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Rhicadhesin-Mediated Attachment and Virulence of an *Agrobacterium tumefaciens chvB* Mutant Can Be Restored by Growth in a Highly Osmotic Medium

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Cyclic β-1,2-glucan is considered to play a role in osmoadaptation of members of the family *Rhizobiaceae* in hypotonic media. *Agrobacterium tumefaciens chvB* mutants, lacking β-1,2-glucan, exhibit a pleiotropic phenotype, including nonmotility, attachment deficiency, and avirulence. Here we report that by growth of *chvB* mutant cells in tryptone-yeast extract medium supplemented with 7 mM $CaCl_2$ and 100 mM NaCl, the mutant cells become motile, attach to pea root hair tips, and are virulent on *Kalanchoë* leaves. Moreover, whereas *chvB* mutants grown in tryptone-yeast extract medium containing 7 mM $CaCl_2$ do not produce active rhicadhesin, addition of 100 mM NaCl to this medium resulted in restoration of rhicadhesin activity. The presence of $CaCl_2$ appeared to be required for attachment, virulence, and activity of rhicadhesin. The results support a role for cyclic β-1,2-glucan in osmoadaptation and strengthen the notion that rhicadhesin is required for attachment and virulence of *A. tumefaciens*.

Attachment to plant cells is one of the early steps in the infection process of the plant pathogen *Agrobacterium tumefaciens*. Attachment of *A. tumefaciens* is a two-step process. The first step, direct attachment of bacteria to plant cells, is mediated by a bacterial Ca^{2+}-binding protein called rhicadhesin (15). The second step, anchoring of bacteria to each other and to the plant cell surface, is established by bacterial cellulose fibrils (8). Another component reported to be required for agrobacterial virulence and suggested to be involved in attachment is cyclic β-1,2-glucan (12).

Mutants defective in synthesis or export of cyclic β-1,2-glucan, *chvB* and *chvA* mutants, respectively, were found to be avirulent (4). The *chvB* gene encodes a protein in the cytoplasmic membrane that catalyzes the synthesis of cyclic β-1,2-glucan (19). *chvB* mutants show a pleiotropic phenotype. They are avirulent, are attachment deficient, show reduced motility (3), and show some additional alterations, probably all linked to changes in the cell envelope (17). Moreover, *chvB* mutants lack active rhicadhesin (18). Miller and coworkers (9) proposed a role for cyclic β-1,2-glucan in osmoadaptation. These authors observed an accumulation of cyclic β-1,2-glucan in the periplasmic space when wild-type bacteria were grown under hypo-osmotic conditions. If lack of cyclic β-1,2-glucan results in inadequate osmoadaptation, this may be the cause of the various cell envelope deficiencies in *chvB* mutants. Those deficiencies, especially lack of active rhicadhesin, could yield the avirulence and attachment deficiency phenotypes of *chvB* mutants. If so, growth under high-osmotic-strength conditions may restore attachment ability and virulence of *chvB* mutants.

We therefore investigated the effect of osmotic strength on growth, motility, attachment, rhicadhesin activity, and virulence of *chvB* mutants.

Growth and motility. Bacteria were grown at 28°C in 100-ml Erlenmeyer flasks containing 50 ml of medium on a rotary shaker (180 rpm). When grown in standard growth medium, that is, 0.5% tryptone–0.3% yeast extract supplemented with 7 mM $CaCl_2$ (TYC medium) (1), wild-type *A. tumefaciens* LBA1010 (6) had a doubling time of 2.1 h and reached a final A_{620} value of 1.2 to 1.3. With a doubling time of 3.4 h and reaching a final A_{620} value of 0.8 to 0.9, *A. tumefaciens chvB* mutant ME117 (4) appeared to grow more slowly in TYC medium than the wild-type strain. The doubling time and the final cell number of strain ME117 were restored to wild-type values by the addition of 100 mM NaCl to the TYC medium. Since addition of 200 mM melezitose had a similar effect, this wild-type-like behavior is probably due to an increase in osmotic strength of the medium rather than to ionic influences. Thus far, these results are in agreement with the results reported by Cangelosi and coworkers (2). However, in contrast to the results obtained by these authors, we found that motility also appeared to be affected by the osmotic strength of the growth medium. Whereas cells of *chvB* mutant strain ME117 were nonmotile when grown in TYC medium, cells of strain ME117 grown in TYC supplemented with 100 mM NaCl were motile, as judged from inoculation of 0.3% agar swarm plates and from light microscopic observation. An explanation for this discrepancy could be that Cangelosi and coworkers (2) used a four- to five-times-higher concentration of solutes. From *Escherichia coli*, it is known that production of flagella can be inhibited by high concentrations of sucrose, NaCl, and other salts (7). Although they did not give specific details, Cangelosi and coworkers reported that the motility of the wild-type strain varied with the medium composition. Even if these authors did find normal motility for the wild-type strain, this does not imply that the concentrations used were not toxic for the *chvB* mutant.

