number of flagella, although panels from B to D in Fig. 9 do not show any flagella. The result suggested that the three strains produced a limited number of flagella regardless of the presence or absence of As.

DISCUSSION

The avirulent mutant strain (M3 strain) isolated was shown to be a mutant with a Tn5 insertion in the *chvA* gene on a chromosome which had been isolated earlier^{2,5)}. The *chvA* gene was isolated from an avirulent mutant with a reduced ability to attach to plant surfaces⁵⁾. The mutant had a defect in production of β -1,2-glucan, one of the superficial polysaccharides of *A. tumefaciens*. When the mutant was complemented, the ability to produce the glucan was restored. Thereafter, the *chvA* gene was shown to be involved in transport of β -1,2-glucan across the inner membrane²⁾. The *chvA* gene was cloned and its complete sequence was determined²⁾.

The gene product of *chvA* (ChvA protein, 64.7 kDa) is localized in the inner membrane²⁾. However, a role for β -1,2-glucan in attachment to the host plant and in virulence was suspected by Swart *et al.*²⁴⁾. They showed that β -1,2-glucan could not restore virulence and attachment ability of the mutant with a mutation in the *chvB* gene, which is responsible for β -1,2-glucan synthesis. Instead, the virulence and attachment ability of the mutant was restored by the addition of ricadhesin, a Ca-binding protein. Thus, β -1,2-glucan does not seem to be directly involved in the attachment to host and virulence. Nevertheless, a mutation in the *chvA* gene did completely knock out virulence on all plants tested, as shown in this paper and previous⁵⁾ paper. Therefore, we speculated that lack of β -1,2-glucan might cause the change in cell surface architecture of *A. tumefaciens* which in turn affects its virulence.

As has long been known to induce the *vir* genes on the Ti-plasmid during gall induction, in addition, some other processes might be induced by As during tumorigenesis. As a result, T-DNA transfer from the three strains to tobacco BY2 cells were examined in media with and without As (Fig. 5). As expected, A208 strain efficiently

transferred T-DNA into the host plant cells in the medium with As but not in the medium without As. Neither M3 nor B119 strain transferred T-DNA into the media, with or without As. However, T-strand (an intermediate form of T-DNA for transfer) was produced normally in the M3 strain as well as in the A208 strain (Fig. 6). Therefore, the processes until T-strand formation must have proceeded normally in M3 strain as shown with B119 strain previously¹⁰⁾. A blockage must occur in some stage following T-strand formation for both M3 and B119 strains.

Evidence has accumulated that the T-strand, formed inside *A. tumefaciens*, is transported into the cytoplasm of host cells through pili on *A. tumefaciens* cells^{1,7,11)}. Recently, Lai and Kado isolated the pilin subunit of the promiscuous conjugative pilus which is responsible for T-DNA transfer¹¹⁾. The pilin subunit was the 7.2 kDa processed product of the VirB2 protein.

According to the results obtained in this study, the M3 and B119 strains very likely have a defect in pilus formation. Tricine-SDS-PAGE of the outer membranes from the three strains revealed that M3 and B119 strains lacked some protein bands in the molecular weight range (5–15 kDa) corresponding to the pili proteins (panel A, Fig. 7). Pili were seen with A208 strain cultured with As but not with A208 strain cultured in the medium without As. Some pilus-like structures were seen on B119 strains when cultured in medium with As, but not when cultured in medium without As. No pili were seen on the M3 strain cultured in both media with and without As (Fig. 8 and 9). The pilus-like structures on B119 strain were regarded as “imperfect” pili based on the following two facts. First, they are thinner (2–3 nm in diameter) than that (4 nm in diameter) of the “perfect pili” of A208 strain (Fig. 9). Second, tricine-SDS-PAGE of the outer membrane fraction of B119 strain showed a decreased number of protein bands in the 5 and 15 kDa range (panel A, Fig. 7).

