Research Note

Attachment to Roots and Virulence of a *chvB* Mutant of *Agrobacterium tumefaciens* Are Temperature Sensitive

Ryan Bash and Ann G. Matthyse

Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280, U.S.A.

Submitted 10 July 2001. Accepted 15 October 2001.

***Agrobacterium tumefaciens chvB* mutants are unable to produce β -1,2 glucan. They are nonattaching and avirulent and show reduced motility at room temperature. At lower temperatures (16°C), *chvB* mutants became virulent on *Bryophyllum daigremontiana* and *Lycopersicon esculentum* and were able to attach to *L. esculentum*, *Arabidopsis thaliana*, *Daucus carota*, and *Tagetes erecta* roots. The mutant bacteria also recovered wild-type motility at lower temperatures. Two other nonattaching mutants of *A. tumefaciens*, AttR and AtrA, were unaffected by the lowered temperature, remaining nonattaching and avirulent.**

Additional keywords: temperature-sensitive mutants, β -1,2-D-glucans.

Agrobacterium tumefaciens induces crown gall tumors in wound sites on dicotyledonous plants. All currently known, nonattaching mutants of *A. tumefaciens* are avirulent. This includes *chvB* mutants, which are deficient in β -1,2 glucan synthesis due to a Tn5 insertion. These mutants do not attach to plant cells or induce tumors at wound sites when assayed at room temperature. However, a recent study by Banta and associates (1998) found that *chvB* mutant A1020 is virulent at temperatures below 22°C. This finding raises the question of the dependence of virulence on attachment. Is attachment not required for virulence at low temperature, or are *chvB* mutants able to attach to plant cells at low temperature? The results reported below show that the *chvB* mutants were able to attach to plant root segments at 16°C. They were also virulent when inoculated into wounds on *Bryophyllum daigremontiana* leaves and *Lycopersicon esculentum* stems at the same temperature.

The ability of wild-type strain C58 and of several nonattaching mutants of this strain to bind to roots was examined. Two *chvB* mutants that differ from each other and from the mutant studied by Banta and associates (1998) in the location of the Tn5 insertion were studied. In order to determine whether the recovery of binding to the host surface at temperatures below room temperature was particular to *chvB* mutants or a general property of all nonattaching mutants, we examined the behavior of two other nonattaching avirulent mutants. Nonattaching mutants of *A. tumefaciens* can be divided into two groups, those that appear to be altered in signal exchange between the bacterium and the plant host and those that appear to be altered at some later step in attachment. The first group can be complemented by conditioned medium and the second group can not. One mutant from each group was chosen for study. *attR*

mutants lack a gene that encodes an acetyltransferase necessary for the production of acetylated capsular polysaccharide. This polysaccharide appears to be required for attachment (Reuhs et al. 1997). This mutant (A205) is not complemented by conditioned media (Matthyse et al. 2000). *atrA* mutants lack a gene required for signaling between the bacteria and the host. This mutation (M211) results in lack of attachment and avirulence and is complemented by conditioned medium (Matthyse et al. 2000). The bacterial strains used are shown in Table 1. They were grown in Luria agar or broth with 50 mg of carbenicillin per liter or 60 mg of neomycin per liter as appropriate.

Bacterial attachment to axenic root segments was examined. To obtain axenic roots, seeds were surface sterilized by soaking in 80% ethanol for 1 min, followed by 20 min in 1.05% sodium hypochlorite solution containing a drop of Tween 80 as a wetting agent, after which they were rinsed four times with sterile water and placed in sterile water at room temperature to germinate. Numbers of viable bacteria that bound to root segments were determined using the attachment assay of Matthyse and associates (1978) with the exception that eight tomato root segments 1 to 2 cm long were placed in 5 ml of a 1:10 dilution of Murashige-Skoog (MS) salts (Gibco BRL, Grand Island, NY, U.S.A.) containing 0.4% sucrose. Bacteria were added to a final concentration of 3×10^3 to 3×10^4 bacteria/ml. At varying times after the addition of the bacteria, root segments and attached bacteria were separated from free bacteria by filtration through Miracloth (Calbiochem, La Jolla, CA, U.S.A.). Viable cell counts were made on the free bacteria (filtrate) directly and on the attached bacteria after releasing them from the roots by sonication in a glassware cleaning sonicator for 2 min (Matthyse and McMahan 1998) or by grinding in a sterile Waring blender. Attachment assays were conducted at room temperature (22 to 25°C) and at 16°C.

