

Video Article

Adherence of Bacteria to Plant Surfaces Measured in the Laboratory

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Abstract

This manuscript describes a method to measure bacterial binding to axenic plant surfaces in the light microscope and through the use of viable cell counts. Plant materials used include roots, sprouts, leaves, and cut fruits. The methods described are inexpensive, easy, and suitable for small sample sizes. Binding is measured in the laboratory and a variety of incubation media and conditions can be used. The effect of inhibitors can be determined. Situations that promote and inhibit binding can also be assessed. In some cases it is possible to distinguish whether various conditions alter binding primarily due to their effects on the plant or on the bacteria.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56599/>

Introduction

The measurement of bacterial binding to plant surfaces has become important in three disparate situations. The first situation is the examination of the transmission of human pathogens on plant surfaces^{1,2,3}. The goal here is to prevent bacterial binding or to remove or kill bound bacteria and thus to reduce the transmission of disease by plant material. The second situation is the examination of the binding of plant pathogens to plant surfaces⁴. Once again the goal here is to prevent binding or to remove or kill bound bacteria and thus to reduce disease. The third situation is the examination of the binding of symbiotic or plant-growth-promoting bacteria^{5,6}. The goal here is to promote bacterial binding and thus to increase plant health and crop yields.

The techniques for measuring bacterial binding to plant surfaces described in this article are inexpensive and relatively easy to carry out. The only requirements are a microscope and materials generally found in a bacteriology laboratory. For some techniques a bath sonicator is useful. The techniques described are designed for binding experiments carried out using relatively small sample sizes. Measurements of binding are made in the laboratory, although it may be possible to modify some of these techniques for use in the greenhouse or in the field.

These techniques have been used to measure bacterial binding to roots, sprouts, cut leaves, cut fruits, and intact cherry tomatoes in the laboratory^{7,8,9,10,11,12,13,14,15}. They have also been used to measure root colonization of plants growing in soil or sand in the laboratory¹⁶. The techniques have been used with many bacterial species including *Agrobacterium tumefaciens*, *Sinorhizobium meliloti*, *Escherichia coli*, *Salmonella enterica*, and *Pseudomonas fluorescens*. A useful description of methods for assessing the interaction of *A. tumefaciens* with surfaces can be found in Morton and Fuqua (2012)¹⁷. In all cases the sample sizes involved were small, generally less than 25 - 50 plants. The techniques described are suitable for use with human pathogens which need to be kept contained during the experiments.

Protocol

1. Preparation of Axenic Plant Material

1. Prepare seedlings grown in water.
 1. Place a small number of seeds (less than 30) in a 30, 50, 100, or 150 mL glass beaker. We have used tomato, alfalfa, *Arabidopsis thaliana*, pea, bean, tobacco, lettuce, and carrot seeds with this protocol.
NOTE: To determine how many seeds can be sterilized together without contamination spreading from one seed to another, see step 1.1.2.
 2. Cover the seeds with 80% ethanol and swirl briefly. Let the seeds soak for 1 min.
 3. Pour off the ethanol and cover the seeds with a solution of 50% by volume commercial bleach (NaClO) and 0.1% Triton X-100 in tap water. Let the seeds soak for 20 min.
NOTE: If the seeds are large, such as bean seeds, it may be necessary to lengthen the soaking time to completely kill the fungi on the seed surface.
 4. Pour off the bleach mixture and wash the seeds 3 times with sterile water. Let the seeds soak in the water for 1 min for each wash.