After considering the results and discussion together, we propose the mechanisms for the avirulent phenotypes of M3 and B119 strains as follows: the *chvA* gene, which functions to transport β -1,2-glucan across the inner membrane, is mutated in the M3 strain, thus inhibiting the accumulation of β -1,2-glucan on the cell surface. This decrease or loss of β -1,2-glucan, in turn, inhibits the formation of pili on the cell surface that are the channel for T-DNA transfer into the host plant cell. As a result, the M3 strain cannot transfer T-DNA into the host cell, although the T-strand is normally produced in the cell. In the B119 strain, the *acvB* gene, which produces the AcvB protein (47 kDa) localized in the periplasm, is mutated. The lack of the AcvB protein in the periplasm, in turn, might affect the formation of pili, resulting in the formation of imperfect pili. Thereby, the B119 strain cannot transfer the T-strand produced inside the cell to the host cell.

These proposed mechanisms for the avirulence of the M3 and B119 strains are also supported by our previous

results⁶⁾. A208 strain could transfer T-DNA to tobacco BY2 cells regardless of the presence or absence of cell walls. B119 could transfer T-DNA to protoplasts of BY2 cells, but could not transfer T-DNA to BY2 cells having cell walls. However, M3 could not transfer T-DNA to BY2 cells, regardless of the presence or absence of cell walls. These results are consistent with the model and the results presented in this paper; the A208 strain produced "perfect" pili, the B119 strain produced "imperfect" pili and the M3 strain produced no pili.

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和 文 摘 要

P. MAJUMDER・高木健一郎・塩入秀成・野末雅之・小島峯雄：
Agrobacterium tumefaciens の染色体上の病原性遺伝子，*acvB*
および *chvA* の transposon 5 挿入非病原性変異菌を用いた機
能解析

トランスポゾン (Tn5) 突然変異法を用いて，*A. tumefaciens*
(A208 菌，病原性) より非病原性変異菌 (M3 菌) を分離した。
M3 菌はニンジン，ジャガイモ等，供試植物すべてに非病原性を
示した。M3 菌は染色体上の病原性遺伝子 (*chvA*) に Tn5 が 1
個挿入されたために非病原性を示すことがわかった。この M3
菌と我々が以前分離した B119 菌 (染色体上病原性遺伝子 *acvB*
に Tn5 が 1 個挿入されたために非病原性を示す) および親株
(A208 菌) を用いて，両遺伝子の機能解析を行った。上記 3 菌株
とタバコ BY2 細胞を共培養したところ，T-DNA のタバコ細胞
核内への効率的転移はアセトシリンゴン (As) を含む培地中で，
A208 菌でのみ起こった。しかし，As 添加培地中で，非病原性菌

M3 と B119 菌でも T-DNA の転移中間体型である T-strand の
形成までの反応は正常に進行していた。3 菌株を T-DNA の転
移が起こる As 添加培地と，転移が起こらない As 無添加培地で
培養し，それぞれの菌から外膜，ペリプラズム可溶性タンパク
質，スフェロプラストを調製し，tricine-SDS-ポリアクリルアミ
ドゲル電気泳動でタンパク質成分を分析した。As 添加培地中で
培養した菌の外膜画分において，3 菌株間に顕著な差が認めら
れた。線毛の構成タンパク質 (pilin) が含まれると推定される
5~15kDa の領域に，M3 菌は淡い 2 本，B119 菌は鮮明な 2 本，
A208 菌は 7~8 本のタンパクバンドが検出された。上記の菌株
細胞と外膜を電子顕微鏡で観察したところ，As 添加培地中で
A208 菌は“完全な”線毛を，B119 菌は“不完全な”線毛を形成
していた。一方，M3 菌ではまったく線毛が観察されなかった。
線毛は，T-DNA の通過経路であることが明らかにされつつあ
り，このことと上記の結果を考えあわせると，*A. tumefaciens* の
染色体上の病原性遺伝子，*acvB* と *chvA* は T-DNA の宿主植物
細胞への通過経路である線毛の形成に対する影響を通して病原
性に関与していると推定される。