Approximately 21% of the *A. tumefaciens* of wild-type strain C58 inoculated bound to root segments of *L. esculentum* cv. Marglobe after 2 h at room temperature. Under the same conditions, 1% of the mutant strain A1045 was bound. Longer incubation times (up to 6 h) at room temperature did not increase the binding of A1045. Binding at 16°C was slower than at room temperature for wild-type *A. tumefaciens*. At 16°C, 35% of the strain C58 inoculated were bound after 6 h. Under the same conditions, 24% of the strain A1045 inoculated were bound (Table 2). Thus, strain A1045 showed a marked increase in its ability to attach to tomato root segments at the lower temperature.

The binding of bacteria to root segments was also observed in the light microscope. For microscopic observations, four or five root segments approximately 1 cm long were placed in 2

ml of a 1:10 dilution of MS salts containing 0.4% sucrose. Bacteria were added to a final concentration of approximately 1×10^6 bacteria/ml and were incubated with the root segments for approximately 24 h at room temperature or 16°C. The root segments were then mounted in water using a probe-clip press-seal incubation chamber (Sigma-Aldrich Co., St. Louis) with the exception of *A. thaliana* root segments, which were small enough to be mounted using an ordinary coverslip. Root seg-

Table 1. Bacterial strains used

Strain	Relevant characteristics ^a	Source and/or reference
C58	Wild type, virulent, attaching	Lab collection
A1045	C58 <i>chvB</i> ::Tn5, avirulent, nonattaching, Neo ^r	Nester (Douglas et al. 1982)
A1038	C58 <i>chvB</i> ::Tn5, avirulent, nonattaching, Neo ^r	Nester (Douglas et al. 1982)
A205	C58 <i>attR</i> ::Tn3HoHo1, avirulent, nonattaching, Carb ^r	Lab collection (Reuhs et al. 1997)
M211	C58 <i>atrA</i> ::Tn3HoHo1, avirulent, nonattaching, Carb ^r	Lab collection (Reuhs et al. 1997)

^a ^r indicates resistance.

Table 2. Effect of temperature on bacterial attachment

Temperature	Percentage of bacterial inoculum attached to tomato root segments	
	Strain C58	Strain A1045
Room temperature ^a	21 ± 1 ^b	1 ± 1
16°C ^c	35 ± 1	24 ± 1

^a Room temperature was between 22 and 25°C. Assays were run for 2 h. Longer incubation times did not result in increased attachment of A1045.

^b Percent attachment ± the standard deviation of a minimum of three measurements.

^c Assays at 16°C were run for 6 h.

ments were examined and photographed using a Zeiss Photo-scope 2 with Nomarski optics (Carl Zeiss, Göttingen, Germany).

Wild-type strain C58 bacteria visibly bound to *L. esculentum* cv. Marglobe root segments within a 24-h incubation period at room temperature. Large numbers of bacteria were observed bound to the epidermis, root hairs, and the cut ends of each root segment (Fig. 1). In general, cut ends were preferentially covered when compared with the intact epidermis. Very few individuals (less than 30 bacteria per cm of root length) of the mutant strains tested (A1045, A1038, A205, and M211) were observed at any location on the surface of the root after 24 h of incubation at room temperature. When incubation was performed at 16°C for 24 h, strain C58 as well as the *chvB* mutants A1045 and A1038 were visibly bound to the epidermis, root hairs, and cut ends of *L. esculentum* (Fig. 1). Binding of the *att* mutants A205 and M211 remained barely detectable (less than 10 bacteria per cm of root).

The same analysis was performed using *L. esculentum* cv. Rutgers, *A. thaliana*, *D. carota*, and *T. erecta* root segments. At room temperature, wild-type C58 visibly bound to epidermis, root hairs, and cut ends of all species tested. Strain A1045 and strain A205 binding was negligible at room temperature. At 16°C, C58 and A1045 were both visibly bound. Strain A205 binding remained insignificant (Fig. 2).