5. To obtain axenic seedlings add a small amount of sterile water between 5 and 25 mL depending on the size of the seeds. Pour the seeds and water into a sterile Petri dish for seed germination.
NOTE: The water should cover the bottom of the dish but not cover the seeds to encourage the formation of root hairs. Axenic describes a culture in which only a single species of organism is present.
 6. Incubate in the dark until the seedlings reach the desired size between 1 cm and 10 cm (about 5 days for tomato and *A. thaliana* and 1 to 3 days for alfalfa). For larger seeds use a covered sterile container with a capacity of more than 100 mL such as a glass dish covered with foil.
2. Determine the frequency of contamination of a particular seed lot with microorganisms which remain viable on or within the seeds after surface-sterilization.
NOTE: This is necessary because occasional seeds carry microorganisms under the seed coat which cannot be killed by surface-sterilization. Use the frequency of contaminated seeds in a seed lot to determine how many seeds to sterilize at one time without a high risk of the group of seeds becoming contaminated due to one seed with bacteria or fungi which survive the treatment.
 1. Carry out steps 1.1.1 through 1.1.3.
 2. Place the seeds on a nutrient agar Petri dish. Put about 10-30 seeds in each dish spacing them out so that they can be scored individually. Seal the dishes with tape or sealing film.
 3. Incubate for 3 - 5 days at 25 °C scoring each day for visible outgrowth of microorganisms from the seeds.
 3. Prepare seedlings grown in sand. Seedlings can be grown in sand for use in bacterial adhesion experiments.
 1. Carry out steps 1.1.1 through 1.1.3.
 2. Sterilize quartz or sea sand in the autoclave. Both of these contain some organic material. If this will affect the experiment, wash the sand by covering it with twice its volume of 0.1 M HCl. Mix for 10 min. Allow the sand to settle and pour off the liquid. Rinse the sand 3 times with water, and once with 80% ethanol followed by two additional water rinses using the same protocol as for the 0.1 M HCl. Sterilize the washed sand in the autoclave.
 3. Sterilize containers by submerging them in 50% bleach for 5 min and rinse them 5 times with sterile water by submersion. Allow them to dry and seal the bottom with sealing film. The film as obtained from the manufacturer is generally free of microorganisms on the inside side which should be placed facing the inside of the container.
 4. Mix the sterile sand with enough sterile water to wet it enough that the sand sticks together. The amount will depend on how dry the sand is. Usually 10 - 35% of the volume of the sand is sufficient. Place the wet sand in the container allowing sufficient depth for the length of roots needed and sufficient space above the sand for growth of the shoot. The exact distances depend on the species and variety of plant being used.
 5. Plant seeds.
 1. Make a shallow hole in the sand with a sterile glass rod just deep enough to place the seed below the surface of the sand (roughly 1 - 5 mm). Place the seed in the hole. Cover it with a thin layer of sand. Seal the top of the container with sealing film to prevent loss of water and entrance of additional microbes.
 2. Grow the plants in the lab under a light or in the greenhouse at an appropriate temperature and day-length for the species and variety of plant. For tomato, alfalfa, or *Arabidopsis thaliana* use room temperature and 12 h light/dark cycles.
 6. Plant seedlings in a hole in the sand (1 - 2 mm in diameter and deep enough that the root will be covered with sand). Make the hole with a sterile glass rod. Guide the root carefully into the hole using a sterile stainless-steel crochet hook if needed and fill the sides of the hole with sand.
 4. Alternatively, grow plants axenically on or in agar containing a salts mixture such as MS salts. Use shoots of axenic plants grown in agar directly. Avoid roots grown with agar as agar sticks to both the plant surface and the bacteria. This may result in a false impression of bacterial adhesion to the plant surface.

2. Preparation of Other Plant Material

1. Wash plant material grown in the greenhouse with water before use to reduce the number of indigenous microbes. There will be bacteria and fungi on the plants. Protozoa will be present in the soil, on the roots, and possibly on the leaves. Washing with bleach or ethanol will alter the plant surface and is not recommended.
 1. If after water washing significant numbers of microbes remain, treat the plants with diluted liquid hand soap or dilute hydrogen peroxide (0.01%) to remove or kill microbes. This is generally less damaging to the plant than bleach or ethanol.
2. As plant material purchased at a local market is difficult to decontaminate, choose material which does not appear to have been subject to long storage. Avoid parts of the material which appear brown or damaged.
 1. Wash it with water and make fresh cuts on the ends of material which have previously been cut before using it, unless the interactions of the test bacteria with the bacteria (and eukaryotes) which will have accumulated at cut sites are of interest. Do not wash with bleach or ethanol as they will alter the plant surface.
3. Treat wound sites. Wound sites often provide bacteria access to the interior of plant material¹⁸.
 1. Block or reduce bacterial attachment to, and movement through, wound or cut sites created in preparing the material by dipping the cut edge in melted paraffin or by painting the site with melted paraffin using a small brush. Do not use a spatula or any sharp implement as this may damage the tissue.
 2. Seal stem scars on fruit such as tomatoes with paraffin. Some bacteria will swim into the area under the paraffin, but their numbers are generally small.

3. If bacterial entry into wound sites is of interest, estimate the distance that bacteria can move carried by water streams or diffusion by observing the movement of a dye added to the solution. Mark motile bacteria by introducing a gene encoding a fluorescent protein such as green fluorescent protein (GFP) into them and trace them using fluorescence microscopy described in step 6.1.2¹⁹.

