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# Attachment of Agrobacteria to Grape Cells†

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**The presence of the Ti plasmid favorably influences the attachment of agrobacteria to grape callus cells, especially during the early stages of a 2-h incubation. *Agrobacterium* strains attached to a similar extent to both the crown gall-resistant cultivar (Catawba), *Vitis labruscana*, and the crown gall-susceptible cultivar (Chancellor), *Vitis* sp. Attachment of the virulent strain to grape callus cells is blocked by the avirulent strain HLB-2 in both the tissue culture cell suspension and the seedling root systems.**

*Agrobacterium tumefaciens*, a soil-borne phytopathogenic bacterium, infects many higher plants through wounds. A large tumor-inducing (Ti) bacterial plasmid is essential for tumorigenesis (for reviews, see references 4 and 29). Two clusters of genes on the Ti plasmid, the *vir* region and the T-DNA, play critical roles during the infection process. Wounded tissues release phenolic compounds (32), which activate a sequence of *vir* genes that prepares the T-DNA for transfer to plant cells and for its subsequent integration into the genome. The expression of T-DNA in the infected plant results in the overproduction of growth hormones, which leads to undifferentiated plant cell proliferation (29).

The binding of bacteria to plant cells is one of the earliest steps in the transformation (tumorigenesis) process (for reviews, see references 1, 7, and 23). In vitro studies revealed that binding of *A. tumefaciens* to plant cells is temperature dependent (26), that a pH of 6 is optimal (25, 26), and that divalent cations had little effect on attachment (15, 26). Quantitative analysis has been performed on plant cell suspensions by either counting viable bacterial cells or detecting isotope-labeled bacteria. The former uses filtration to separate free bacterial cells from those attached to the plant cells (20, 22). The latter measures radioactivity retained on plant cells (9, 25, 26).

*A. tumefaciens* biovar 3 is the predominant pathogen in vineyards worldwide (5, 19, 27, 34) and has not yet been successfully controlled in practice. Chen and Xiang (8) reported that an antagonist, *Agrobacterium radiobacter* HLB-2, suppresses crown gall bacteria in grapevines. However, the inhibitory mechanism has not been identified. Our preliminary data indicated that HLB-2 cells rather than culture filtrates attenuated the numbers and sizes of tumors formed on grape explants. We hypothesized that HLB-2 might interfere with infection by blockage of the infectable sites. The attachment process has been studied with various plant tissues, such as pinto bean leaves (18), potato discs (13, 28), carrot (20, 24), tobacco (9, 13), and even some monocot cells (10, 14, 26, 37). However, the attachment of *Agrobacterium* strains to grape cells has not been well studied. The objective of this study was to investigate the attachment of *Agrobacterium* strains to grape cells in a callus suspension and the competitive attachment between *A. radiobacter* HLB-2 and *A. tumefaciens* in both a callus suspension and a root system.

## MATERIALS AND METHODS

**Preparation of grape callus cell.** Grape calli of cultivars Catawba and Chancellor were derived from stem internodes. The grape explants were sterilized with 2.5% sodium hypochlorite; a sterile distilled water rinse followed. Trimmed pieces of explants, ca. 0.5 cm in length, were grown on half-ionic-strength Gamborg's B5 (Sigma) tissue culture medium (12) supplemented with α-naphthalene acetic acid and kinetin (both at 0.5 μg/ml). Callus from the rims of the explants was subcultured onto fresh medium. Grape calli, 20 to 25 days old, were homogenized in 0.07 M phosphate buffer (pH 6.3). The homogenized cell suspension was filtered through a double layer of cheesecloth. One milliliter of the filtrate was diluted 10-fold, and cells were counted in a Sedgewick-Rafter chamber. This cell suspension was diluted in the same buffer to a final concentration of ca. $10^4$ cells per ml.

**Preparation of bacterial cells.** The bacterial strains used are listed in Table 1. Overnight cultures were pelleted, washed once with phosphate buffer, resuspended, and diluted to the required final concentrations before inoculation.