In order to correlate the results of binding studies with bacterial virulence, the ability of the parent and mutant strains to form tumors at room temperature and 16°C were determined. The bacteria were inoculated into leaves of greenhouse-grown *B. daigremontiana* via toothpick wounds and into the stems of *L. esculentum* cv. Marglobe via vertical wounds about 3 mm long made with a needle. Between 10^9 and 10^{10} bacteria were inoculated into each wound. *B. daigremontiana* inoculated at the lower temperature were restored to room temperature (22 to 25°C) after 4 h. In a few cases, the plants were kept at the lower temperature for 24 h; this longer incubation at the lower

Fig. 1. Light micrographs of *Lycopersicon esculentum* cv. Marglobe root segments incubated with various *Agrobacterium tumefaciens* strains. **A**, C58 at room temperature; **B**, C58 at 16°C; **C**, A1045 at room temperature; **D**, A1045 at 16°C; **E**, A205 at room temperature; and **F**, A205 at 16°C. Note that A1045 is unable to bind at room temperature, but does bind at 16°C, apparently having regained functionality. Similar results to those obtained with strain A1045 were also seen with strain A1038. Mutant A205 was unable to bind at either temperature. Similar results to those obtained with strain A205 were seen with strain M211. Thus, lower temperature increased the binding of *chvB* mutants but not the other nonattaching mutants.

temperature had no effect on the percentage of sites developing tumors. Four hours was chosen because previous experiments suggested that it was sufficient time for the initiation of tumor formation by C58 on *B. daigremontiana* (Sykes and Matthysse 1986). Tomato inoculated at the lower temperature was restored to room temperature after 24 h. After the initial treatment, all inoculated plants were grown on a 14-h light cycle at room temperature and checked weekly for tumor formation.

Wild-type strain C58 produced tumors at 99% of the inoculated sites on *B. daigremontiana* after 10 weeks at both temperatures tested. All of the tumors caused by C58 were large, multilobed, and pale green. At room temperature, A1045 produced tumors at 3% of the inoculation sites after 10 weeks (Table 3). These tumors were small, white, and dry. At 16°C, 65% of the sites inoculated with A1045 developed tumors. These were generally larger than the tumors produced by A1045 at room temperature. Strain A205 did not induce any tumors at either temperature. Another *chvB* mutant, A1038, also recovered virulence at 16°C suggesting that the recovery of

Table 3. Effect of temperature on bacterial virulence

Bacterial strain	% Inoculated sites developing tumors	
	Room temperature	16°C
<i>Bryophyllum daigremontiana</i> ^a		
C58	99% ± 1 ^b	100% ± 1
A1045	3% ± 3	65% ± 10
A205 ^c	0% (50)	0% (13)
Tomato ^d		
C58	100% ± 2	100% ± 1
A1045	10% ± 5	40% ± 5
A205 ^c	0% (25)	0% (15)

^a Tumor formation after 10 weeks on leaves of *B. daigremontiana*.
^b Average percentage of inoculated sites forming tumors ± the standard deviation of a minimum of 20 different inoculations for C58 and A1045 and 13 different inoculations for A205.
^c Strain A205 never formed tumors. Numbers in parentheses indicate the total number of sites inoculated.
^d Tumor formation after 8 weeks on tomato stems.

virulence at lower temperatures is a general property of *chvB* mutants.

Similar results were obtained when the same bacterial strains were inoculated into the stems of *L. esculentum* cv.

Fig. 3. Photographs of *Lycopersicon esculentum* cv. Marglobe stems inoculated with *A. tumefaciens* strains C58, A1045, and A205 and allowed to grow for 8 weeks. **A**, Tomato stems inoculated at room temperature. **B**, Tomato stems inoculated at 16°C and restored to room temperature after 24 h. Note the formation of large tumors on stems inoculated with strain C58 and the relatively small tumors induced by strain A1045.

Fig. 2. Light micrographs of various plant root segments incubated in the presence of *Agrobacterium tumefaciens* strain A1045. *Arabidopsis thaliana* at **A**, room temperature and **B**, 16°C. *Daucus carota* at **C**, room temperature and **D**, 16°C. *Tagetes erecta* at **E**, room temperature and **F**, 16°C. Note that on all species tested, A1045 did not bind to the root segment at room temperature but did bind at 16°C, indicating that restoration of attachment at lower temperature was not host specific.

Marglobe (Table 3). Strain C58 formed large, multilobed, pale green tumors after 8 weeks at 99% of the inoculation sites at both temperatures tested. Strain A1045 formed small, white, dry tumors at 10% of the room temperature inoculation sites and at 40% of the 16°C inoculation sites. Strain A205 did not induce tumors on tomato stems at either temperature (Fig. 3).