3. Preparation of the Bacteria

1. Grow the bacteria. Use a medium that most closely approximates the conditions the bacteria are likely to have been exposed to immediately prior to encountering the plant in the real world outside the laboratory. Carbon and nitrogen sources as well as the presence of ions, particularly divalent cations (Ca, Mg, Fe, Mn, and Zn) and phosphate, and the pH of the medium are important.
 1. Use minimal AB medium or Luria broth for *A. tumefaciens* and other soil bacteria²⁰. For *E. coli*, which may originate in the gut, use Luria broth.
 2. Add inducers such as root exudates or commercial plant extracts such as soytone or sugars such as sucrose or xylose to the medium²¹. If these substances are used as inducers, add a low concentration, for example 0.01%. If they are used carbon sources, add a higher concentration, for example 0.1%.
2. Prepare the bacterial inoculum. Dilute the bacteria in sterile water or in the medium in which the incubation will be carried out and add them to the plant material. The appropriate dilution is discussed in steps 4.2 and 4.3.
 1. To remove the growth media before using the bacteria, centrifuge the bacterial suspension at 10,000 x g for 2 min, pour off the supernatant and resuspend the bacteria by vortexing them in the same medium which will be used for incubation with the plant material. This method may remove or decrease the number or amount of extracellular material and appendages such as exopolysaccharides, capsules, flagellae, and/or pili. If it is important that these surface structures remain intact, then simply dilute the bacteria before inoculating them or use the alternative method described in step 3.2.2.
 2. As an alternative method to remove growth medium, collect the bacteria on a nitrocellulose or polycarbonate filter with a pore size of 0.2 μm or less. Wash the bacteria with the incubation medium and resuspend them by gentle shaking or vortexing of the filter in a sterile container of the medium.

4. Inoculation of the Bacteria

1. Determine the number of bacteria to be inoculated with reference to the measurement to be used to determine bacterial adhesion and the length of the incubation time.
2. For microscopic studies involving incubation times less than 1 day inoculate relatively large numbers of bacteria. Add an amount of culture to reach a final bacterial concentration of more than 10^6 bacteria per mL. For longer incubation times decrease the bacterial inoculum size.
3. For studies in which bacterial adhesion will be measured by viable cell counts, add an amount of culture to reach a final bacterial concentration of 10^3 to 10^6 bacteria per mL.
4. Avoid adding so many bacteria that their metabolism changes the pH or oxygen concentration in the incubation medium. Measure pH using pH paper or an electrode. Measure oxygen concentration using an oxygen electrode.
5. For plants grown in sand, inoculate in three possible ways.
 1. Inoculate the seed with the bacteria before planting by soaking the seed for 1 min in a suspension in water of about 10^6 bacteria per mL.
 2. Inoculate the root by germinating axenic seedlings as described in section 1 and when the seedling root is about 1 cm long dipping it or placing the whole seedling in a suspension of 10^6 bacteria per mL in water for 1 min.
 3. Inoculate sand by mixing the bacteria with the sand before planting to give a final concentration of about 10^3 bacteria per mL or by watering the seedling with a suspension of 10^6 bacteria per mL after planting.

5. Incubation of the Bacteria with the Plant Material

1. For incubation in liquid media, incubate the bacteria with the plant material in sterile water or sucrose and mineral salts or plant tissue culture medium (such as a 1:10 dilution of MS salts)^{22,23}.
 1. Use a container to which the bacteria do not adhere. Try to keep the plant surface covered continuously either by submersion or by gentle agitation. Vigorous agitation may prevent adhesion or even remove bacteria from the plant surface.
 2. Observe the plant material in the light microscope after varying time intervals to determine when to stop the incubation and make measurements. A time course of adhesion is often valuable with samples taken every 1 to 4 h or every day depending on the speed of the interaction.
2. To incubate in sand, apply the same considerations described for incubation in liquid medium in step 5.1.

6. Measurement of Adhesion Using Microscopy

1. Make microscopic measurements with Nomarski or phase-contrast optics for easy viewing of the bacteria on surfaces. However, any bright-field microscope with magnification of 20X or higher can be used.
 1. Use microscopic observation to determine if the bacteria are randomly distributed or located in specific sites. Also check whether they are bound singly or in clusters. Look for the presence of microcolonies suggesting bacterial growth or entrapment after adhesion. Check whether the bacteria seem to form a biofilm on the surface.

NOTE: A biofilm is a large number of bacteria bound to the surface and surrounded by an extracellular matrix^{23,24,25}. The structure may be smooth and uniform or have a more complicated architecture. Methods for studying biofilms associated with plant surfaces have been described¹⁷.

2. Use bacteria tagged with a fluorescent marker. If other bacteria are present and the bacteria of interest are identified by a fluorescent tag such as green fluorescent protein, use fluorescence microscopy to determine the presence of the tagged bacteria in clusters of other bacteria²⁶. For GFP, use a filter with 490 nm excitation and 520 nm emission.

1. Check that the fluorescent tag is not adversely affecting the bacteria by observing the adherence to axenic material of an equal mixture of wild type bacteria and tagged bacteria of the same strain using both Nomarski and fluorescence optics. If the fluorescent and dark bacteria are randomly mixed and present in equal numbers then the tag did not interfere with the assay.

2. Determine the number of attached bacteria.

NOTE: It is very difficult to determine the number of attached bacteria in the microscope. When the binding is to irregular plant surfaces, it is generally not possible to obtain a quantitative measurement. Scanning electron microscopy (not discussed in this article) can be used to make such measurements.

