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Osmosensitivity Phenotypes of *Agrobacterium tumefaciens* Mutants That Lack Periplasmic β-1,2-Glucan

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The periplasmic cyclic β-1,2-glucan of *Agrobacterium tumefaciens* is believed to maintain high osmolarity in the periplasm during growth of the bacteria on low-osmotic-strength media. Strains with mutations in the *chvA* or *chvB* gene do not accumulate β-1,2-glucan in their periplasm and exhibit pleiotropic phenotypes, including inability to form crown gall tumors on plants. We examined the effects of medium osmolarity to determine whether some or all of these phenotypes result from suboptimal periplasmic osmolarity. The mutants grew more slowly than wild-type cells and exhibited altered periplasmic and cytoplasmic protein content when cultured in low-osmotic-strength media, but not when cultured in high-osmotic-strength media. These observations support a role for periplasmic glucan in osmoadaptation. However, the mutants were avirulent and exhibited reduced motility regardless of the osmolarity of the medium. Therefore, β-1,2-glucan may play roles in virulence and motility that are unrelated to its role in osmoadaptation.

The chromosomal *chvA* and *chvB* genes of *Agrobacterium tumefaciens* are required for effective attachment of the bacteria to plant cells, an essential step in crown gall tumorigenesis on plants (3). Strains with mutations in these loci are avirulent, exhibit reduced motility, and are resistant to some phages (1, 3).

chvB codes for a large inner membrane protein that binds UDP-glucose and catalyzes the synthesis of a cyclic β-1,2-glucan (16). The role of ChvB in virulence is apparently related to glucan synthesis, since a large portion of the protein that is dispensable for glucan synthesis is also dispensable for virulence (16).

Wild-type *A. tumefaciens* cells accumulate β-1,2-glucan in their periplasmic space and extracellularly (14), and most of this glucan is modified by the addition of anionic substitutions (10). When spheroplasts are made from wild-type cells, most of the cellular β-1,2-glucan is released (9). *chvA* mutants synthesize but do not secrete β-1,2-glucan extracellularly (2, 12), and mutant spheroplasts retain most or all of their cellular glucan (2) in unsubstituted form (6). Since the mutant spheroplasts release the polysaccharide upon mild sonication, it appears that they accumulate the glucan in the cytoplasm or loosely bound to the inner membrane, rather than in the periplasm. The *chvA* locus maps very close to *chvB* and codes for a protein that is homologous to a family of membrane proteins involved in secretion (2, 13). Thus, ChvA may export cyclic glucan across the inner membrane or export an enzyme that catalyzes the release of membrane-bound glucan into the periplasm.

Since mutants defective in cyclic glucan synthesis or export are avirulent, the polysaccharide appears to play a direct or indirect role in attachment and virulence. This role has not been identified. One possibility is that extracellular β-1,2-glucan interacts directly with host plant cells, mediating attachment. However, O'Connell and Handelsman (12) showed that purified neutral glucan, added exogenously, does not stimulate attachment and tumorigenesis by the mutants. We have made the same observations by using purified neutral as well as anionic glucan isolated by size fractionation and crude preparations of conditioned supernatants obtained by incubating wild-type *A. tumefaciens* cells with plant tissues (data not shown). These observations argue that extracellular glucan does not play a role in virulence and suggest that periplasmic glucan may be more important.

Miller et al. (9) proposed a physiological function for periplasmic glucan which may indirectly affect virulence. They observed that synthesis of periplasmic glucan is partially suppressed when *A. tumefaciens* cells grow on media of high osmolarity. This led to the proposal that the polysaccharide is synthesized during growth on low-osmotic-strength media to maintain high osmolarity in the periplasm, thereby minimizing the gradient in osmolarity across the inner membrane. A similar function has been proposed for the osmoregulated membrane-derived oligosaccharides of enteric bacteria (7, 8). Fiedler and Rotering (4) observed that *Escherichia coli* mutants lacking membrane-derived oligosaccharides exhibit pleiotropic phenotypes, including reduced flagellum production and increased slime production on dilute media, and alterations in expression of osmoregulated outer membrane proteins. However, such mutants grow as well as wild-type cells on dilute media (8).

