Differences in Attachment of the Biotypes of *Agrobacterium tumefaciens* and *A. rhizogenes* to Carrot Suspension Culture Cells

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Accepted for publication 3 May 1988.

ABSTRACT

Sykes, L., and Matthysse, A. G. 1988. Differences in attachment of the biotypes of *Agrobacterium tumefaciens* and *A. rhizogenes* to carrot suspension culture cells. Phytopathology 78:1322-1326.

Attachment of *Agrobacterium tumefaciens* to cells of plant hosts is one of the early steps in the bacterial induction of tumors. We compared the effects of alterations in the incubation medium on attachment to carrot cells of bacterial strains belonging to the three biotypes of *A. tumefaciens* and *A. rhizogenes*. Attachment of biotype 1 bacteria was not affected by the composition of the medium. Aggregation of carrot cells by biotype 1 bacteria also was insensitive to medium composition. Attachment of biotype 3 bacteria also was not affected by alterations in the medium. However, the ability of biotype 3 bacteria to aggregate carrot cells was inhibited by 0.25 M NaCl. Attachment of biotype 2 strains to carrot cells in 2 hr occurred only at ionic strengths less than 0.09 M. A small amount of binding of these strains to carrot cells in Murashige and Skoog medium was observed microscopically after 24 hr. Biotype 2 strains failed to aggregate carrot cells at ionic strengths of 0.09 M or more. The differences in binding among the biotypes appeared to be due to differences in chromosomal rather than plasmid genes.

Additional keywords: bacterial attachment, crown gall, *Daucus carota*, suspension culture cells.

One of the early steps in the induction of tumors on plants by *Agrobacterium tumefaciens* is the attachment of the bacteria to the plant cell surface. This attachment appears to be required for tumor formation by strains C58 and A6. Mutants of these bacteria that fail to bind to plant cells are avirulent (1,7,15). Revertants of the nonattaching mutants selected for their ability to attach to plant cells simultaneously recover both the ability to bind to plant cells and virulence, suggesting that both phenotypes are due to the same mutation (7).

Agrobacteria can be divided into three biotypes using various growth and metabolic properties of the bacteria as criteria (4,5,13). Biotype 1 strains include those with a wide host range that grow in the presence of 2% NaCl and that induce undifferentiated tumors (4). Most studies of bacterial attachment have been done with biotype 1 strains such as A6, B6, and C58. Biotype 2 strains generally have a more limited host range than biotype 1 strains. They fail to grow in the presence of 2% NaCl. Biotype 2 strains include most isolates of *A. rhizogenes*, which induces root-forming tumors (4). Attachment of biotype 2 strains to plant tissue culture cells has not been studied. Biotype 2 strains have been observed to attach to root cap cells incubated in water (3). Most biotype 3 strains have been isolated from tumors on grapes, although they can form tumors on other plants. They grow in the presence of 2% NaCl (5,13).

The binding of biotype 1 strains to cultured plant cells has been studied extensively. It is not strongly dependent on the composition of the medium (2,8,11,12). The bacteria bind to carrot suspension cells in 4% sucrose, in Murashige and Skoog (MS) medium (10), in the presence of chelating agents such as ethylenediaminetetraacetic acid, and in 0.25 M NaCl (2). When a casual examination of the binding of *A. rhizogenes* strain A4PC (a biotype 2 strain) to carrot cells was made, it was apparent that this bacterium bound to carrot cells only under much more restricted conditions than those that would support the binding of biotype 1 strains (Table 1). To determine whether this was a general difference among the different biotypes or a special peculiarity of strain A4PC, a comparison of the binding of strains of *Agrobacterium* belonging to the three biotypes was made. The results suggest that various strains from the same biotype differ only slightly from each other with respect to binding to plant cells. However, the three biotypes of *Agrobacterium* appear to differ significantly from each other in the conditions that affect their binding to plant cells.