***Agrobacterium* cell recovery by different techniques.** Aliquots of 1 ml of bacterial suspension prepared from phosphate buffer, ca. $10^3$ cells per ml, were centrifuged at $17 \times g$ for 2 min (Beckman Microfuge model 11). One-hundred-microliter aliquots of the supernatant solution were spread onto a nutrient yeast dextrose agar (NYDA) plate (17) in triplicate. Meanwhile, aliquots of 10 ml of the bacterial suspension were filtered through Miracloth or Whatman no. 1 filter paper. One-hundred-microliter aliquots of the filtrate were spread on NYDA plates in triplicate. As a control, a 0.1-ml bacterial suspension without filtration or centrifugation was spread on the same medium in triplicate. All of the plates were incubated at 28°C. The CFU were determined, and the efficiency of bacterial recovery was calculated and compared with that of the control. The experiment was conducted twice. Statistical analysis was done by using Fisher's protected least significant difference (LSD) method (33).

**Bacterial attachment to grape cells.** Grape cell and bacterial cell suspensions were mixed at a ratio of 10:1, which gave a final bacterial concentration of $10^3$ cells per ml and a plant cell concentration of $10^4$ cells per ml. The mixtures were

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TABLE 1. Bacterial strains used in this study

| Strains | Biovar | Other characteristics[a] | Source (reference) |
|---|---|---|---|
| *A. tumefaciens* | | | |
| C58 | 1 | NOC | Nester |
| A136 | 1 | C58 cured of pTi, Rif$^r$ | Nester (39) |
| A348 | | A136(pTiA6), OCC | Nester |
| A854 | | A136(pTiAg63), OCC | Nester |
| NT1 | | Same as those for A136 except Rif$^s$ | Pueppke (39) |
| NT1(pTi15955) | | NT1(pTi15955) | Pueppke |
| FACH | 1 | Isolated from grape | Goodman |
| Ag63R | 3 | Mutant of Ag63; Str$^r$ Rif$^r$ | Goodman (36) |
| A5129 | | Tn*5* mutagenesis of A348, ExoC$^-$ | Nester (10) |
| *A. radiobacter* | | | |
| HLB-2 | 1 | | Xiang (12) |
| K84 | 2 | | Kerr |
| *E. coli* K-12 | | Wild type | Chatterjee |

[a] NOC, nopaline catabolism; OCC, octopine catabolism.

TABLE 2. Comparison of bacterial recovery from bacterial cell suspension by various techniques in absence of plant cells

| Strain | Bacterial recovery (% of control[a] ± SEM) after: | | |
|---|---|---|---|
| | Low-speed centrifugation | Filtration through: | |
| | | Miracloth | Whatman paper |
| A136 | 98.7 A[b] ± 1.3 | 92.3 A ± 4.6 | 59.0 C ± 1.5 |
| A348 | 99.7 A ± 0.3 | 71.7 B ± 0.3 | 47.3 C ± 5.2 |
| A854 | 98.7 A ± 1.3 | 98.7 A ± 1.3 | 80.7 B ± 3.3 |
| C58 | 99.3 A ± 0.7 | 97.3 A ± 1.2 | 83.7 B ± 1.9 |
| FACH | 92.3 A ± 2.4 | 87.3 AB ± 4.3 | 78.0 B ± 0.6 |
| Ag57 | 100 A ± 0 | 74.7 B ± 8.1 | 73.2 B ± 9.5 |
| Ag63 | 97.1 A ± 2.5 | 95.2 A ± 4.7 | 75.5 B ± 6.8 |
| HLB-2 | 98.7 A ± 1.3 | 87.3 B ± 1.7 | 77.7 C ± 3.5 |
| K84 | 96.7 A ± 3.3 | 50.3 C ± 1.2 | 47.0 C ± 4.5 |

[a] Means of three measurements. Each strain was tested three to five times and gave similar results.

[b] Means followed by the same letter are not significantly different at $P < 0.05$ as determined by Fisher's protected LSD test. Analysis was performed on square root-transformed data.

placed in Erlenmeyer flasks, two to three flasks per mixture, and the flasks served as blocks. The mixtures were gently agitated for a few seconds and incubated at room temperature. After regular time intervals, aliquots of 1 ml of the mixture were pipetted into a 1.5-ml microcentrifuge tube and centrifuged at 17 × *g* for 2 min. This low-speed centrifugation allows the grape callus cells with the attached bacterial cells to sediment, while the unattached bacterial cells remain in the supernatant. Thus, bacterial cells attached to grape cells were separated from the unattached ones. One-hundred-microliter aliquots of the supernatant were spread onto NYDA plates in triplicate, and colonies were counted as described before. For controls, viable bacterial cells maintained in phosphate buffer with and without grape cells also were counted. Percentages of attached bacterial cells were calculated by subtracting percentages of unattached cells recovered from the supernatant. The procedures were repeated three to five times, with each experiment set up in a randomized complete block design. The differences in means between strains at each time point were analyzed by the LSD test (33).