Defects in osmoadaptation may explain the defective virulence and motility of *chvA* and *chvB* mutants of *A. tumefaciens*. The activity of cell surface proteins directly related to virulence or motility may be sensitive to suboptimal periplasmic osmolarity. If so, high-osmotic-strength growth conditions may be permissive for these phenotypes. We studied the effects of osmotic strength on growth, protein content, virulence, and motility in mutant and wild-type cells, to (i) genetically test in *A. tumefaciens* the proposed role for periplasmic glucan in osmoadaptation and (ii) determine the relationship between osmoadaptation and avirulence in the mutants.

The mutants grew normally on yeast-mannitol (YM) medium, a low-osmotic-strength medium which was reported to derepress glucan synthesis in wild-type cells (9). However, the mutants grew more slowly than wild-type cells on the extremely dilute medium YPL (0.1% yeast extract, 0.1% peptone, and 5 mM glucose) (Table 1). The osmolarity of

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TABLE 1. Effect of added solutes on growth rate in dilute medium (YPL)

Addition	Doubling time, h (σ)[a]				
	A348 (wild type)	ME42 (*chvA*)	ME66 (*chvA*)	ME73 (*chvB*)	A1038 (*chvB*)
None	2.0 (0.3)	4.4 (0.5)	4.4 (0.4)	3.1 (0.4)	3.0 (0.3)
0.1 M NaCl	2.1 (0.2)	2.0 (0.2)	2.1 (0.1)	2.1 (0.3)	1.9 (0.1)
0.15 M sucrose	1.7 (0.2)	1.8 (0.2)	2.2 (0.1)	2.2 (0.1)	1.9 (0.3)
0.15 M mannitol	1.8 (0.4)	1.8 (0.3)	2.1 (0.4)	2.1 (0.5)	1.9 (0.3)
0.15 M $MgSO_4$	2.2 (0.3)	2.4 (0.2)	2.6 (0.5)	2.1 (0.3)	2.1 (0.3)
0.05 M K_2SO_4	2.7 (0.2)	2.8 (0.1)	3.1 (0.2)	2.8 (0.3)	3.0 (0.2)

[a] Results are means of three experiments. The doubling time was determined before bacteria reached an optical density at 600 nm of 0.4, the density at which they entered stationary phase on YPL.

YPL is lower than 0.03 osM, compared with 0.08 osM for YM (measured on a Wescor 5100C vapor pressure osmometer with NaCl and KCl solutions as standards). Growth of the mutants on YPL was restored to wild-type rates by the addition of either 0.1 M NaCl, 0.15 M sucrose, 0.15 M mannitol, 0.15 M $MgSO_4$, or 0.05 M K_2SO_4 (Table 1). The uniform effect of these diverse solutes suggests that low osmolarity, rather than a specific nutritional limitation, slowed the growth of the mutants on YPL. These observations provided genetic evidence supporting a role for periplasmic glucan in osmoadaptation by *A. tumefaciens*. Growth of both mutant and wild-type cells on YPL ceased when cultures reached an optical density (600 nm) of approximately 0.4 (compared with about 2.0 on YM). This is presumably due to specific nutrient limitation and not to low medium osmolarity, since the addition of solutes did not affect cell yield (data not shown).

It is interesting that *chvA* mutants were affected more severely by low osmolarity than *chvB* mutants. This may be due to accumulation of β-1,2-glucan in the cytoplasm of *chvA* mutants (2), which may result in a greater osmotic gradient across the inner membrane of these mutants than that of *chvB* mutants. An alternative explanation is that ChvA but not ChvB may be required for accumulation of periplasmic osmotic effectors other than cyclic glucan.