MATERIALS AND METHODS

Bacterial strains and culture medium. The sources of *A. tumefaciens* strains A6, C58, and NT1 (strain C58 cured of pTi) are as previously described (9). *A. tumefaciens* strains Ag20, Ag222, Ag57, Ag5, and Ag123 were obtained from Greg Cleveland, University of Missouri, Columbia. *A. rhizogenes* strains A4PC and 15834 and *A. tumefaciens* strains Ag 63, FHT14 (strain NT1 with pRi15834b), R1000 (strain NT1 with pRiA4b), and NT1 (pTiAg63) were obtained from Mary-Dell Chilton, Ciba Geigy, Research Triangle Park, NC. All bacteria were maintained at 28 C on Difco nutrient agar. Liquid cultures were grown overnight in

TABLE 1. Effect of the medium on attachment of *Agrobacterium* strains to carrot cells[a]

Bacterial strain	Percent bacterial inoculum bound in:		
	4% sucrose	MS[b]	0.25 M Nacl
Biotype 1			
A6	60 ± 5[c]	50 ± 7	54 ± 8
C58	30 ± 5	24 ± 6	30 ± 6
Ag20	25 ± 10	28 ± 8	55 ± 5
Ag222	20 + 8	23 ± 7	8 ± 4
Biotype 2			
A4PC	15 ± 4	0 ± 2	0 ± 0
Ag5	17 ± 2	0 ± 9	0 ± 1
15834	19 ± 6	0 ± 1	3 ± 3
Biotype 3			
Ag57	34 ± 12	20 ± 14	NT[d]
Ag63	24 ± 5	20 ± 7	12 ± 2
Ag123	11 ± 6	10 ± 5	15 ± 2

[a] $2–3 \times 10^3$ bacteria/ml were incubated with $2–5 \times 10^5$ carrot cells/ml. The number of attached and free bacteria was measured after 2 hr of incubation.
[b] Murashige and Skoog medium (10) containing 4% sucrose and having an ionic strength of 0.09 M.
[c] Mean ± standard deviation of a minimum of three experiments.
[d] NT = not tested.

nutrient broth on a roller drum at 25 C. The biotypes of the strains used have been described previously (4,5,13).

Attachment assays. Attachment assays were carried out as previously described (9). Briefly, early stationary phase cells from a liquid suspension culture of *Daucus carota* grown in MS medium (10) on a shaker at 25 C were collected on Miracloth filters. The carrot cells were resuspended in the desired medium at a concentration of approximately 10^5 cells/ml, and between 10^3 and 10^4 bacteria/ml were added. After various incubation times, the free bacteria were separated from the carrot cells and any attached bacteria by filtration through Miracloth. The number of free and bound bacteria was determined by viable cell counts on nutrient agar. The transfer of the bacteria from broth to MS medium represents a shift down in nutrition. The bacteria show a lag in growth lasting 2–4 hr (9). Cultures were scored for visible aggregation of the carrot cells after 2 days of incubation as previously described (8).

Virulence assays. Virulence assays on carrot disks were carried out as described by Klein and Tenenbaum (6) except that the orientation of the carrot disk (basal or apical end down) was recorded and each assay was done in both orientations. We felt this modification to be necessary because the results of Ryder et al (14) showed that orientation of the carrot disk can determine the result obtained in these assays.