1. When the bacteria are bound to a smooth surface such as a root hair, count the number of bacteria bound to the edge of the root hair per mm root hair length. Take care to use root hairs of roughly the same size and shape in comparing measurements.
2. To determine the size of objects in the microscope, use a commercial slide with measured markings on it. Observe and photograph this slide at the same settings as used for the experimental material and use the resulting images to determine the size of objects in the photomicrographs.

3. Prepare the sample for microscopy.

1. Wash the sample. Move the sample to a drop of water or incubation medium on a microscope slide and observe it directly. NOTE: This has the advantage that if there was no bacterial growth or actual bacterial death there are unlikely to be many free bacteria. Take the absence of free bacteria as a warning sign that there may have been bacterial death or bacterial binding to the container in which the incubation was carried out. The effect of washing the sample is shown in **Figure 1**.

2. Wash the sample gently in water or incubation medium by placing it in a vial of liquid and inverting the vial gently. Then place the sample on the microscope slide in fresh liquid for observation.

3. Mount the sample in liquid using an ordinary cover slip and microscope slide.

1. If the sample is thick and so would make a bulge under the cover slip, use a press-apply cover slip. These cover slips have a ring of rubber or plastic around the edge of the cover slip. Place the liquid and sample in the well in the cover slip and then place the slide on top and press down gently to seal the cover slip to the slide. Invert and examine.
2. Alternatively use an algae counting slide and cover slip in a similar manner. Note that slides with this depth cannot generally be examined with an objective lens of greater than 20X magnification.

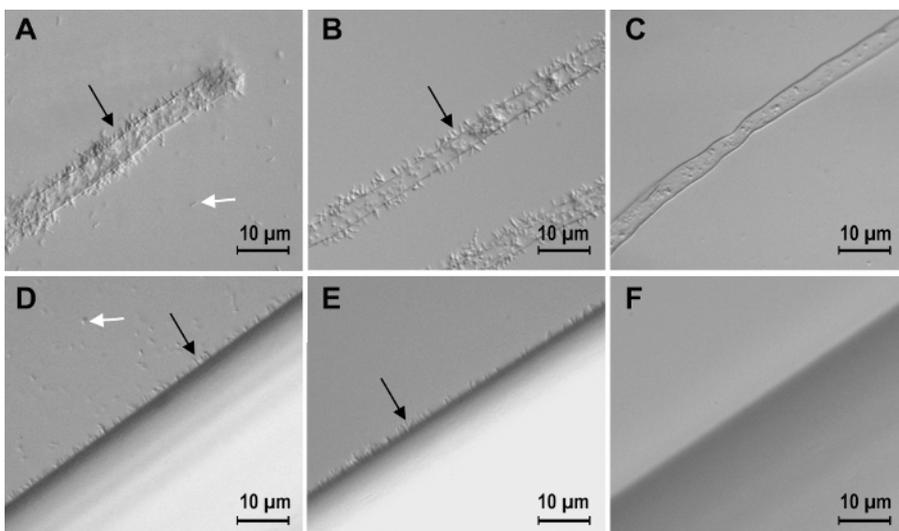


Figure 1: Steps in the preparation of a sample for determining the number of bound bacteria. A. *tumefaciens* binding to tomato root hairs (A, B, and C) and to nylon threads (D, E, and F). In samples mounted in water without washing both bound bacteria (black arrows) and free bacteria (white arrows) can be seen (A and D). After washing the bound bacteria remain but the free bacteria are no longer present (B and E). After sonication the bound bacteria have been removed from the sample surface (C and F). [Please click here to view a larger version of this figure.](#)

4. If fluorescent bacteria were used, examine the sample in the microscope after the sand has been removed. Roots of plants grown in sand are generally not suitable for microscopy as the sand particles interfere with the observation of the bacteria.

1. Remove the plant from a growth container as per step 7. Place the roots in a container of water and gently mix to remove the sand which will settle to the bottom of the container.
2. Remove the plant from the washing water and mount the sample as in step 6.3.3.