Attachment. For attachment assays, bacteria were grown to early stationary phase, centrifuged, and resuspended in 25 mM potassium phosphate buffer, pH 7.5, to obtain 3×10^8 bacteria per ml. Seeds of pea plants (*Pisum sativum* cv. Finale; purchased from Cebeco, Rotterdam, The Netherlands) were surface sterilized and cultivated as described previously (13). After incubation of lateral roots from 7-day-old axenically grown pea plants in this suspension for 2 h, the roots were washed in phosphate buffer and screened for attached bacteria

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TABLE 1. Influence of culture medium on attachment of cells of *A. tumefaciens* wild-type strain LBA1010 and of *chvB* mutant strain ME117 to pea root hair tips[a]

Strain	Culture medium[b]	Root pretreatment[c]	% Attachment for class[d]:		
			1	2	3
LBA1010	TY	None	81	14	5
	TYC	None	16	24	60
	TYC	Rhicadhesin	45	32	23
ME117	TY	None	90	8	2
	TY + NaCl	None	87	11	2
	TYC	None	92	8	0
	TYC + NaCl	None	3	40	57
	TYC + NaCl	Rhicadhesin	61	29	10

[a] Bacteria were incubated with the roots as described previously (13).
[b] Media were used with or without 100 mM NaCl.
[c] Where indicated, roots were pretreated with 10 ng of rhicadhesin per ml for 60 min before bacteria were added.
[d] Class 1, no attached bacteria; class 2, few attached bacteria, attached directly to the root hair; class 3, many attached bacteria, forming two or more layers of bacteria or a cap-like aggregate at the tip of the root hair (cap formation). The variability of the test is about 10% and depends largely on the condition of the roots.

TABLE 2. Influence of culture medium[a] on the inhibitory activity of rhicadhesin isolated from *A. tumefaciens* wild-type strain LBA1010 and from *chvB* mutant strain ME117 on attachment of strain LBA1010 to pea root hairs[b]

Root pretreatment[c]	% Attachment for class[d]:		
	1	2	3
None	6	37	57
Rhicadhesin isolated from:			
LBA1010 grown in TYC medium	45	32	23
ME117 grown in TYC medium[e]	6	36	58
ME117 grown in TYC + NaCl medium	50	29	21
ME117 grown in TY + NaCl medium[e]	10	39	51

[a] See Table 1, footnote *b*.
[b] See Table 1, footnote *a*.
[c] See Table 1, footnote *c*.
[d] See Table 1, footnote *d*.
[e] In addition, up to three times the concentration used for wild-type rhicadhesin was tested with similar results.