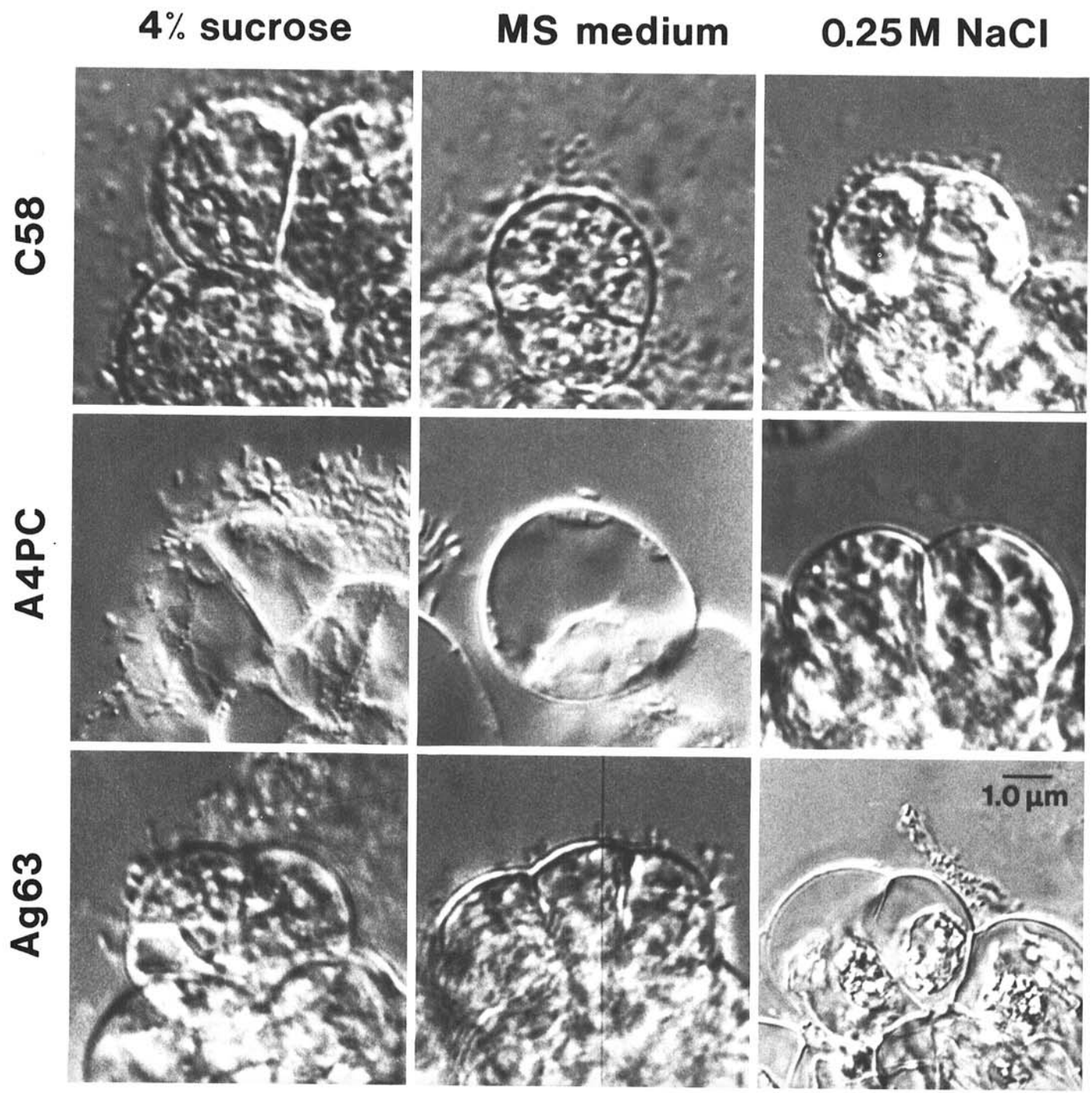

Fig. 1. Binding of strains of *Agrobacterium* belonging to each of the three biotypes to carrot cells as seen in the light microscope after 24 hr of incubation of 10^7 bacteria/ml with 10^5 carrot cells/ml. Cells were incubated in 4% sucrose, Murashige and Skoog (MS) medium (10), or 0.25 M NaCl. Binding of biotype 1 strain C58, biotype 2 strain A4PC, and biotype 3 strain Ag63 are shown. Note that biotype 1 and biotype 3 bacteria bound in all three media. Biotype 2 bacteria bound in 4% sucrose and showed some binding after 24 hr in MS medium. No binding of biotype 2 strains in 0.25 M NaCl was observed. Results similar to those shown here were obtained with the other strains of each biotype.

RESULTS

Attachment of biotype 1 strains to carrot cells. All biotype 1 strains tested bound to carrot cells in the medium in which the carrot cells were grown (Table 1). These bacteria also bound to the plant cells in 4% sucrose, the carbon source in MS medium. Increasing the ionic strength of the medium also did not prevent the binding of biotype 1 strains to carrot cells. These bacteria bound to carrot cells in the presence of 0.25 M NaCl. Four biotype 1 strains were tested. There were differences among the various strains in the percentages of the bacteria that bound to the plant cells. However, greater than 20% of the bacterial inoculum of all the biotype 1 strains bound to the carrot cells after 2 hr incubation in 4% sucrose or in MS medium. Strains A6, C58, and Ag20 also bound more than 20% of the bacterial inoculum in 0.25 M NaCl. Strain Ag222 showed a much reduced, but still significant, binding to carrot cells in 0.25 M NaCl. Thus biotype 1 strains appear to be able to bind to carrot cells regardless of the ionic strength of the medium.