7. Measurement of adhesion using viable cell counts

1. Determine the number of viable attached bacteria using a sonicator.
 1. Remove the unbound bacteria. Place the sample in a vial with sufficient water, washing buffer or incubation medium to cover the plant material and invert the vial several times.
 1. If there were more than 10^3 free bacteria per mL present in the initial incubation, perform sequential washings to remove all of them. Check in a light microscope (see step 6.3) to determine the presence of free bacteria.
 2. Wash until there is a substantial reduction in the number of free bacteria but remember that there is an equilibrium between bound and unbound bacteria so the number of free bacteria may never decrease to zero.
 2. Remove the sample from the vial of washing liquid with a forceps or spatula.
 3. Determine the number of bound bacteria in incubations of plants in liquid.
 1. Suspend the washed sample in a vial and cover it with a measured volume of water, incubation medium, or washing buffer. Use enough liquid to cover the sample. Place the vial in a bath sonicator and sonicate it for one min.
 2. Remove the sample and examine it under the light microscope to determine whether the bound bacteria have been removed from the plant. The removal of bacteria from a sample by sonication is shown in **Figure 1**. If there are still bound bacteria present continue sonicating the sample until such a time that no bound bacteria remain on the sample surface. If the bound bacteria cannot be removed by sonication, add 1 - 10 mg/mL sterile quartz sand and repeat the sonication and microscopic examination.
 3. Determine that the sonication procedure which appears most effective in step 7.1.3.2 does not reduce the viability of the bacteria by suspending bacteria from a liquid culture in the solution to be used for sonication (add quartz sand, if it was necessary). Determine the viable cell count. Sonicate the bacteria and determine the viable cell count again.
NOTE: If there is a reduction in viable cell count, modify the procedure by changing the composition of the liquid and/or sonication time until the treatment has no effect on viable cell count.
 4. Determine that the dilution buffer used for viable cell counts does not affect the viability of bacteria which have been incubated under the conditions used by comparing viable cell counts made using different dilution buffers and/or water.
2. Determine the number of viable attached bacteria using homogenization.
 1. Place the sample in a sterile mortar or blender or other homogenization device. Cover it with a measured volume of sterile water, incubation medium, or washing buffer²⁷. Use a volume sufficient to cover the sample. For a blender use 100 mL.
 2. Grind the sample until it is well homogenized. Check the pH after homogenization to be sure that acid released from the plant tissue has not caused a sharp drop in pH below 7. If the pH has dropped use a buffer such as phosphate buffer in the homogenization liquid to maintain the pH.
 3. Determine the viable cell count.
3. Use bacteria marked with antibiotic resistance.
 1. In situations in which more than one type of bacterium is present, mark bacterial strains using spontaneous resistance to antibiotics (typically rifampicin and nalidixic acid).
 1. Determine the level of antibiotic to use. If cultures of the other organisms expected to be present in the incubation are available, plate these cultures grown under the conditions of the intended incubation with plants on plates containing a range of concentrations of the chosen antibiotic. Determine the lowest concentration which does not allow growth of any of these organisms. This is the lowest concentration of antibiotic to which the test bacteria will need to be resistant.
 2. Obtain spontaneous antibiotic resistant mutants. Grow a culture of the bacteria in rich medium to late log or stationary phase. Plate 0.1 mL undiluted onto a plate containing the desired antibiotic at an appropriate concentration. Determine the concentrations to use as described in step 7.3.1.1. Keep and purify the bacteria which grow and are thus resistant to the antibiotic.
 3. Determine that antibiotic resistance does not reduce the growth of the bacteria by doing a growth curve in liquid medium of the parent and antibiotic resistant strains. Determine that the antibiotic resistant bacteria show the same level of colonization of axenic plant material as the parent strain, if this is possible.
4. Determine the number of viable bacteria bound to plants grown in sand.
 1. Remove the plant from the container and sand. To remove plants from containers, first remove sealing material on the top and bottom of the container. Place the container over a piece of sterile paper and gently remove the entire sand or soil and plant as one large tapered cylinder by gently knocking the container against a surface to loosen the material.
 1. If necessary, use a spatula or rod to loosen the material around the edges going in through the bottom of the container.
 2. When the cylinder of sand or soil containing the plant is free on the paper, split it down the middle to reveal the plant root. If desired, take samples of the sand from near the edge of the container as well as near the root. This may be useful to determine the spread (and accumulation and growth) of the bacteria.
 3. Measure the length of the root. Pick up the root and remove the sand and bacteria adhering loosely to the root (the rhizosphere material) by dipping the root in a measured volume of water or buffer and gently shaking it. Determine the viable cell count of the bacteria in the resulting suspension by plating on a suitable medium such as Luria agar. This represents the number of bacteria loosely associated with the root.
5. Remove the tightly bound bacteria by sonication and determine their numbers as described in step 7.1.
6. Alternatively, to determine the location on the root of the tightly bound bacteria place the washed root on the surface of a Petri dish containing nutrient agar or other suitable medium. Observe the location of bacterial colonies on the root over the next 3 days using a dissecting microscope or magnifying glass.

7. Express results as number of bacteria per plant, per cm^2 of surface area, per cm root length, or per gram fresh weight of tissue. Do multiple replicates on the same day and also do replicates on different days using different bacterial cultures and different lots of plant material.