**Measurement of competitive attachment to callus cells.** *A. tumefaciens* Ag63R and *A. radiobacter* HLB-2 were mixed in equal volumes with the grape callus suspension ($10^4$ cells per ml), giving a final concentration of $10^3$ CFU of each bacterium per ml. The mixtures were set up and incubated, and 1-ml aliquots were centrifuged as described above. Aliquots of 0.1 ml of supernatant were spread onto NYDA plates and NYDA plates supplemented with streptomycin and rifampin at 1,000 and 10 μg/ml, respectively. Unattached Ag63R cells were counted as colonies that developed on the medium supplemented with the antibiotics following plating of aliquots of the supernatant, and unattached HLB-2 cells were obtained by subtracting recovered Ag63R from the total colonies recovered from NYDA plates without antibiotics. The experiments were performed four times to produce a complete block design. Data were analyzed by the LSD test (33).

**Competitive attachment of agrobacteria to grape seedling roots.** Grape plantlets (cv. Chancellor) were regenerated from embryoids produced by anther callus cultures. Roots of aseptic grape plantlets were rinsed with sterile distilled water and immersed in the phosphate buffer with Ag63R alone or a mixture of Ag63R and HLB-2. The final concentration of each strain was ca. $5 \times 10^6$ cells per ml. After 1, 2, or 3 h of incubation, the roots were rinsed thoroughly with sterile distilled water, and excess water was absorbed with sterile Whatman filter paper. The roots were then excised, and the root surface area was measured with a portable area meter (model L1-300, Lambda Instruments Corp.). The total root surface area was obtained by multiplying the projected area by π, assuming that the measured roots are circular in cross section (2). The roots were triturated in a sterile mortar with 10 ml of sterile distilled water. One-hundred-microliter aliquots of 10-fold dilutions were spread onto Roy-Sasser's selective medium (30) supplemented with streptomycin and rifampin in triplicate. The plates were incubated at 28°C, and the colonies were counted. The number of attached bacterial cells per area of root surface was determined. Each treatment consisted of three to four grape plantlets. The experiment was repeated once. Data were log transformed, and because of significant strain versus time interactions, data were analyzed by orthogonal contrasts to determine whether significant differences existed between treatments at each time (33).

## RESULTS

**Comparison of bacterial recovery by various techniques.** Attachment has been routinely measured by filtering a mixture of plant and bacterial cells through Miracloth or Whatman filter paper (21), which permits the passage of bacteria but not plant cells. In this study, we found that the filtration provided erratic bacterial recovery, while low-speed centrifugation provides nearly perfect recovery from bacterial cell supernatant in the absence of plant cells (Table 2). Of all bacterial strains tested, four of nine strains were recovered by Miracloth filtration at 90% of the control levels, but three of nine strains were recovered below 80% of the control levels. Whatman filter paper gave the lowest bacterial recoveries of the three techniques. Since the low-speed centrifugation sediments grape cells (personal observation) but not free bacterial cells (Table 2), it was substituted for filtration because it permitted accurate detection of

