JOURNAL OF BACTERIOLOGY, May 1983, p. 906–915
0021-9193/83/050906-10$02.00/0

Vol. 154, No. 2

Role of Bacterial Cellulose Fibrils in *Agrobacterium tumefaciens* Infection

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Received 29 July 1982/Accepted 12 February 1983

During the attachment of *Agrobacterium tumefaciens* to carrot tissue culture cells, the bacteria synthesize cellulose fibrils. We examined the role of these cellulose fibrils in the attachment process by determining the properties of bacterial mutants unable to synthesize cellulose. Such cellulose-minus bacteria attached to the carrot cell surface, but, in contrast to the parent strain, with which larger clusters of bacteria were seen on the plant cell, cellulose-minus mutant bacteria were attached individually to the plant cell surface. The wild-type bacteria became surrounded by fibrils within 2 h after attachment. No fibrils were seen with the cellulose-minus mutants. Prolonged incubation of wild-type *A. tumefaciens* with carrot cells resulted in the formation of large aggregates of bacteria, bacterial fibrils, and carrot cells. No such aggregates were formed after the incubation of carrot cells with cellulose-minus *A. tumefaciens*. The absence of cellulose fibrils also caused an alteration in the kinetics of bacterial attachment to carrot cells. Cellulose synthesis was not required for bacterial virulence; the cellulose-minus mutants were all virulent. However, the ability of the parent bacterial strain to produce tumors was unaffected by washing the inoculation site with water, whereas the ability of the cellulose-minus mutants to form tumors was much reduced by washing the inoculation site with water. Thus, a major role of the cellulose fibrils synthesized by *A. tumefaciens* appears to be anchoring the bacteria to the host cells, thereby aiding the production of tumors.

Infection of dicotyledonous plants with *Agrobacterium tumefaciens* results in the formation of crown gall tumors. The first step in the infection process is the site-specific attachment of the bacteria to the plant host (7). We have demonstrated specific attachment of *A. tumefaciens* species to tissue culture cells (13). During the course of this attachment, the bacteria synthesize cellulose fibrils that cover the surface of the plant cells (11). We have proposed that these fibrils serve to anchor the bacteria to the plant cell surface and that, as the fibrils grow longer, they entrap other bacteria, forming the large clusters observed on the host cell surface (11). Eventually, plant cells, attached and entrapped bacteria, and bacterial cellulose fibrils form large aggregates visible to the unaided eye (11). To examine the role of bacterial cellulose fibrils in these processes, we have isolated transposon mutants of *A. tumefaciens* with altered ability to synthesize cellulose fibrils. This paper describes the construction and properties of the mutants.

MATERIALS AND METHODS

Strains. Bacteria were grown and viable cell counts were determined as previously described (13). A virulent strain of *A. tumefaciens* A6 was obtained from A. Braun, Rockefeller University, New York, N.Y. *Escherichia coli* 1830(pJB4JI), Gm4(pHM5), and RV1(Mu cts62) were obtained from F. Ausubel, Harvard University, Boston, Mass. *E. coli* RR1(pNW31c-2,19-1), whose plasmid contains the cloned *Bam*HI fragments 2 and 19 from pTi-A6, was obtained from M. Thomashow, University of Washington, Seattle (17). Suspension cultures of *Daucus carota* were obtained from W. Boss, North Carolina State University, Raleigh, and maintained as previously described (11).

Construction of mutants. To introduce transposon Tn*5* into *A. tumefaciens*, the auxotrophic (*pro met*) *E. coli* 1830(pJB4JI) was mated with *A. tumefaciens* A6 at 28°C by the procedure described by Beringer et al. (1). pJB4JI is a wide host range conjugative P1 plasmid containing Mu, Tn*5* (which confers neomycin resistance), and gentamycin resistance. It was constructed for use in introducing Tn*5* into *Rhizobium* and *Agrobacterium* species (1). The plasmid itself is unstable in these hosts and is generally not maintained in them. Transconjugants were selected for their ability to grow on minimal medium with 60 μg of neomycin per ml. Neither parent grew under these conditions. The resulting colonies were screened for cellulose synthesis by examining their fluorescence under UV light after they were grown on minimal medium with 3 mg of glucose per ml, 60 μg of neomycin per ml, and 200 μg of Calcofluor (American Cyanamid Co.) per ml. Calcofluor is a fluorescent stain for cellulose (4). Any colonies with apparently altered staining properties

were screened for fluorescent staining with Calcofluor by examining their growth on minimal medium (10) with 2 mg of glucose per ml, 60 μg of neomycin per ml, and 200 μg of Calcofluor per ml and on Luria broth agar (13) containing 60 μg of neomycin per ml, 200 μg of Calcofluor per ml, and 200 μg of Soytone (Difco Laboratories) per ml. Soytone was used because, under some conditions, its presence in the medium causes increased cellulose synthesis by *A. tumefaciens* species (11). Bacterial colonies that showed altered cellulose synthesis on both of these solid media were picked and transferred to 5 ml of Luria broth containing 0.02% Soytone and grown in a tube on a roller drum to stationary phase. Samples of the cultures were examined for the presence of cellulose by Calcofluor staining and fluorescence microscopy as previously described (11). Approximately 10^4 transconjugants were originally examined on minimal agar containing Calcofluor. Of these, seven did not produce any detectable cellulose under any of the growth conditions described above, and three produced colonies that stained more brightly for cellulose than did the parent strain. None of the transconjugants was gentamycin resistant, confirming that pJB4JI was not maintained in *A. tumefaciens*.