We also examined the effects of osmotic strength on periplasmic and cytoplasmic proteins. Since YPL medium does not allow sufficient cell yields for these experiments, bacteria were grown on YM medium alone or supplemented with 0.4 M sucrose or 0.4 M NaCl. These solutes added to YM were reported to repress glucan synthesis (9). During growth on unsupplemented YM, a periplasmic protein of >115 kilodaltons (kDa) was overexpressed and a cytoplasmic protein of 28 kDa was underexpressed in the mutants but not in wild-type cells (Fig. 1). The addition of the solutes restored these proteins to normal levels, except in the case of the 28-kDa cytoplasmic protein in *chvB* mutants. In addition to these proteins, a periplasmic protein of approximately 40 kDa appeared to be osmoregulated in the mutants in a reverse pattern relative to the wild type. These observations further correlate periplasmic glucan with osmoadaptation in *A. tumefaciens*. The function of these proteins is unknown. Since preparation of periplasmic fractions did not include a centrifugation step, it is possible that some of the proteins present in these fractions may be outer membrane proteins. Altered osmotic regulation of outer membrane proteins was among the phenotypes observed in membrane-derived oligosaccharide mutants of *E. coli* (4).

We next studied the effects of osmotic strength on virulence.

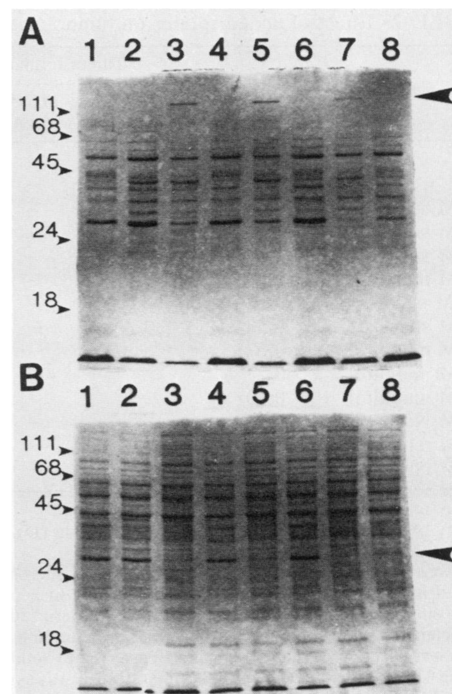

FIG. 1. Effects of added solutes on periplasmic and cytoplasmic proteins. Bacteria were grown on YM or YM with 0.4 M sucrose. Periplasmic fractions were prepared by lysozyme treatment as described previously (2), and proteins were concentrated with 10% trichloroacetic acid. Cytoplasmic fractions were prepared by mild sonication of spheroplasts (2). Results from sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis and Coomassie blue staining are shown, with positions of molecular mass standards (kDa) on the sides. Similar results were observed in periplasmic and cytoplasmic fractions when 0.4 M NaCl was substituted for 0.4 M sucrose. (A) Periplasmic fractions. Lanes: 1, 3, 5, and 7, bacteria grown on YM; 2, 4, 6, and 8, bacteria grown on YM with 0.4 M sucrose; 1 and 2, wild-type strain A348; 3 and 4, *chvA* mutant ME42; 5 and 6, *chvA* mutant ME66; 7 and 8, *chvB* mutant ME73. Arrow indicates the position of the >115-kDa protein. (B) Cytoplasmic fractions. Lanes are numbered as in panel A. The arrow indicates the position of the 28-kDa protein.

The effects of osmotic strength on attachment to plant cells were impossible to assess, because cells grown on high-osmotic-strength media grew in clumps, resulting in seemingly elevated attachment by mutant and wild-type cells in quantitative assays (data not shown). Virulence was assessed by incubating bacteria with *Nicotiana glauca* leaf slices in various liquid media (Table 2). Wild-type *A. tumefaciens* cells formed tumors over a wide range of osmotic strengths. Tumorigenesis was optimum when MS medium (11) was used at 0.77 osM or less. Inoculation in YM resulted in relatively poor but still easily detectable tumorigenesis. Extremely high-osmotic-strength media inhibited tumorigenesis and resulted in visible damage to the leaf slices. *chvA* and *chvB* mutants did not form tumors in any of the media (Table 2).