8. Determining whether an effect of incubation conditions on adhesion is due to a response of the bacteria or the plant

1. Use dead or killed plant material.
 1. Subject the plant material to a variety of chemicals, fixatives, or other treatments such as heat in order to kill it. Wash the plant material thoroughly in water and incubation medium after the use of any of these treatments. Then inoculate the bacteria. This will not destroy the plant surface but it will make it metabolically inactive so that it cannot respond to the bacteria.
 2. Measure bacterial adhesion as described in steps 6 and/or 7. Also determine the number of viable cells added to the incubation with the plant material at the beginning of the incubation and the number of viable cells present at the end of the incubation to ensure that no toxic chemicals were present during the incubation.
2. Use inanimate material.
 1. Use bacterial adherence to inanimate material to determine if an effect seen on bacterial adherence to plant material is due to an effect on the plant or the bacteria. Choose an inanimate material to which the bacteria bind and which is similar in shape and size to the plant material studied. Possibilities include filter papers of all types (cellulose, nitrocellulose, glass fiber, polycarbonate, etc.), threads (nylon, cotton, polyester, glass wool, etc.), glass or plastic coverslips, stainless steel coupons, and dialysis membranes.
 2. Wash the inanimate material thoroughly in water and the medium in which the incubation will be carried out and sterilize it before use. Most of the materials listed in step 8.2.1 are stable to sterilization by autoclaving.
 3. Incubate the inanimate material under the desired conditions and score as described in steps 6 and 7. An example of the use of nylon threads to determine that the reduced binding of *A. tumefaciens* to root hairs at high calcium concentrations is due (at least in part) to an effect of calcium on the bacteria is shown in **Figure 4**²⁸.

Representative Results

A. tumefaciens colonizes root surfaces. In order to determine whether bacterial production of cellulose plays a role in root colonization, the effects of bacterial mutations which prevent cellulose synthesis were examined¹⁶. The techniques described in steps 1.3 and 7.1 were used. Tomato seeds were surface sterilized and germinated in sterile water. When the roots were approximately 2 cm long they were dipped in a suspension of 10^5 bacteria per mL and planted in pasteurized soil in containers. The plants were grown for 14 days at 25 °C on a 12 h light/12 h dark cycle. After the indicated times the plants were removed from the containers. The roots were washed and sonicated in a bath sonicator to remove bound bacteria. Bacterial numbers were determined using viable cell counts. **Figure 2** shows the effect of two different cellulose-minus mutations on the ability of the bacteria to colonize tomato roots. Although the standard deviations of some measurements were as high as 0.9 \log_{10} (a common problem with this type of measurement) the reduction in binding of the cellulose-minus mutants is clearly evident and we can conclude that bacterial production of cellulose aids the bacteria in the colonization of tomato roots.

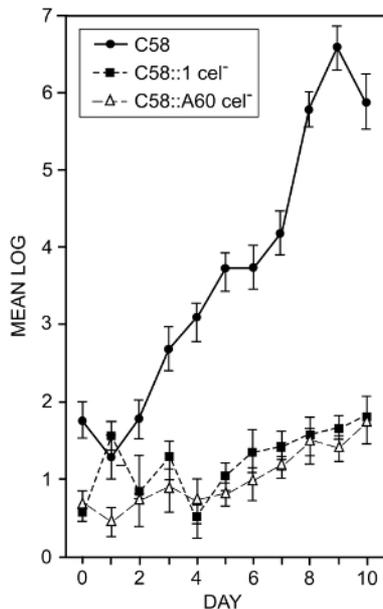


Figure 2: Root colonization by wild type and cellulose-minus mutants of *A. tumefaciens*. Log₁₀ total number of bacteria per cm root length recovered from tomato roots inoculated with wild type *A. tumefaciens* strain C58 and cellulose-minus mutants C58:1 and C58:A60. The numbers shown are the means from a minimum of four separate experiments. Bars indicate standard deviations of the means. Roots were inoculated by dipping them in a suspension of 10⁵ bacteria per mL for one min. The plants were grown in containers and the loosely adherent bacteria were removed by washing the roots in washing buffer in a glass vial. Tightly adherent bacteria were removed using a bath sonicator and the resulting suspension plated to determine viable cell counts¹⁶. This figure has been modified from Matthyssse and McMahan. [Please click here to view a larger version of this figure.](#)

The role of exopolysaccharides in the binding of *E. coli* and other bacteria to alfalfa sprouts was examined. Some outbreaks of diarrheal disease due to *E. coli* O157:H7 have been traced to contaminated alfalfa sprouts. Binding of the wild-type bacteria and mutants unable to make various exopolysaccharides was measured using the methods described in steps 1.1, 5.1, and 7.2. Alfalfa sprouts were surface sterilized and germinated for one day in sterile water at 25 °C in the dark. Four sprouts with attached seed coats were placed in sterile plastic dishes containing 5 mL of water. Bacteria grown in Luria broth were added to a final concentration of approximately 5 × 10³ per mL. The inoculated sprouts were incubated at 25 °C in the dark for 3 days. The sprouts were washed twice in 5 mL sterile water in a vial by vigorous inversion and homogenized in washing buffer using a motor-driven Teflon glass homogenizer. Previous experiments using bacteria marked with a plasmid carrying the GFP gene showed no internalized bacteria although surface bacteria were easily observed. The results are shown in **Table 1**. Two strains of *E. coli* O157:H7 were examined. In both strains the production of poly-β-1,6-glucuronic acid (PGA) appeared to make the largest contribution to the binding of pathogenic *E. coli* to plant surfaces. Colonic acid also played a significant role in binding. While the reduction in binding in cellulose-minus mutants was significant it was not as large as that for the other two polysaccharides.