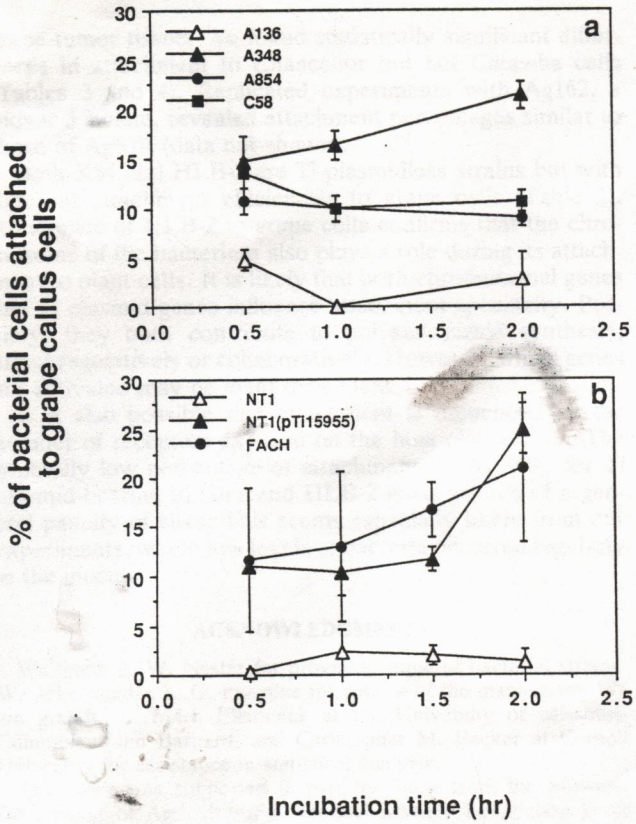


FIG. 1. Attachment of agrobacteria with A136 chromosome (a) and NT1 background (b) to grape cv. Chancellor callus cells. FACH is a grape isolate used as a positive control. Data in panel a are means of 12 measurements from one representative experiment, and data in panel b are means of six measurements from three independent experiments. Analysis was performed on square root-transformed data. Bars represent standard errors of the means.

the unattached bacterial cells that remained in the supernatant and, in turn, the number of attached bacterial cells could be calculated.

**Influence of Ti plasmid on attachment to grape cells.** *Agrobacterium* strains with the same chromosomal background were tested for their attachment to grape cells. A136, a Ti-plasmidless strain, attached to grape cells poorly even after 2 h of incubation (Fig. 1a). However, the strains with the A136 chromosome, but containing pTiA6 and pTiAg63, attached to grape cells more rapidly and in greater numbers than A136. The differences between A136 and A348 or A136 and A854 were significant at 0.5, 1.0, and 2.0 h of incubation ($P < 0.05$ by Fisher's LSD test). The parental strain C58, which has a nopaline-type Ti plasmid, also attached to grape cells at a significantly higher level than A136 ($P < 0.05$). Similarly, NT1, an analog of A136, attached to grape cells sparingly, whereas the strain NT1 containing pTi15955 permitted a significantly higher attachment efficiency than NT1 (Fig. 1b; $P < 0.05$). FACH, a biovar 1 grape isolate that induces large tumors on grapevines, attached to grape cells to a similar extent as that of the Ti plasmid-containing strain NT1(pTi15955).

Attachment of *A. radiobacter* to grape cells was also tested. *A. radiobacter* K84, which failed to suppress grape crown gall, scarcely adhered to grape callus cells after 2 h of incubation. However, another strain of *A. radiobacter*,

TABLE 3. Attachment of *Agrobacterium* spp. to grape cv. Chancellor tissue culture cells

| Strain | % Attached bacteria[a] ± SEM after 2 h of incubation |
|---|---|
| FACH | 25.97 A[b] ± 2.7 |
| Ag63R | 9.7 B ± 2.1 |
| HLB-2 | 9.5 B ± 1.0 |
| A5129 | 0 C ± 0 |
| K84 | 0.2 C ± 0.2 |
| *E. coli* K-12 | 0 C ± 0 |

[a] Means of three measurements. Each strain was tested three to five times and gave similar results.

[b] Means followed by the same letter are not significantly different at $P < 0.05$ as determined by Fisher's protected LSD test. Analysis was performed on square root-transformed data.

HLB-2, which has been reported to inhibit grape isolates in vitro and in planta (8), attached to grape cells with greater efficiency than K84 (Table 3). FACH, as a positive control, attached to Chancellor cells in greater numbers than *A. radiobacter* HLB-2 and K84. Ag63R is a mutant of biovar 3 strain Ag63 (36) and is also highly tumorigenic to cultivar Chancellor. It attached to Chancellor cells at the same level as HLB-2. A5129, a mutant of A348, is attachment defective on *Zinnea* leaf mesophyll cells (6). Serving as negative controls, neither A5129 nor *Escherichia coli* attached to grape cells.

**Cultivar specificity of agrobacterial attachment to grape callus cells.** As shown in Table 4, the Ti-plasmidless strain A136 did not attach to Catawba callus cells during 0.5- and 2-h incubation periods. The highly virulent strains FACH and Ag63R adhered to Catawba cells after 0.5 h of incubation, and their attachment had doubled after 2 h. *A. radiobacter* HLB-2 attached to Catawba callus cells at the same levels as the pathogenic strains.

**Competitive attachment of *A. tumefaciens* and *A. radiobacter* to grape callus cells and to plantlet root surfaces.** Attachment of Ag63R to grape callus cells was affected by HLB-2 when they were coinoculated. The virulent strain Ag63R alone is able to attach to grape cells to about the same extent as HLB-2 (Fig. 2). However, when coinoculated with HLB-2, the attachment efficiency of HLB-2 did not change, whereas the attachment efficiency of virulent strain Ag63R was significantly reduced ($P < 0.01$ by LSD test) at each sampling interval.