In similar experiments, we grew and washed wild-type and *chvA* and *chvB* mutant bacteria as described in footnote *c* of Table 2 and suspended them in 2 ml of MS, MS with 0.2, 0.3, or 0.4 M sucrose, or YM with 0.4 M sucrose. Filter paper disks were soaked in these suspensions and applied to fresh wounds on *Kalanchoe* leaves. The leaves were then wrapped in plastic wrap to minimize evaporation. After 36 h

TABLE 2. Effect of added solutes on tumorigenesis

Medium[a]	Osmolarity[b] (osM)	Tumor formation[c]		
		A348 (wild type)	ME45 (*chvA*)	ME73 (*chvB*)
YM	0.08	+*	−	−
MS	0.12	+	−	−
MS + 0.1 M sucrose	0.23	+	−	−
MS + 0.2 M sucrose	0.33	+	−	−
MS + 0.3 M sucrose	0.46	+	−	−
MS + 0.4 M mannitol	0.52	+	−	−
YM + 0.4 M sucrose	0.52	+*	−	−
MS + 0.4 M sucrose	0.58	+	−	−
MS + 0.5 M mannitol	0.67	+	−	−
MS + 0.6 M mannitol	0.77	+	−	−
MS + 0.7 M mannitol	0.90	+*	−	−
MS + 0.4 M NaCl	0.95	+*	−	−
MS + 0.8 M mannitol	1.03	−	−	−
MS + 0.6 M NaCl	1.45	−	−	−

[a] In order of increasing osmolarity.

[b] Osmalarity of media was calculated from reference data (15), except for YM, which was measured as described in the text.

[c] Bacteria were grown on 4 ml of YM containing 0.4 M sucrose, washed, and suspended in 15 ml of the indicated medium. These suspensions were transferred to sterile petri plates containing four surface-sterilized *N. glauca* leaf slices (rectangles averaging 3 cm^2). After incubation for 36 h with gentle agitation, the leaf slices were blotted dry and transferred to solid MS plates (11) containing 0.3 mg of cefataxime per ml. Tumorigenesis was assessed after incubation for 14 days in the dark (leaf pieces were aseptically transferred to fresh plates on days 3 and 6). +, Significant tumors; +*, smaller than average tumors; −, indistinguishable from uninoculated controls. Identical results were obtained in replicate experiments when *chvA* mutant ME42 and *chvB* mutant A1038 were substituted for the indicated strains.

of incubation in the dark, plastic wrap and filter disks were removed, and the plants were incubated for 14 days. Tumors formed on all wounds inoculated with wild-type cells, but no tumors formed with the mutant strains.

Motility was assessed in triplicate experiments by inoculating swarm agar plates containing 0.3% agar (Sigma Chemical Co.) with 0.005 ml of overnight cultures as described previously (5). YM alone or YM supplemented with 0.4 M sucrose, 0.4 M NaCl, 0.5 M mannitol, or 0.27 M K_2SO_4 was used. The diameters of the swarms varied with the media, but *chvA* and *chvB* mutants (ME42, ME66, ME73, and A1038) always produced swarms about 60 to 80% the diameter of wild-type swarms. On YPL medium, the mutants formed swarms about 30% the diameter of wild-type swarms, but we could not discern whether this was due to differences in motility or in growth rate.