The increased attachment and virulence seen with *chvB* mutants at low temperatures could be due to an accumulation of revertants at this temperature. In order to examine this possibility, *chvB* mutant bacteria that bound to root segments at 16°C were isolated as described above and tested for retention of resistance to neomycin and ability to bind to tomato root segments and virulence at room temperature. No revertants of the original mutation were detected.

At room temperature, *chvB* mutants are pleiotropic. They are nonattaching and avirulent and also exhibit reduced motility. In order to determine if motility was also restored at lower temperatures, C58 and A1045 were inoculated into motility agar (Langer et al. 1980), the plates incubated at room temperature and at 16°C, and the diameter of the colonies formed measured after 4 and 6 days, respectively. *chvB* mutants were reduced in motility at room temperature when compared with the wild type (average colony diameters of 14.2 ± 0.5 mm and 26.4 ± 0.6 mm, respectively) but recovered motility at lower temperatures (28.2 ± 0.5 mm and 27.6 ± 0.4 mm colony diameters).

Thus the pleiotropic effects of the *chvB* mutation at room temperature (loss of virulence, failure to attach to plant surfaces, and lower motility) are all reversed at lower temperature. The nature of the temperature-sensitive substance in *chvB* mutants is not known. It is thought that many of the effects of mutations in osmoregulated periplasmic glucans, including β -1,2-D-glucans, are due to alterations in membrane properties in the absence of these compounds (Bohin 2000; Miller and Wood 1996). The observation that these same properties of *chvB* mutants (avirulence, lack of motility, and lack of attachment to plant surfaces) can be reversed by growing the bacteria in high osmolarity medium (Swart et al. 1994) is in agreement

with the possibility that the effects of the *chvB* mutation are on the stability of the membrane. Thus, it may be that, in *chvB* mutants, the membrane is unstable in a temperature-dependent osmolarity-sensitive manner and that the temperature and osmolarity sensitivity of the various phenotypes caused by the mutation is due to alterations in membrane properties.

LITERATURE CITED

- Banta, L. M., Bohme, J., Lovejoy, S. D., and Dostal, K. 1998. Stability of the *Agrobacterium tumefaciens* VirB10 protein is modulated by growth temperature and periplasmic osmoadaptation. *J. Bacteriol.* 180:6597-6606.
- Bohin, J.-P. 2000. Osmoregulated periplasmic glucans in Proteobacteria. *FEMS (Fed. Eur. Biochem. Soc.) Microbiol. Lett.* 186:11-19.
- Douglas, C. J., Halperin, W., and Nester, E. W. 1982. *Agrobacterium tumefaciens* mutants affected in attachment to plants. *J. Bacteriol.* 152:1265-1275.
- Langer, R., Fefferman, M., Gyska, P., and Bergman, K. 1980. A simple method for studying chemotaxis using sustained release of attractants from inert polymers. *Can. J. Microbiol.* 26:274-278.
- Matthysse, A. G., and McMahan, S. 1998. Root colonization by *Agrobacterium tumefaciens* is reduced in *cel*, *attB*, *attD*, and *attR* mutants. *Appl. Environ. Microbiol.* 64:2341-2345.
- Matthysse, A. G., Wyman, P. M., and Holmes, K. V. 1978. Plasmid-dependent attachment of *Agrobacterium tumefaciens* to plant tissue culture cells. *Infect. Immun.* 22:516-522.
- Matthysse, A. G., Yarnall, H. A., Boles, S. B., and McMahan, S. 2000. A region of the *Agrobacterium tumefaciens* chromosome containing genes required for virulence and attachment to host cells. *Biochim. Biophys. Acta* 1490:208-212.
- Miller, K. J., and Wood, J. M. 1996. Osmoadaptation by rhizosphere bacteria. *Ann. Rev. Microbiol.* 50:101-136.
- Reuhs, B. L., Kim, J. S., and Matthysse, A. G. 1997. The attachment of *Agrobacterium tumefaciens* to carrot cells and *Arabidopsis* wound sites is correlated with the production of a cell-associated, acidic polysaccharide. *J. Bacteriol.* 179:5372-5379.
- Swart, S., Lugtenberg, B. J., Smit, G., and Kijne, J. W. 1994. Rhicadhesin-mediated attachment and virulence of an *Agrobacterium tumefaciens chvB* mutant can be restored by growth in a highly osmotic medium. *J. Bacteriol.* 176:3816-3819.
- Sykes, L., and Matthysse, A. G. 1986. Time required for tumor induction by *Agrobacterium tumefaciens*. *Appl. Environ. Microbiol.* 52:597-598.