Effects of Mutations in Exopolysaccharide Production Genes on Binding of <i>E. coli</i> O157:H7 to sprouts and open seed coats			
Bacterial strain	Mutation or genotype (relevant phenotype)	Log ₁₀ number of bacteria bound per sprout or seed coat	
		Alfalfa sprouts ^b	Open seed coats
86-24	None (wild type)	4.7 ± 0.6	5.6 ± 0.2
8624N	<i>yhjN</i> (cellulose-minus)	2.9 ± 0.7 ^c	3.5 ± 0.6 ^c
8624C	<i>wcaD</i> (colonic acid-minus)	1.8 ± 0.7 ^c	2.4 ± 0.5 ^c
8624P	<i>pgaC</i> (PGA-minus)	<1.0 ^c	1.0 ± 1.0 ^c
DEC4A	None (wild type)	5.6 ± 0.2	6.1 ± 0.3
DEC4AN	<i>yhjN</i> (cellulose-minus)	4.8 ± 0.8 ^d	4.1 ± 0.8 ^d
DEC4AC	<i>wcaD</i> (colonic acid-minus)	3.9 ± 0.5 ^c	4.8 ± 0.8 ^d
DEC4AP	<i>pgaC</i> (PGA-minus)	<1.0 ^c	1.2 ± 0.7 ^c

a mean ± standard deviation of a minimum of three measurements of the number (log₁₀) of bacteria bound after 3 days.

b Sprouts were washed before measurement.

c Significantly different from the wild type: P <0.01.

d significantly different from the wild type: P <0.05.

This table has been modified from Matthyse, Deora, Mishra, and Torres¹⁰.

Table 1: Effects of mutations in exopolysaccharide production genes on binding of *E. coli* O157:H7 to sprouts. In order to determine the role of various exopolysaccharides and lipopolysaccharide in the binding of pathogenic *E. coli* O157:H7 strains to alfalfa sprouts, the binding of a set of mutants to the sprouts and open seed coats was examined using the methods described in step 6. The results showed that poly-β-1, 6-*N*-acetyl-D-glucosamine (PGA) appears to be essential for binding to sprouts and that both cellulose and colanic acid are required for maximum binding of *E. coli* O157. This table has been modified from Matthyse, Deora, Mishra, and Torres¹⁰.

In order to determine if the production of PGA is sufficient to cause bacterial binding to plant surfaces, a plasmid (pMM11) carrying the cloned operon encoding the genes required for PGA production was introduced into two different bacteria which would not ordinarily be able to bind to tomato roots¹⁰. *A. tumefaciens* A1045 is a mutant of the wild type strain C58 which fails to make cyclic-β-1,2 glucan and also fails to bind to plant surfaces²⁹. *Sinorhizobium meliloti* 1021 which forms root nodules on alfalfa fails to bind to non-legumes including tomato¹². The techniques described in steps 1.1, 5.1, 6.3, and 7.1 were used to determine if the ability to make PGA generally increased bacterial binding to root surfaces. Tomato seeds were surface sterilized and germinated in sterile water. Roots were cut into segments 1 cm in length and placed in sterile water and the bacteria were inoculated. As these two species of bacteria grow at different rates, binding was measured at different times to allow for roughly equal amounts of bacterial growth. The presence of the plasmid pMM11 caused a similar significant increase in the number of bound bacteria of both species (**Table 2**)¹⁰. A significant increase in binding was also seen in the light microscope but the binding was very different for the two species (**Figure 3**). *A. tumefaciens* A1045 bound as individual bacteria to the root surface. *S. meliloti* bound in large clusters in which only a few of the bacteria were directly attached to the root and the majority of the bacteria were attached to other bacteria. This example shows that simply analyzing the numbers of bacteria bound without including microscopic observations can give a misleading impression of the results of an experiment. Although both methods (viable cell counts and microscopic observations) show that pMM11 increased bacterial binding to tomato roots, the type of binding caused by the production of PGA was different for the two bacterial species¹⁰.