The cellulose mutants were maintained in Luria broth or agar with neomycin. Under these conditions, six of the seven cellulose-minus strains appeared to be stable. When plated on Calcofluor-containing medium, the seventh strain (Ce-13) gave rise to occasional fluorescent colonies. One of these revertants was isolated and examined as described below. In contrast, the three mutants that showed brighter fluorescence with Calcofluor than did the parent strain were not stable in liquid medium and gave rise to revertants at a high frequency. These mutant bacteria grew as an aggregate in liquid medium, and 10% or more of the bacteria not contained in the aggregate were revertants which showed normal fluorescence with Calcofluor. Thus, there may be a strong selection against increased bacterial cellulose synthesis under these conditions.

DNA isolation and hybridization. To determine the location of Tn*5* in these transposon mutants, bacterial plasmid and chromosomal DNAs were separated by lysing the bacteria in the well of an agarose gel and separating plasmid and chromosomal DNAs by electrophoresis by the procedure of Taylor et al. (R. Taylor, A. G. Wood, D. H. Hubbell, and D. E. Duggan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, H31, p. 118). The DNA was transferred from the gel to nitrocellulose filter paper (16) and hybridized to radioactive Tn*5* DNA prepared by nick translation (9) of the plasmid pHM5, which contains Tn*5* (14). pHM5 DNA was isolated by the method of Klein et al. (6) or Clewell and Helinski (2). The bacterial mutants were also examined for the presence of Mu DNA (which could have arisen from pJB4JI) by hybridization to nick-translated Mu DNA prepared from *E. coli* RV1 (Mu *c*ts62) by the procedure of Miller (15). Neither pHM5 nor Mu DNA showed any detectable hybridization to wild-type *A. tumefaciens* A6. To identify the Ti plasmid unambiguously, Southern blots of gels were also hybridized to pNW31c-2,19-1 labeled by nick translation. Hybridizations, washing, and autoradiography were carried out by the method of Wahl et al. (18).

To determine whether the site of Tn*5* insertion in the different mutants isolated was the same or different, we examined the hybridization of Tn*5* to Southern blots of *Eco*RI digest fragments of DNA isolated from the various mutants. DNA from *A. tumefaciens* was prepared by lysing the bacteria in 50 mM Tris-hydrochloride (pH 8)–20 mM EDTA (pH 8)–0.5 mg of pronase per ml–1% sodium dodecyl sulfate–2 μg of RNase A per ml at 37°C (3). The lysate was extracted twice with phenol, and the DNA was precipitated by spooling after the addition of an equal volume of isopropyl alcohol. The precipitate was dissolved in 1 mM Tris-hydrochloride (pH 8) and dialyzed extensively against 1 mM Tris-hydrochloride (pH 8). Restriction endonuclease *Eco*RI was purchased from New England BioLabs and used under the conditions specified.

Interaction of bacteria with plant cells. Measurements of the ability of the bacteria to attach to carrot cells and to cause the aggregation of carrot cell suspension cultures were made as previously described (11, 13). Bacterial virulence was determined by infecting *Nicotiana tabacum* cv. Coker 319, *Bryophyllum daigremontiana*, and *Phaseolus vulgaris* cv. Long Tendergreen. All plants were grown in the greenhouse. Tobacco stems and *B. daigremontiana* leaves were infected by wounding the plant with a toothpick containing a colony of *A. tumefaciens*. Bean leaves were infected with 0.05 ml of a suspension of bacteria in phosphate-buffered saline, as described by Lippincott and Heberlein (8).

Preparation of carrot cell protoplasts and examination of bacterial attachment to the protoplasts by scanning electron microscopy was carried out as previously described (12).

The ability of the various bacterial strains to remain attached to wound sites during a water wash was determined by infecting toothpick wounds on *B. daigremontiana* leaves with one bacterial colony per wound site. Each leaf was infected at 16 to 20 sites, of which at least 6 sites were inoculated with the parent wild-type A6 strain, and the remaining sites were inoculated with various mutants. Some leaves were washed 2 h after inoculation for 10 s by pouring 60 ml of water slowly over the leaf surface. Leaves were scored for tumor formation during the succeeding 5 weeks.

RESULTS

Characteristics of bacterial mutants. We obtained seven Tn*5*-induced bacterial mutants that did not synthesize any Calcofluor-fluorescent material when grown on Calcofluor-containing minimal agar or Luria broth with Soytone agar. These strains also did not synthesize any material that showed fluorescent staining with Calcofluor by the criterion of fluorescence microscopy. The parent A6 strain showed bright Calcofluor-fluorescent staining under all of these conditions. None of these strains formed aggregates when grown in Luria broth with Soytone, whereas the parent strain formed large clumps (Fig. 1 and Table 1) that contained many cellulose fibrils when examined with a fluorescent