Binding of the bacteria to plant cells also was examined by light microscopy after 6 and 24 hr of incubation of a bacterial inoculum of 10^7/ml with 10^5 carrot cells/ml. Biotype 1 strains bound to carrot cells in 4% sucrose, MS medium, and 0.25 M NaCl after 6 hr (Fig. 1). After a 24-hr incubation with the bacteria, the carrot cells were aggregated by the attached bacteria and associated cellulose fibrils (Fig. 2). Biotype 1 strains were virulent when inoculated on carrot disks (Fig. 3) inducing the formation of undifferentiated tumors.

Attachment of biotype 2 strains to carrot cells. In contrast to biotype 1 strains, the binding of biotype 2 strains to carrot cells was highly dependent on the medium. None of the biotype 2 strains tested showed significant binding to carrot cells after 2 hr incubation in MS medium or 0.25 M NaCl (Table 1). All of the strains bound significantly in 4% sucrose (0.12 M), although the binding was generally less than that seen with biotype 1 strains. The failure of the biotype 2 strains to bind to carrot cells in MS medium was not due to toxicity of the medium because the bacteria grew when incubated in MS medium with carrot cells.

To examine the cause of the inhibitory effect of MS medium on the attachment of the biotype 2 strains, binding to carrot cells of the biotype 2 strain *A. rhizogenes* A4PC in diluted MS medium and in 0.05 M NaCl was measured. The bacteria bound to carrot cells in diluted MS medium but failed to bind in the presence of 0.05 M NaCl (Table 2). The ionic strength of undiluted MS medium is 0.09 M. The molarity of 4% sucrose is 0.12 M, and the bacteria bound to carrot cells in 4% sucrose, suggesting that it is ionic rather than osmotic strength that affects the binding of the bacteria. Thus it appears that the inhibition of binding of biotype 2 bacteria seen in MS medium may be due to the ionic strength of the medium rather than to any specific constituent of the medium.

When the binding of biotype 2 strains to carrot cells was examined by light microscopy, bacteria bound to carrot cells were seen after 6 hr of incubation in 4% sucrose. Very few attached bacteria (less than 1 per 10 carrot cells) were seen after 6 hr in MS medium or 0.25 M NaCl. After 24 hr, bacteria were attached to carrot cells in MS medium, but no attached bacteria were observed