by phase-contrast microscopy, as described previously (13). Attachment behavior of *chvB* mutants grown in TYC medium and TYC medium supplemented with 100 mM NaCl was investigated. Attachment was quantified by randomly screening at least 100 root hairs in the zone of developing root hairs by using a phase-contrast light microscope. Attachment to root hairs was ranked into three classes: class 1, no attached bacteria; class 2, a few bacteria, attached directly to the root hair; and class 3, many attached bacteria, forming two or more layers of bacteria or a cap-like aggregate on top of the root hair. *A. tumefaciens chvB* mutants show an impaired attachment behavior when grown in TYC medium. However, addition of NaCl to the growth medium resulted in restoration of attachment ability (Table 1). Only when calcium was present in the medium was attachment restored to wild-type levels. Omission of calcium (TY medium) resulted in low attachment levels in both the presence and absence of the 100 mM NaCl supplement (Table 1). Apparently, the presence of calcium in the growth medium is required for attachment ability of *chvB* mutants. Wild-type *A. tumefaciens* cultured in TY medium also requires the presence of calcium for attachment to pea root hairs in the test system used (14) (Table 1). To investigate whether osmotically restored attachment of the *chvB* mutant strain was mediated by rhicadhesin, inhibition of attachment of ME117 cells to pea root hair tips by rhicadhesin was tested as described previously (15). Lateral pea roots were preincubated with wild-type rhicadhesin (10 ng/ml of 25 mM potassium phosphate buffer, pH 7.5) for 1 h prior to addition of bacteria. Attachment of ME117 cells grown in TYC supplemented with 100 mM NaCl appeared to be inhibited by this pretreatment of the roots with wild-type rhicadhesin, indicating that this attachment is rhicadhesin dependent (Table 1).

For restoration of attachment of ME117, it was necessary to culture the bacteria in TYC supplemented with NaCl. Addition of 100 mM NaCl to the attachment buffer did not result in restoration of attachment ability of *chvB* mutant strain ME117. This indicates that NaCl is not directly responsible for the observed attachment enhancement.

Rhicadhesin activity. *chvB* mutants grown in TYC medium do not produce active rhicadhesin. Purified rhicadhesin from these bacteria is not able to inhibit attachment of wild-type agrobacteria (18). Also, *chvB* mutants cultured in TY medium do not produce active rhicadhesin (data not shown). This is in agreement with observations of Smit and coworkers (16), who reported the involvement of Ca^{2+} in the activity of rhicadhesin and its anchoring to the bacterial surface. Since attachment of *chvB* mutants was restored by addition of 100 mM NaCl to the TYC medium and this attachment appeared to be rhicadhesin dependent, production and activity of rhicadhesin from *chvB* mutants grown in TYC medium supplemented with 100 mM NaCl were investigated. The protocol for the purification of rhicadhesin and the assay for determination of rhicadhesin activity were described previously (15). Rhicadhesin isolated from ME117 cells grown in TYC supplemented with 100 mM NaCl was able to inhibit attachment of wild-type *A. tumefaciens*, whereas rhicadhesin from ME117 cells grown in TYC without the NaCl supplement was not (Table 2). Since no β-1,2-glucan could be detected in these bacteria (data not shown), it was concluded that cyclic β-1,2-glucan is not necessary for production of active rhicadhesin. *chvB* mutant bacteria cultured in TY medium supplemented with 100 mM NaCl did not produce active rhicadhesin (Table 2). Apparently, the presence of calcium in the growth medium is required for the production of active rhicadhesin.

Virulence. To test our hypothesis that *chvB* mutants are avirulent because they lack active rhicadhesin, the effect of addition of NaCl to TYC medium on the virulence of the bacteria was studied. *Kalanchoë daigremontiana* plants were cultivated as described previously (11), and leaves were wounded with a toothpick. Cells of early stationary-phase *A. tumefaciens* cultures of strain LBA1010 or strain ME117, grown in TYC or TY medium, were harvested and resuspended in phosphate buffer to an A_{620} value of 1.0. Ten microliters of this suspension was added per wound site. Tumor formation was quantified 3 weeks after inoculation. ME117 cells grown in TY or TYC medium appeared to be avirulent. Hardly any tumor formation on wounded leaves could be observed (Fig. 1B and D). However, addition of 100 mM NaCl to TYC medium resulted in restoration of virulence in that tumor formation on *Kalanchoë* leaves was restored to wild-type levels (Fig. 1E). *chvB* mutant bacteria grown in TY medium supplemented with NaCl did not show wild-type virulence (Fig. 1C). Apparently, presence of calcium in the growth medium of *chvB* mutants is required for tumor formation, as it is for attachment and for the production of active