The osmosensitivity phenotypes of *chvA* and *chvB* mutants genetically correlate periplasmic glucan with osmoadaptation in *A. tumefaciens*. We did not detect effects of osmolarity on virulence or motility. It is possible that such effects require very specific environments that are difficult to provide. However, these experiments covered an extensive range of osmotic conditions, including those which affect cytoplasmic and periplasmic protein content (Fig. 1) and modulate glucan synthesis (9). Therefore, it is possible that periplasmic glucan performs functions in addition to osmoadaptation. In *Agrobacterium* species, repression of glucan synthesis by high osmolarity appears to be incomplete (9). Thus, *Agrobacterium* species may always synthesize enough β-1,2-glucan to perform functions unrelated to osmoadaptation, and it may be these functions that exert direct or indirect effects on attachment, virulence, and motility.

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LITERATURE CITED

1. **Bradley, D. E., C. J. Douglas, and J. Peschon.** 1984. Flagella-specific bacteriophages of *Agrobacterium tumefaciens*: demonstration of virulence of non-motile mutants. Can. J. Microbiol. **30**:676–681.
2. **Cangelosi, G. A., G. Martinetti, J. A. Leigh, C.-C. Lee, C. Theines, and E. W. Nester.** 1989. A role for *Agrobacterium tumefaciens* ChvA protein in export of β-1,2-glucan. J. Bacteriol. **171**:1609–1615.
3. **Douglas, C. J., R. J. Staneloni, R. A. Rubin, and E. W. Nester.** 1985. Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. J. Bacteriol. **161**: 850–860.
4. **Fiedler, W., and H. Rotering.** 1988. Properties of *Escherichia coli* mutants lacking membrane-derived oligosaccharides. J. Biol. Chem. **263**:14684–14689.
5. **Hawes, M. C., L. Y. Smith, and J. Howarth.** 1988. *Agrobacterium tumefaciens* mutants deficient in chemotaxis to root exudates. Mol. Plant-Microbe Interact. **1**:182–186.
6. **Inon de Iannino, N., and R. A. Ugalde.** 1989. Biochemical characterization of avirulent *Agrobacterium tumefaciens chvA* mutants: synthesis and excretion of β-1,2-glucan. J. Bacteriol. **171**:2842–2849.
7. **Kennedy, E. P.** 1982. Osmotic regulation and the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **79**:1092–1095.
8. **Kennedy, E. P.** 1987. Membrane-derived oligosaccharides, p. 672–679. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
9. **Miller, K. J., E. P. Kennedy, and V. N. Reinhold.** 1986. Osmotic adaptation by gram-negative bacteria: possible role for periplasmic oligosaccharides. Science **231**:48–51.
10. **Miller, K. J., V. N. Reinhold, A. C. Weissborn, and E. P. Kennedy.** 1987. Cyclic glucans produced by *Agrobacterium tumefaciens* are substituted with *sn*-1-phosphoglycerol residues. Biochim. Biophys. Acta **901**:112–118.
11. **Murashige, T., and F. Skoog.** 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**:473–497.
12. **O'Connell, K. P., and J. Handelsman.** 1989. *chvA* locus may be involved in export of neutral cyclic β-1,2 linked D-glucan from *Agrobacterium tumefaciens*. Mol. Plant-Microbe Interact. **2**: 11–16.
13. **Stanfield, S. W., L. Ielpi, D. O'Brochta, D. R. Helinski, and G. S. Ditta.** 1988. The *ndvA* gene of *Rhizobium meliloti* is required for β-(1,2)glucan production and has homology to the ATP binding export protein HlyB. J. Bacteriol. **170**:3523–3530.
14. **Sutherland, I. W.** 1985. Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharides. Annu. Rev. Microbiol. **39**:243–270.
15. **Weast, R. C., and M. J. Astle (ed.).** 1980. CRC handbook of chemistry and physics, 61st ed. CRC Press, Inc., Boca Raton, Fla.
16. **Zorreguieta, A., R. A. Geremia, S. Cavaignac, G. A. Cangelosi, E. W. Nester, and R. A. Ugalde.** 1988. Identification of the product of an *Agrobacterium tumefaciens* chromosomal virulence gene. Mol. Plant-Microbe Interact. **1**:121–127.