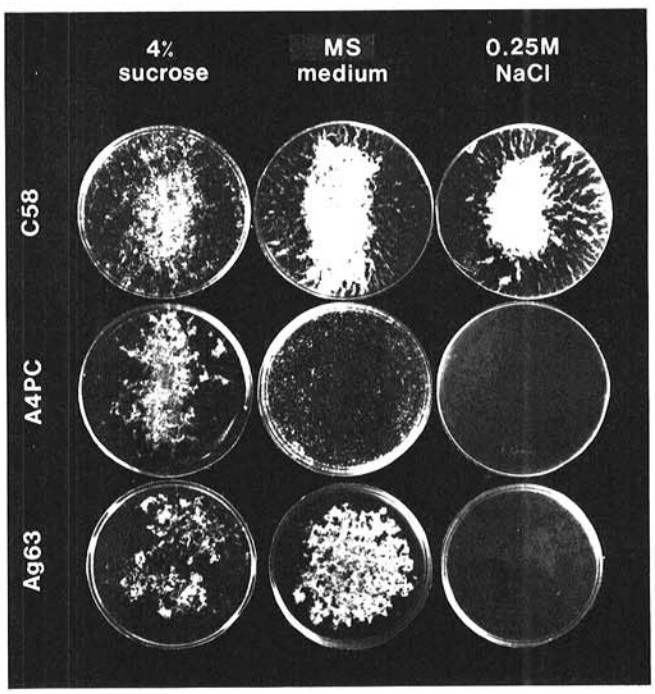

Fig. 2. Aggregation of carrot cells by strains of *Agrobacterium* belonging to each of the three biotypes as seen after 24 hr of incubation. The cultures were poured into petri dishes for photography. Cells were incubated in 4% sucrose, Murashige and Skoog (MS) medium (10), or 0.25 M NaCl. Aggregation by biotype 1 strain C58, biotype 2 strain A4PC, and biotype 3 strain Ag63 are shown. Bacteria belonging to all biotypes aggregated carrot cells in 4% sucrose. In MS medium, bacteria of biotypes 1 and 3 aggregated carrot cells; biotype 2 bacteria caused only a slight aggregation of carrot cells. In 0.25 M NaCl, only bacteria belonging to biotype 1 aggregated carrot cells. Results similar to those shown here were obtained with the other strains of each biotype.

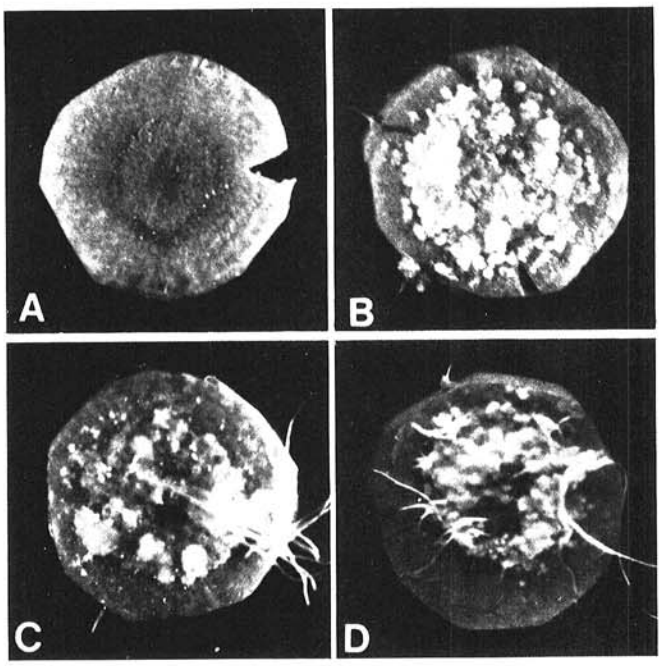

Fig. 3. Virulence of *Agrobacterium* strains on carrot disks. A suspension of 10^9 bacteria was inoculated onto the surface of freshly cut carrot disks. The tumors were photographed after 4 wk. **A,** Avirulent strain NT1; **B,** biotype 1 strain C58; **C,** biotype 2 strain A4PC; **D,** biotype 3 strain Ag63. Biotype 1 strains induced the formation of undifferentiated tumors. Both biotype 2 and biotype 3 strains induced the formation of tumor tissue accompanied by roots. Similar results were obtained with the other strains of each biotype.

TABLE 2. Effect of the medium on the attachment of *Agrobacterium rhizogenes* strain A4PC to carrot cells

Medium[a]	Ionic strength	Percent bacterial inoculum bound[b]
4% sucrose	0	19 ± 6[c]
4% sucrose plus tenth strength MS	0.01 M	20 ± 7
MS	0.09 M	2 ± 2
0.05 M NaCl	0.10 M	1 ± 2
0.15 M NaCl	0.30 M	2 ± 1

[a] MS = Murashige and Skoog medium (10).
[b] 2–3 × bacteria/ml were incubated with 2–5 × 10^5 carrot cells/ml. The number of attached and free bacteria was measured after 2 hr of incubation.
[c] Mean ± standard deviation of a minimum of three experiments.

in 0.25 M NaCl (Fig. 1). The bacteria aggregated the carrot cells in 4% sucrose after 24 hr, but no aggregation was seen in MS medium or 0.25 M NaCl (Fig. 2). The lack of carrot cell aggregation by strain A4PC appeared to be due to the ionic strength of the incubation medium. These bacteria aggregated carrot cells in 24 hr in 4% sucrose, but not in 4% sucrose plus 0.05 M NaCl (Fig. 4). The MS medium did not appear to be toxic to the bacteria because the motile biotype 2 strains A4PC and 15834 remained motile after 24 hr of incubation with carrot cells in MS medium. The biotype 2 strains tested were all virulent on carrot disks, inducing the formation of ageotropic roots (Fig. 3).