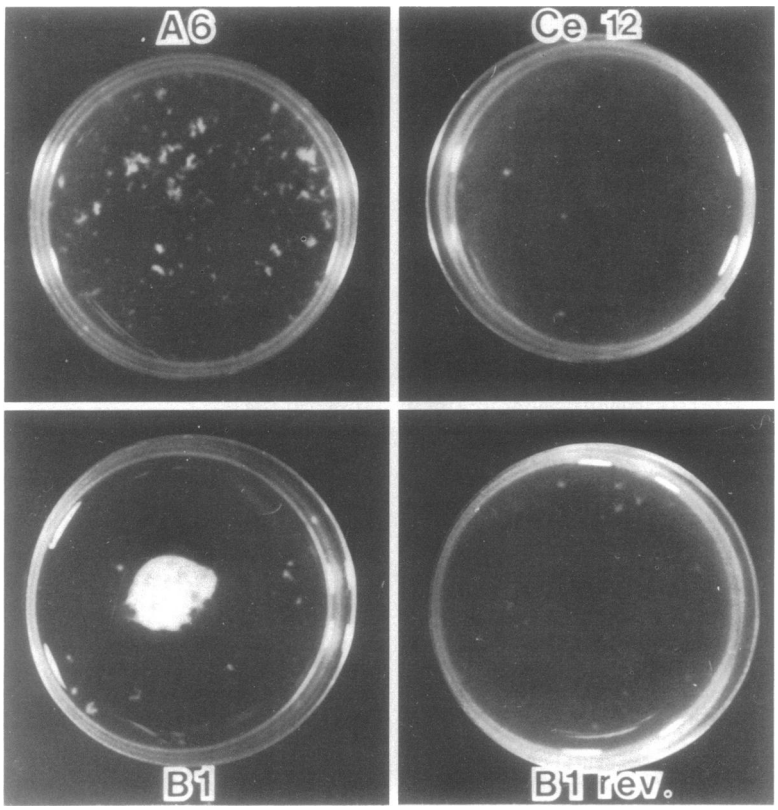

FIG. 1. Aggregation of bacteria grown in Luria broth with Soytone. A6, Parent strain, cellulose positive; Ce-12, cellulose-minus mutant; B1, Calcofluor-bright mutant; B1 rev, revertant of B1 that showed normal staining with Calcofluor. The parent strain aggregated when grown in Luria broth with Soytone. None of the cellulose-minus mutants showed any aggregation. The three Calcofluor-bright mutants all grew as large aggregates. Revertants of these mutants to normal Calcofluor staining also showed more normal growth in Luria broth with Soytone, although the amount of aggregation was sometimes less than that seen with wild type.

light microscope or a scanning electron microscope (11). Thus, these mutant strains appeared to be unable to synthesize significant amounts of cellulose. One of these strains (Ce-13) gave rise to occasional revertant colonies that showed fluorescent staining with Calcofluor. This revertant also formed aggregates when grown in Luria broth with Soytone.

An unexpected product of the introduction of Tn*5* into *A. tumefaciens* was three bacterial mutants which showed brighter fluorescent staining with Calcofluor than did the A6 parent. These strains grew as clumps in Luria broth or minimal medium and were difficult to work with because of their extreme tendency to aggregate (Fig. 1 and Table 2).

All of the mutant strains were gentamycin sensitive, and none showed any evidence of retention of pJB4JI when plasmid DNA preparations from them were examined by gel electrophoresis. Two of the cellulose-minus strains, Ce-4 and Ce-13. appeared to have retained at least some Mu DNA, as judged by hybridization with Mu. None of the other mutants showed any hybridization with Mu DNA. All of the mutants contained Tn*5* DNA, as judged by hybridization. In six of the seven cellulose-minus mutants and in the three Calcofluor-bright mutants, the Tn*5* appeared to be located in the bacterial chromosome, suggesting that many of the genes required for cellulose synthesis are chromosomal genes in *A. tumefaciens*. This is consistent with the observation that two strains of *A. tumefaciens* that lack the Ti plasmid (NT1 and ACH5C3) synthesize cellulose (unpublished observation). However, in one mutant, Ce-12, the transposon appeared to be located in the large cryptic plasmid of A6 (Fig. 2). Ti plasmid DNA, purified from Ce-12, showed no differences in the band patterns resulting from digestion with *Sma*1 and *Hpa*I, confirming that the Ti plasmid did not contain the transposon (data not shown).

To determine whether Tn*5* was integrated in different or similar locations in the mutants,

TABLE 1. Characteristics of cellulose-minus bacterial strains

Strain	Characteristic									
	Cellulose synthesis	Presence of Mu	Location of Tn*5*	Aggregation in Luria broth + Soytone	Attachment to carrot cells	Ability to aggregate carrot cells	Virulence			Cellulose synthesis by bacteria recovered from tumor
							B. daigremontiana	*N. tabacum*	*P. vulgaris*	
A6 parent	+	No	−	+	+	+	+	+	+	+
Ce-1	−	No	Chr[a]	−	ND[b]	−	+	+	+	−
Ce-2	−	No	Chr	−	+	−	+	+	+	−
Ce-3	−	No	Chr	−	ND	−	+	+	NT[c]	−
Ce-4	−	Yes	Chr	−	+	−	+	+	NT	−
Ce-5	−	No	Chr	−	ND	−	+	+	NT	+ and −
Ce-12	−	No	Plasmid	−	+	−	+	+	+	−
Ce-13	−	Yes	Chr	−	ND	−	+	+	+	+ and −
Ce-13 revertant	+	Yes	Chr	+	+	+	+	NT	NT	+

[a] Chr, Chromosome.
[b] ND, Not detectable in a kinetic assay (<5%). All strains showed attachment in a microscopic assay in which 0.5% attachment could be detected.
[c] NT, Not tested.