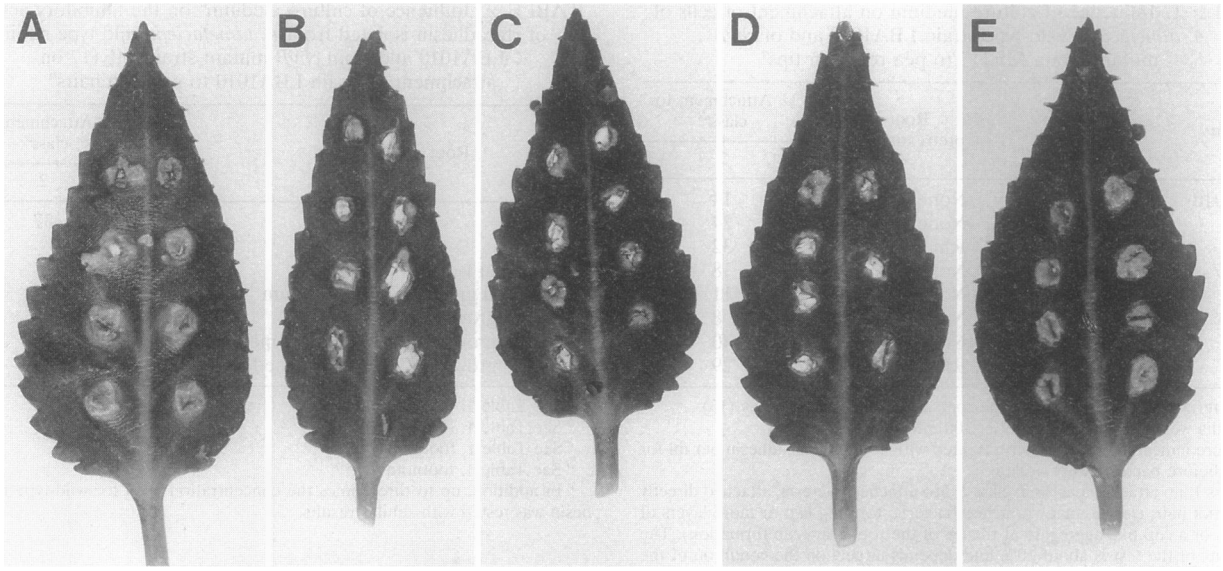

FIG. 1. Influence of culture medium of cells of *chvB* mutant strain ME117 on virulence on *K. daigremontiana* leaves. Wound sites were inoculated with *A. tumefaciens* wild-type strain LBA1010 cultured in TYC medium (A) or with *chvB* mutant strain ME117 cultured in either TY medium (B), TY medium with 100 mM NaCl (C), TYC medium (D), or TYC medium with 100 mM NaCl (E).

rhicadhesin. This conclusion is consistent with results obtained by Cangelosi and coworkers (2), who used media with a low calcium content and did not detect effects of medium osmolarity on virulence of *chvB* mutants.

The pleiotropic phenotype of *chvB* mutants corresponds to changes in the cell envelope (3, 17, 18). Presumably, presence of 100 mM NaCl or 200 mM melezitose is sufficient to increase the osmolarity of the medium to a level at which cyclic β-1,2-glucan is no longer required for osmoadaptation. The results support a role for cyclic β-1,2-glucan in osmoadaptation. They also support the suggestion of O'Connell and Handelsman (10) that cyclic β-1,2-glucan is not required for attachment and virulence but only indirectly influences these properties through an osmoregulatory effect. Presumably, cyclic β-1,2-glucan is needed to maintain high osmolarity in the periplasmic space, thereby preventing disassembly or impaired assembly of outer membrane components. A few plant species, including *Solanum tuberosum*, were reported to be susceptible to infection by *chvB* mutants (5). A putatively highly osmotic environment in wound sites of such plants may cause this susceptibility.

The results of this investigation support the hypothesis that *chvB* mutants, cultured in media of low osmolarity or at low calcium concentrations, are avirulent because they lack active rhicadhesin. Moreover, the results provide further evidence that rhicadhesin is a major determinant in attachment and virulence of *A. tumefaciens*. Calcium probably plays a role in anchoring, stabilizing, and/or activation of rhicadhesin (16).

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