Attachment of biotype 3 strains to carrot cells. The binding of biotype 3 strains was similar to that of biotype 1 strains; that is, it was insensitive to the ionic strength of the medium. Roughly the same percentage of the bacterial inoculum bound to carrot cells in 4% sucrose, MS medium, and 0.25 M NaCl (Table 1). In general the binding of biotype 3 strains to carrot cells was less than the binding of biotype 1 strains. Biotype 3 strains were virulent when inoculated on carrot disks, inducing the formation of tumors and roots (Fig. 3). Biotype 3 strains bound to carrot cells after 6 and 24 hr of incubation in 4% sucrose, MS medium, or 0.25 M NaCl (Fig. 1). The bacteria aggregated the carrot cells after 24 hr in 4% sucrose or MS medium. However, there was little aggregation of the carrot cells in 0.25 M NaCl (Fig. 2).

Role of the chromosome and plasmid in attachment. To determine whether the genes responsible for the observed differences in bacterial attachment were located on the bacterial chromosome or on the Ti or Ri plasmid, the binding of bacteria containing combinations of a chromosome and plasmid of various origins was examined (Table 3). The binding of the biotype 1 strain C58 carrying the C58 pTi and strain C58 carrying a pRi derived from a biotype 2 strain or a pTi from a biotype 3 strain were compared (Table 3). When the Ri plasmid from strain 15834 was introduced into C58, the binding characteristics of the resulting bacteria were similar to those of the C58 parent rather than of the biotype 2 parent. Similar results were obtained by the introduction of the Ri plasmid which contains the T region from A4(pRiA4b), except that the binding of the resulting bacteria in 4% sucrose and in MS medium was extremely variable. The cause of this variability is unknown. When the Ti plasmid from the biotype 3 strain Ag63 was introduced into C58, the resultant bacteria showed binding to carrot cells characteristic of the C58 parent (Table 3). Thus the binding characteristics of the bacteria appear to be determined by the chromosome rather than by the plasmid.

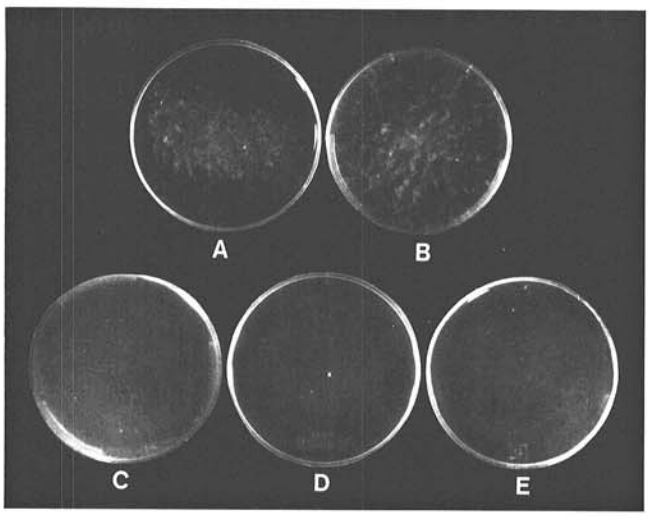

Fig. 4. Effect of ionic strength on the aggregation of carrot cells by biotype 2 strain A4PC. Bacteria (10^7/ml) were incubated with 10^5 carrot cells/ml for 24 hr. The cultures were poured into petri dishes for photography. Cells were incubated in **A,** 4% sucrose (0.12 M osmolarity, ionic strength less than 0.001 M), **B,** Murashige and Skoog (MS) medium (10) diluted 1:10 (ionic strength 0.009 M), **C,** MS medium (ionic strength 0.09 M), **D,** 0.05 M NaCl (ionic strength 0.10 M), and **E,** 0.15 M NaCl (ionic strength 0.30 M). Note that strain A4PC aggregates carrot cells only in medium of ionic strength less than 0.09 M.