TABLE 2. Characteristics of Calcofluor-bright bacterial strains

Strain	Characteristic								
	Cellulose synthesis	Presence of Mu	Location of Tn*5*	Aggregation in Luria broth	Attachment to carrot cells	Ability to aggregate carrot cells	Virulence		Cellulose synthesis by bacteria recovered from tumor
							B. daigremontiana	*N. tabacum*	
A6 parent	+	No	−	+	+	+	+	+	+
B1	++	No	Chr[a]	++	ND[b]	+	+	+	++ and +
B3	++	No	Chr	++	+	+	+	+	++ and +
B4	++	No	Chr	++	+	+	+	+	++ and +
B1 revertant	+	No	Chr	+	NT[c]	+	+	NT	NT

[a] Chr, Chromosome.
[b] ND, Not detectable.
[c] NT, Not tested.

radioactive Tn*5* DNA was hybridized to Southern blots of bacterial DNA digested with the restriction endonuclease *Eco*RI. *Eco*RI was chosen because Tn*5* lacks any *Eco*RI sites (5). The size of the *Eco*RI band that hybridized with Tn*5* was different for each of the cellulose-minus and Calcofluor-bright mutants (Fig. 3). The cellulose-positive revertant of Ce-13 still contained Tn*5* but in a new location, as judged by Tn*5* hybridization to *Eco*RI digests of Ce-13 and Ce-13 revertant. Thus, each of the mutants isolated appears to have resulted from the insertion of Tn*5* at a different location. This suggests that several gene products may be required for cellulose synthesis in *A. tumefaciens*.

Interactions of cellulose mutants with carrot tissue culture cells. When *A. tumefaciens* A6 is added to carrot suspension cultures, the bacteria attach to the surfaces of the plant cells (11, 13).

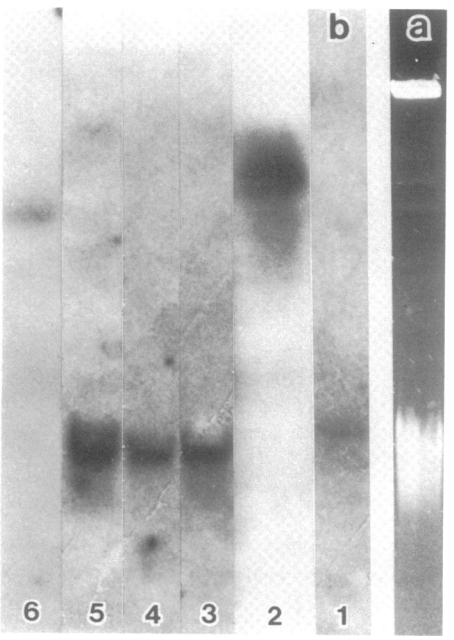

FIG. 2. Hybridization of Tn*5* with plasmid and chromosomal DNA from various cellulose mutants of *A. tumefaciens*. (a) Ethidium bromide-stained agarose gel. Strain A6 bacteria were lysed in the well. The bottom band is chromosomal DNA, and the middle band is Ti plasmid DNA, as determined by hybridization to cloned pTi DNA (lane 6). (b) Autoradiogram of other channels from the gel in (a) hybridized to ^{32}P-labeled Tn*5* DNA. The bacterial strains in various lanes were as follows: lane 1, Ce-1; lane 2, Ce-12; lane 3, B1; lane 4, Ce-13; and lane 5, Ce-13 revertant. Hybridization patterns like those in lanes 4 and 5 were also obtained with mutants Ce-2, Ce-3, Ce-4, Ce-5, and B3. Tn*5* appeared to be located in the chromosomal DNA of all strains except Ce-12, in which the transposon appeared to hybridize to the plasmid DNA from the large cryptic plasmid contained in strain A6.

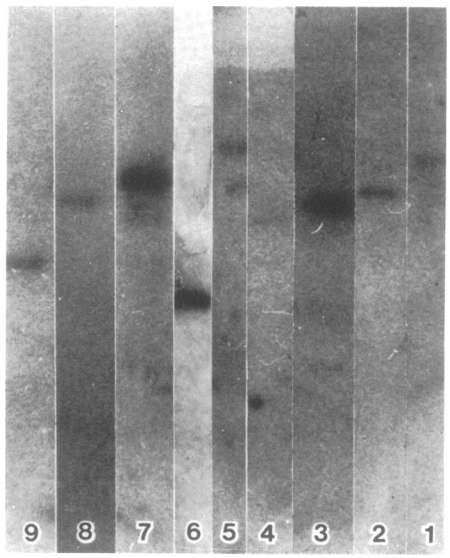

FIG. 3. Location of Tn*5* in the DNA from various cellulose mutants of *A. tumefaciens*. DNA was extracted from various bacterial mutants, digested with restriction endonuclease *Eco*RI, separated by gel electrophoresis, transferred to nitrocellulose paper, and hybridized to ^{32}P-labeled Tn*5* DNA. The bacterial strains in the various lanes were as follows: lane 1, Ce-1; lane 2, Ce-2; lane 3, Ce-3; lane 4, Ce-4; lane 5, Ce-5; lane 6, Ce-12; lane 7, Ce-13; lane 8, Ce-13 revertant; and lane 9, B1. Tn*5* hybridized to a different-sized band in each of the mutant strains.