Competitive attachment of the two strains was also tested on grape roots. Cocultivation with HLB-2 reduced the

TABLE 4. Attachment of agrobacteria to grape cv. Catawba tissue culture cells

| Strain | % Attached bacteria[a] ± SEM after incubation for: | |
|---|---|---|
| | 0.5 h | 2.0 h |
| A136 | 0 A[b] ± 0 | 0 Y ± 0 |
| Ag63R | 7.5 B ± 1.0 | 16.0 Z ± 3.6 |
| FACH | 10.5 BC ± 1.7 | 18.3 Z ± 3.9 |
| HLB-2 | 12.3 C ± 1.2 | 17.5 Z ± 1.4 |

[a] Means of pooled data from three separate experiments with three measurements per experiment.

[b] Means followed by the same letter are not significantly different at $P < 0.05$ as determined by Fisher's protected LSD test. Analysis was performed on square root-transformed data.

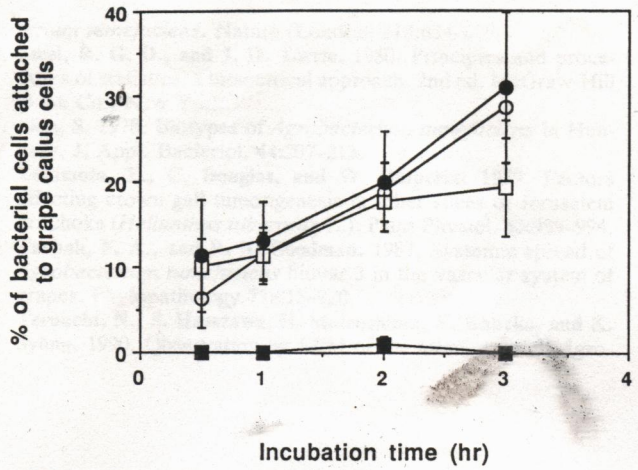


FIG. 2. Competitive attachment of *A. tumefaciens* Ag63R and *A. radiobacter* HLB-2 to grape cv. Chancellor callus cells. Datum points are means of eight measurements from four separate experiments. Analysis was performed on square root-transformed data. The standard errors of the means are shown by bars. Symbols: ○, attachment of HLB-2 without coincubation; □, attachment of Ag63R without coincubation; ●, attachment of HLB-2 in the presence of Ag63R; ■, attachment of Ag63R in the presence of HLB-2.

attachment of virulent strain Ag63R to Chancellor plantlet roots (Fig. 3) during 3 h of incubation. Attachment differences in the presence and absence of HLB-2 were significant at 1 and 2 h ($P < 0.05$) when tested by orthogonal contrasts on log-transformed data. Reduced attachment resulting from cocultivation with HLB-2 was also detected in grape cultivar Chardonnay and Catawba seedling roots (data not shown).

## DISCUSSION

Miracloth and Whatman filter paper have been used to separate unattached bacteria from those attached to plant

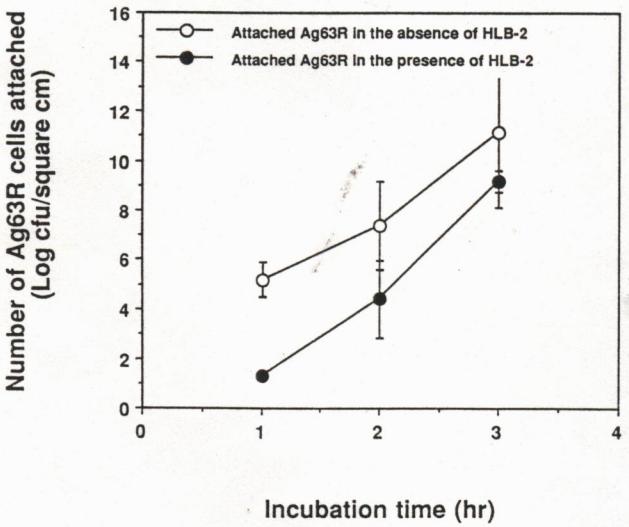


FIG. 3. Inhibition of attachment of *A. tumefaciens* Ag63R by *A. radiobacter* HLB-2 to Chancellor seedling roots. Data are means of three measurements from one representative experiment. Analysis was performed on log-transformed data by orthogonal contrasts. Standard deviations are shown by bars.