DISCUSSION

Although bacterial strains belonging to each of the three biotypes of *Agrobacterium* were virulent on carrot disks, they differed in their attachment to carrot suspension culture cells. Binding of bacteria of biotype 1 generally involved a larger percentage of the bacterial inoculum than binding of biotypes 2 or 3. In contrast to biotypes 1 and 3, binding of biotype 2 strains was inhibited by ionic strengths of 0.09 M or more. The ability of biotype 3 strains to aggregate carrot cells was inhibited by 0.25 M NaCl. This inhibition was associated with an inhibition of the production of material which gave a fluorescent stain with cellufluor (presumably cellulose). Production of cellulose fibrils has been shown to be required for the aggregation of carrot cells by biotype 1 strains of *A. tumefaciens* (6,7).

Although biotype 2 strains showed very little binding to carrot cells in media with an ionic strength greater than 0.09 M, the bacteria are virulent on carrot disks. This suggests that either the ionic strength of the fluid in the wounded tissue is less than 0.09 M or the limited binding seen at higher ionic strength is sufficient for virulence.

Biotype 1 strains of *A. tumefaciens* generally have a wide host range. The binding of these strains to carrot cells occurs over a substantial range of ionic strengths and medium composition. Biotype 2 strains have a more limited host range and are generally less virulent than biotype 1 strains. The binding of these strains also is more affected by the ionic strength of the medium. Biotype 3 strains also have a more restricted host range than biotype 1 strains. They are generally intermediate in virulence between biotype 1 and biotype 2 strains. Binding of these strains to plant cells is not limited by ionic strength, but the binding is generally less than that of biotype 1 strains. It is not known to what extent the ability of the bacteria to bind to plant cells may determine the host range of a particular bacterial strain. Hawes and Pueppke (3) found a correlation between the binding of the bacteria to root cap cells from a variety of plant species and the susceptibility of the species to crown gall disease.

The bacterial genes encoding the observed differences in binding appear to be chromosomal, at least in the case of biotype 1 strain C58. This is not surprising since all of the known bacterial mutants that affect bacterial attachment to host cells are chromosomal (2,7). The nature of the difference in the bacterial binding sites of biotypes 1 and 2 is unclear. Although the growth of biotype 2 strains is inhibited by NaCl, the bacteria grew at an ionic strength of 0.09 M in MS medium. Binding to carrot cells in this medium was minimal. In addition, binding in MS medium was very slow, requiring more than 6 hr. Bacterial binding of all three biotypes occurred in 4% sucrose suggesting that none of the other

TABLE 3. Effect of the medium on attachment of Agrobacteria with varying plasmids[a]

Bacterial strain	Chromosome	Virulence plasmid	Percent bacterial inoculum bound in: 4% sucrose	MS[b]	0.25 M NaCl
C58	C58	pTiC58	30 ± 5[c]	24 ± 6	30 ± 6
FHT14	C58	pRil5834b	43 ± 3	18 ± 13	28 ± 2
R1000	C58	pRiA4b	V[d]	V	32 ± 3
NTl(pTiAg63)	C58	pTiAg63	43 ± 10	37 ± 2	40 ± 2
15834	15834	pRil5834	19 ± 6	0 ± 1	3 ± 3
A4PC	A4	pRiA4	15 ± 4	0 ± 2	0 ± 1
Ag63	Ag63	pTiAg63	11 ± 6	10 ± 5	15 ± 2

[a] $2–3 \times 10^3$ bacteria/ml were incubated with $2–5 \times 10^5$ carrot cells/ml. The number of free and attached bacteria was measured after 2 hr of incubation.
[b] Murashige and Skoog medium (10) containing 4% sucrose and having an ionic strength of 0.09 M.
[c] Mean ± standard deviation of a minimum of three experiments.
[d] V = results extremely variable, ranging from 0 to 25% of the bacterial inoculum bound.

constituents of MS medium was required. The ability of biotype 1 and 3 strains to bind to host cells in 0.25 M NaCl suggests that the binding is not solely dependent on ionic charge interactions. The binding of biotype 2 strains which is affected by ionic strength may involve ionic interactions, perhaps between the negatively charged bacterial surface and the positively charged hydroxyproline-rich glycoproteins on the plant cell surface.

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