The attached bacteria synthesize cellulose fibrils which cover the surface of the host cell. After a few hours, large clumps of bacteria and bacterial fibrils are seen on the plant cell surface. Eventually the bacteria, bacterial fibrils, and carrot

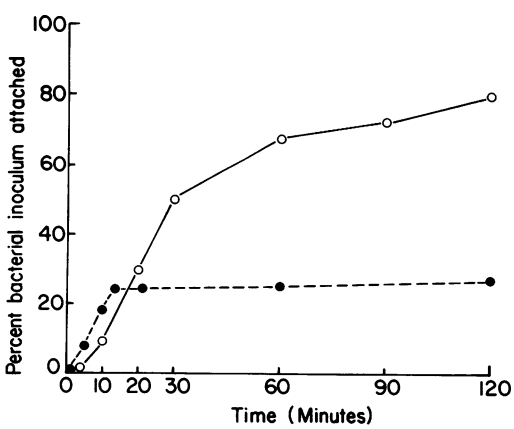

FIG. 4. Time course of attachment of *A. tumefaciens* A6 (○) (cellulose positive) and Ce-12 (●) (cellulose minus) to carrot suspension cells. The cellulose-minus mutant lacked the second phase of attachment seen with the parent strain.

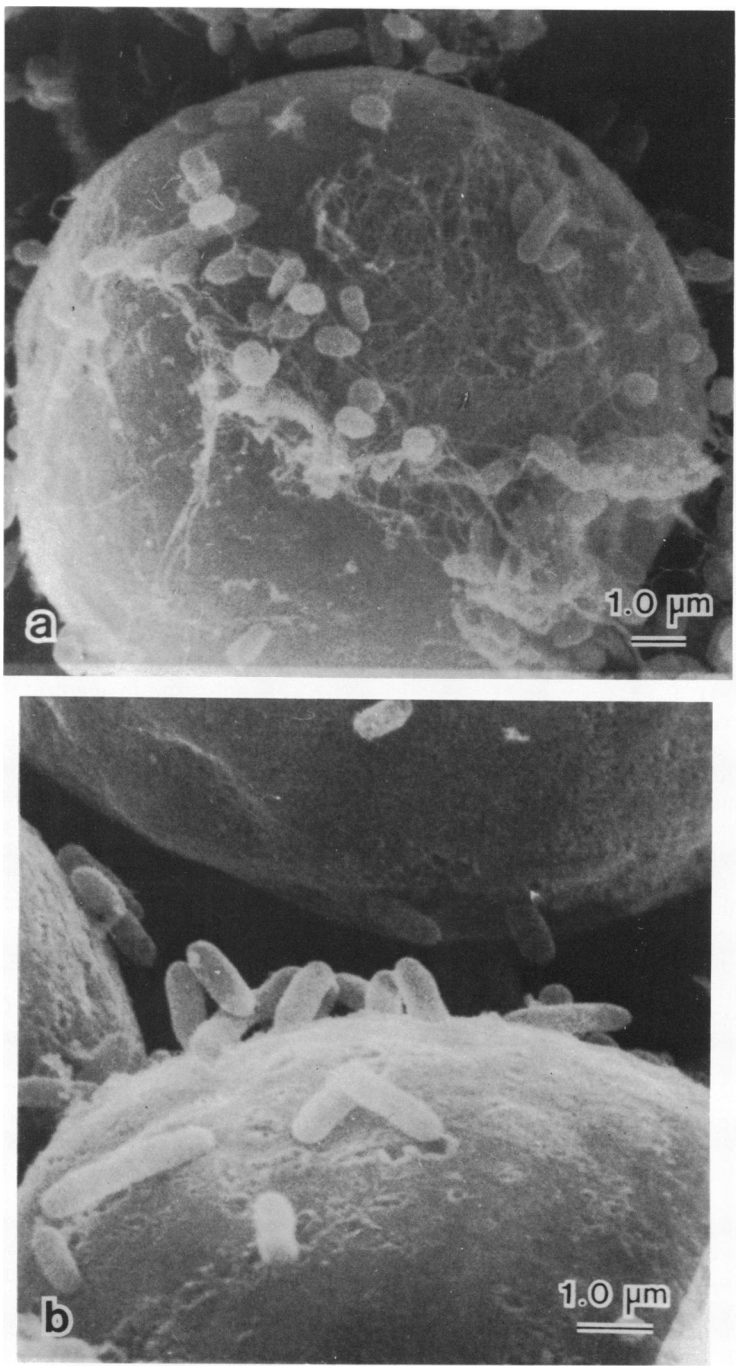

FIG. 5. Scanning electron micrographs of *A. tumefaciens* incubated with carrot protoplasts for 19 h. (a) Strain A6 (cellulose positive). (b) Strain Ce-12 (cellulose minus). Note the numerous fibrils and clusters of bacteria present with A6 which are absent with Ce-12.

cells form a large aggregate that involves most of the carrot cells in the culture flask (11). The kinetics of this bacterial attachment is unusual in that the number of bacteria attached to the host cells does not saturate but continues to increase with increasing length of incubation time (11, 13; Fig. 4).