cells (20, 21). In a preliminary experiment, we found that low-speed centrifugation had no detectable effect on sedimenting free bacterial cells (Table 2) but could pellet grape cells (personal observation). Hence, the bacterial cells that attached to grape cells were readily separated from the unattached bacteria. The agrobacterial population remained at approximately the same level in phosphate buffer during 2 h of coincubation, with or without plant cells (data not shown). Therefore, recovery of unattached bacterial cells from the supernatant was an accurate reflection of the attached bacterial cells that sedimented to the bottom with plant cells.

Hawes and Pueppke (16) demonstrated that a high level of binding of *A. tumefaciens* (B6) was strongly correlated with tumorigenesis in 48 plant species. However, no difference was observed between weakly and highly susceptible plants in adherence of *Agrobacterium* cells to *Helianthus* cotyledons and to potato discs (3). The agrobacterial strains tested in this study attached similarly to the crown gall-resistant cultivar Catawba and the crown gall-susceptible cultivar Chancellor (Tables 3 and 4). It would seem that, as far as cultivar Catawba is concerned, resistance to *A. tumefaciens* is not expressed at this early stage of the infection process.

*A. radiobacter* K84, which failed to suppress grape crown gall infections, attached to grape cells below the limits of detection during 2 h of coincubation. This may be another indication of why K84 is unable to inhibit crown gall in grapes in addition to the insensitivity of grape isolates to agrocin 84. *A. radiobacter* HLB-2 adhered to grape cells at levels similar to virulent strains and competitively prevented the virulent strain Ag63R from adhering to either grape callus cells or plantlet roots (Fig. 2 and 3). HLB-2 was also demonstrated in planta as an antagonist against tumor induction and development (27a). It would appear that avirulent HLB-2 may suppress tumor development by competitive blockage, preventing the attachment of the virulent strain to grape cells.

Genes that have been identified to control agrobacterial attachment appear to be chromosomal (6, 9, 11, 22, 24, 38). However, there is evidence that the Ti plasmid can also contribute to attachment (20, 31, 35, 40). In studies measuring radioactivity as an indication of attachment (9, 25, 26), a high bacterial inoculum ($10^6$ cells per ml) had to be used to obtain a measurable radioactive signal. This might have increased the opportunity for bacterial interactions, such as aggregation or clustering (22). Matthysse (23) observed that a Ti-plasmidless strain (NT1) at high inoculum concentrations attached to tobacco cells, but at low concentrations, attachment was not apparent. Our data support that observation.

The data presented here with low bacterial and plant cell numbers provide evidence that the Ti plasmid influences the attachment of *A. tumefaciens* to grape cells. A136, which has the C58 chromosome, appears to produce polysaccharides in smaller amounts and more slowly than the Ti plasmid-bearing strains with the same chromosomal background, such as A348, A854, and C58 (personal observation). A136 attached poorly to grape cells during 2 h of incubation (Fig. 1) but was able to attach to grape cells after 3 h (data not shown). It seems, therefore, that the Ti plasmid may be critical for early attachment and subsequent transformation.

The possibility that there are comprehensive differences in attachment to grape cells between biovars 1 and 3 was not examined specifically. We did compare a biovar 1 strain, FACH, with a biovar 3 strain, Ag63R, both derived from

grape tumor tissue. We found statistically significant differences in attachment to Chancellor but not Catawba cells (Tables 3 and 4). Replicated experiments with Ag162, a biovar 3 isolate, revealed attachment percentages similar to those of Ag63R (data not shown).

Both K84 and HLB-2 are Ti-plasmidless strains but with different attachment efficiencies to grape cells (Table 3). Adherence of HLB-2 to grape cells confirms that the chromosome of the bacterium also plays a role during its attachment to plant cells. It is likely that both chromosomal genes and Ti plasmid genes influence attachment specificity. Possibly, they both contribute to polysaccharide synthesis, either regulatively or collaboratively. However, which genes are activated may be plant dependent.

It is also possible that attachment is influenced by the number of receptors exposed on the host cell surface. The generally low percentage of attachment, 10 to 25%, for Ti plasmid-bearing isolates and HLB-2 is suggestive of a general paucity of sites. This seems especially likely from our experiments, where low levels of bacteria occurred regularly in the inocula.

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