The attachment of the cellulose-minus mutant bacteria to carrot suspension cultures was examined. When compared to the A6 parent strain, all

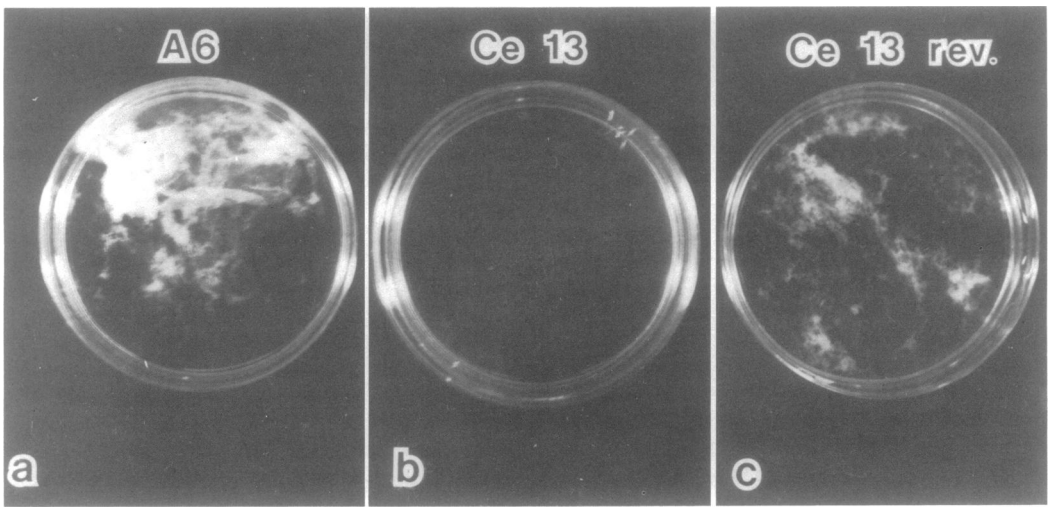

FIG. 6. Aggregation of carrot suspension cultures after incubation with *A. tumefaciens* for 19 h. (a) Strain A6, (cellulose-positive parent). (b) Strain Ce-13 (cellulose minus). (c) Strain Ce-13 revertant (cellulose positive). None of the cellulose-minus mutants caused the aggregation of carrot cells.

of these strains showed reduced attachment. Some strains (Ce-1, Ce-3, Ce-5, and Ce-13) did not attach to carrot cells at a detectable level in a kinetic assay (less than 5% of the bacterial inoculum attached) (Table 1). All of the cellulose-minus mutants showed attachment to carrot cells in a light microscope. The kinetics of attachment of one cellulose-minus strain, Ce-12, to carrot cells was examined in detail (Fig. 4). This mutant appeared to show a normal initial rate of attachment but to lack the continued attachment with increasing incubation times shown by A6. This observation tends to confirm the hypothesis that this second phase of attachment of A6 is due to the entrapment of bacteria in the cellulose fibrils synthesized by previously attached bacteria.

The attachment of the same mutant, Ce-12, to carrot protoplasts was examined in the scanning electron microscope and compared to the attachment of the parent strain A6 (Fig. 5). As reported previously, A6 cells were bound to the plant protoplast cell surface in large clusters of bacteria held together by bacterial fibrils. In contrast, Ce-12 cells were bound to the plant cell surface as individual bacteria; no large bacterial clusters or fibrils were visible. Thus, the formation of large aggregates of bacteria on the surface of the host cell may be dependent on cellulose fibril synthesis by the bacteria.

We next tested the ability of the cellulose-minus bacterial mutants to cause the formation of large aggregates of carrot suspension culture cells. The wild-type parent and the cellulose-positive revertant (Ce-13 *rev*) caused the formation of such aggregates, but incubation of carrot cells for 19 h with the cellulose-minus *A. tumefaciens* did not result in aggregate formation (Fig. 6 and Table 1).

Interactions of cellulose mutants with plants. Virulence of the *A. tumefaciens* mutants was tested on *B. daigremontiana* and tobacco. All mutant strains were virulent on both plants (Tables 1 and 2 and Fig. 7). Since it was possible that an occasional bacterial revertant could have initiated the tumor, bacteria were isolated from the tumors and examined for their ability to synthesize cellulose. Only those bacteria unable to synthesize cellulose were recovered from tumors caused by Ce-1, Ce-2, Ce-3, Ce-4, and Ce-12, suggesting that the cellulose-minus mutant was indeed the cause of the tumor. Strain Ce-13, which gave rise to spontaneous revertants in culture, also gave rise to revertants in the plant, and Ce-5, which has not previously been observed to revert in culture, gave rise to cellulose-positive revertants in the plant. Thus, for these two mutants, it was not possible to be certain whether the mutant itself or only its revertant was virulent. The virulence of four of the mutant strains, Ce-1, Ce-2, Ce-12, and Ce-13, was also measured on leaves of *P. vulgaris*. This virulence assay permits a semiquantitative determination of the number of tumors produced per bacterium inoculated. The assay is roughly linear from 10^6 to 10^8 bacteria inoculated (8). The four mutant strains tested and the parent A6 strain all produced between 30 and 70 tumors per 5×10^7 bacteria inoculated.

However, on *B. daigremontiana*, the cellulose-minus mutants generally gave rise to a somewhat more variable response than did the

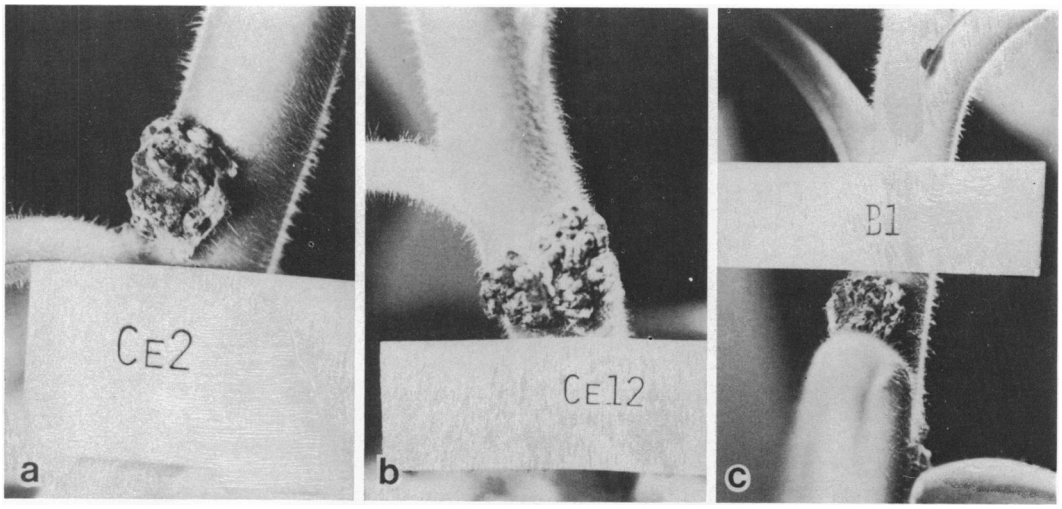

FIG. 7. Virulence of mutant bacterial strains on *N. tabacum*. A bacterial colony was inoculated into a wound site on the stem. (a) Strain Ce-2 (cellulose minus). (b) Strain Ce-12 (cellulose minus). (c) Strain B1 (Calcofluor bright). All of the cellulose mutants were virulent on tobacco.

parent strain (Table 3). The parent strain, A6, caused the formation of tumors at all sites at which it was inoculated. The cellulose-minus mutants varied from Ce-3, which caused tumors at 100% of the inoculation sites, to Ce-4, which caused tumors at an average of only 60% of the inoculation sites, with a large variation between replicate experiments.

The three Calcofluor-bright strains were virulent on both *B. daigremontiana* and tobacco. However, when bacteria were isolated from the tumors, both Calcofluor-bright and normal *A. tumefaciens* were recovered. Thus, it was not possible to be certain that these mutants were truly virulent.

TABLE 3. Effect of washing on tumor formation by cellulose-minus bacterial strains[a]

Bacterial strain	Cellulose synthesis	% Inoculation sites developing tumors ± SD[b]	
		Untreated	Washed after inoculation
A6	+	100 ± 0	86 ± 15
Ce-1	−	90 ± 14	35 ± 44
Ce-2	−	83 ± 5	20 ± 15
Ce-3	−	100 ± 0	59 ± 42
Ce-4	−	60 ± 46	36 ± 44
Ce-5	−	70 ± 42	40 ± 14
Ce-12	−	72 ± 48	5 ± 8
Ce-13	−	76 ± 38	23 ± 20
Ce-13 revertant	+	86 ± 19	87 ± 15

[a] Bacteria were inoculated into toothpick wounds on *Bryophyllum* leaves. Each leaf was washed with 60 ml of water 2 h after inoculation.

[b] Standard deviation of a minimum of three experiments.

All of the virulence measurements reported above were carried out in plants with which precautions were taken to avoid disturbance of the site of bacterial inoculation. Since cellulose fibrils appeared to be involved in the attachment of bacteria to the plant host, the effect of gently washing the inoculation site 2 h after inoculation was measured. (Previous experiments suggest that, by 2 h after infection, *A. tumefaciens* A6 begins to synthesize cellulose fibrils [11]). The percentage of inoculation sites that developed tumors was unaffected by water washing for the cellulose-synthesizing strains A6 and Ce-13 revertant. However, water washing markedly reduced tumor formation by all of the cellulose-minus mutants (Table 3 and Fig. 8), suggesting that bacterial cellulose may be important in the ability of *A. tumefaciens* to adhere to wound sites under adverse conditions such as rainstorms.

DISCUSSION

The isolation of mutants of *A. tumefaciens* that do not synthesize cellulose allows an assessment of the role of cellulose synthesis in the attachment of the bacteria to the plant host cell and in the production of disease. Cellulose synthesis was not required for bacterial attachment to carrot tissue culture cells, but bacterial mutants that did not synthesize cellulose attached only individually to the plant cells and did not form clusters of attached bacteria. The large aggregates of bacteria and carrot cells formed by *A. tumefaciens* were not seen with cellulose-

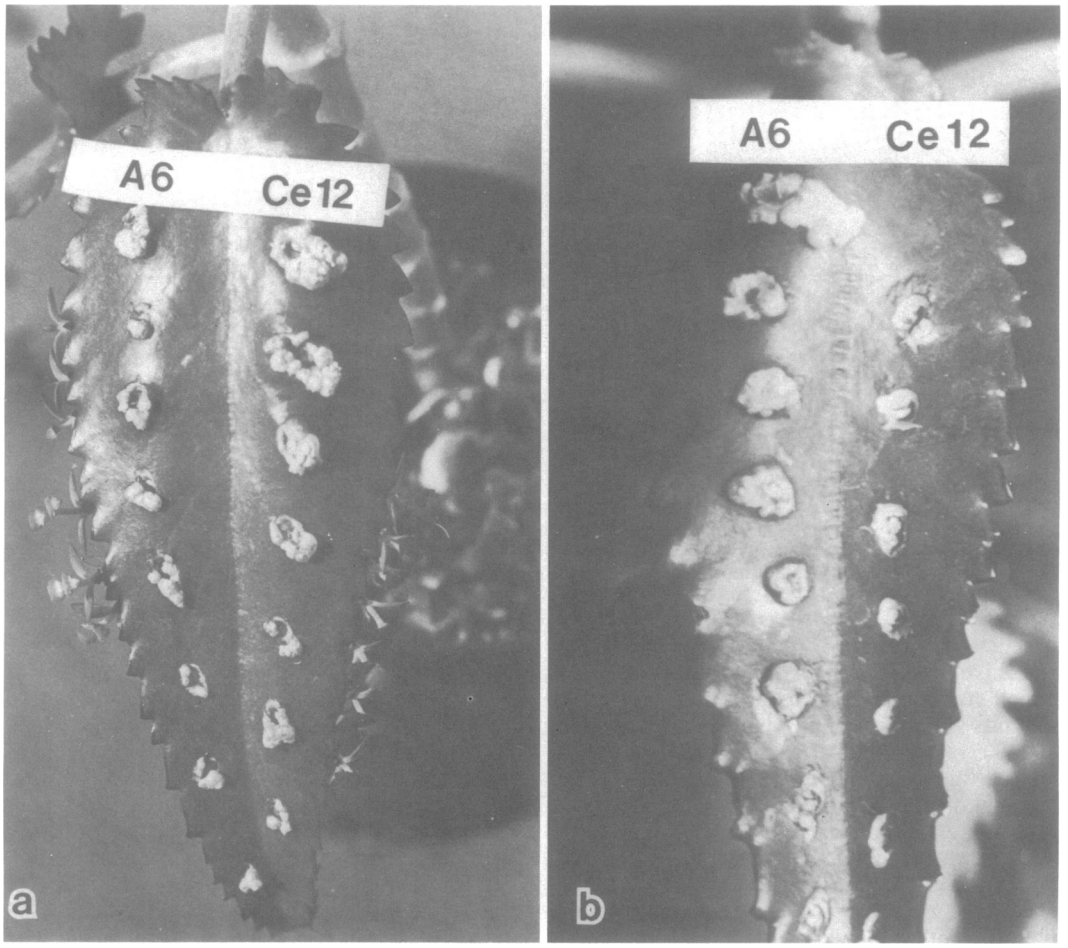

FIG. 8. Effect of water washing on virulence of *A. tumefaciens* A6 and Ce-12. (a) *B. daigremontiana* leaf inoculated with A6 and Ce-12. Both strains induced the formation of tumors at each wound site. (b) *B. daigremontiana* leaf inoculated with A6 and Ce-12 and washed with water 2 h postinoculation. Strain A6 still induced tumors at all inoculation sites, but Ce-12 failed to induce tumors at most inoculation sites.

minus bacterial mutants. The kinetics of bacterial attachment was also altered with cellulose-minus bacterial mutants. Thus, cellulose fibril synthesis by *A. tumefaciens* appears to be responsible for the peculiar kinetics of bacterial attachment and the tendency of the bacteria to attach in large clusters.

Bacterial mutants that were unable to synthesize cellulose and mutants that appeared to synthesize additional (or possibly altered) cellulose were both virulent under laboratory conditions. However, the mutants which synthesized extra cellulose were apparently at a selective disadvantage in liquid broth cultures, which were rapidly taken over by revertants that synthesized less cellulose. Mutants that were unable to synthesize cellulose, although virulent in the laboratory, were unable to adhere to a wound site during a brief water wash. This wash had no effect on the virulence of the cellulose-synthesizing wild-type strain. The cellulose-minus mutants would presumably have reduced virulence under natural conditions. Thus, the principal role of cellulose synthesis by *A. tumefaciens* appears to be the firm attachment of large numbers of bacteria to the host cell.

ACKNOWLEDGMENTS

I thank Patricia W. Lamb for excellent technical assistance and Fred Ausubel and R. Malcolm Brown, Jr., for helpful discussions. I am grateful for the help of Kathryn V. Holmes in performing the scanning electron microscopy.

This research was supported by Public Health Service research grant CA18604 from the National Cancer Institute.

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