The Effect of the Plasmid pMM11 on the Binding of Bacteria to Tomato Roots		
Bacterial strain	Plasmid	Number of bacteria bound per mm root length
<i>A. tumefaciens</i> A1045 ^a	none	0.25 x 10 ³ ± 0.25 x 10 ³
	pBBR1mcs (vector)	0.25 x 10 ³ ± 0.25 x 10 ³
	pMM11 (PGA synthesis)	10 x 10 ³ ± 0.25 x 10 ³
<i>S. meliloti</i> 1021 ^b	none	none detected
	pBBR1mcs (vector)	none detected
	pMM11 (PGA synthesis)	50 x 10 ³ ± 5 x 10 ³

a Bacterial binding was measured after 2 hours

b Bacterial binding was measure after 18 hours

This table has been modified from Matthyse, Deora, Mishra, and Torres¹⁰.

Table 2: The effect of a plasmid carrying genes for the synthesis of PGA on binding of *A. tumefaciens* A1045 and *S. meliloti* 1021 to tomato root segments. In order to examine the ability of poly- β -1, 6-*N*-acetyl-D-glucosamine (PGA) to promote the binding of bacteria to plant roots, the effect of a plasmid conferring the ability to make PGA (pMM11) on the binding of two strains of plant-associated bacteria to tomato roots was examined. Neither strain of bacteria showed significant binding to tomato roots in the absence of the plasmid or in the presence of the plasmid without the genes encoding PGA synthesis (pBBR1mcs). The addition of the plasmid carrying PGA synthesis genes increased binding by both types of bacteria. Because *A. tumefaciens* grows faster than *S. meliloti* binding was measured after 2 h of incubation for *A. tumefaciens* and after 18 h for *S. meliloti*. The techniques used are those described in step 7. This table has been modified from Matthyse, Deora, Mishra, and Torres¹⁰.

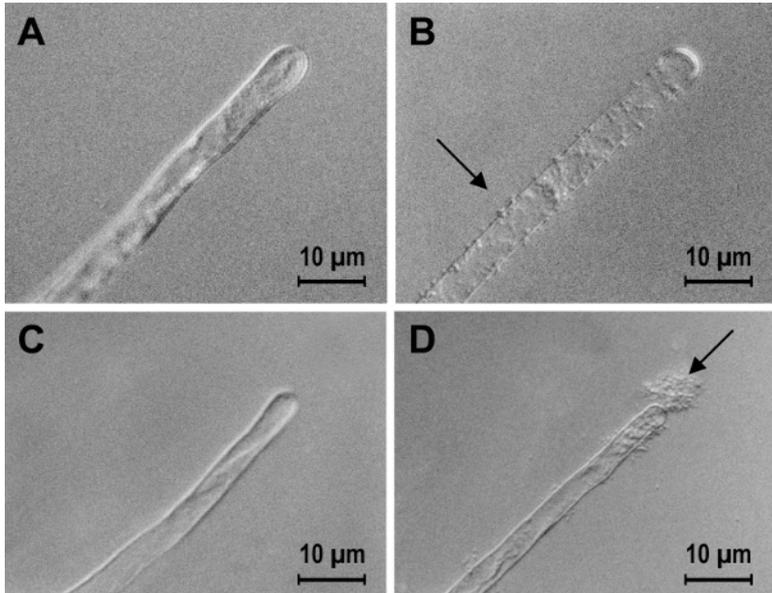


Figure 3: The effect of the plasmid pMM11 carrying the PGA biosynthesis genes on the binding of *A. tumefaciens* A1045 and *S. meliloti* 1021 to tomato root hairs. Binding to tomato root hairs of A) *A. tumefaciens* A1045, B) *A. tumefaciens* A1045 pMM11, C) *S. meliloti* 1021, and D) *S. meliloti* 1021 pMM11. Although the increase in binding of the two bacterial species is roughly similar the details of the binding as seen in the light microscope are quite different. The techniques used are those described in step 6. This figure has been modified from Matthyse, Deora, Mishra, and Torres¹⁰. [Please click here to view a larger version of this figure.](#)

It is sometimes possible to use binding to non-biological surfaces to aid in distinguishing the contribution of the bacteria and of the plant in a specific interaction. The unipolar polysaccharide (UPP) of *A. tumefaciens* has been shown to be able to mediate bacterial binding to a variety of both biological and non-biological surfaces³⁰. Calcium was observed to inhibit the binding of *A. tumefaciens* to plant surfaces mediated by the UPP²⁸. In order to determine whether the inhibition by calcium ions of bacterial binding to plant surfaces is due to an effect on the bacteria or on the plant surface, the binding of the bacteria to nylon threads was examined. The techniques described in step 8.2 were used. Tomato seeds were surface sterilized and germinated in water as described in step 1. The bacteria were grown in minimal medium with sucrose and added to tomato roots or nylon threads at a final concentration of approximately 10^5 /mL as described in step 5.1. The effect of added CaCl_2 on bacterial binding to tomato roots and nylon threads was examined in the microscope. **Figure 4** shows a similar inhibition of binding by calcium using either surface suggesting that the effect of calcium is primarily on the bacteria¹⁰.

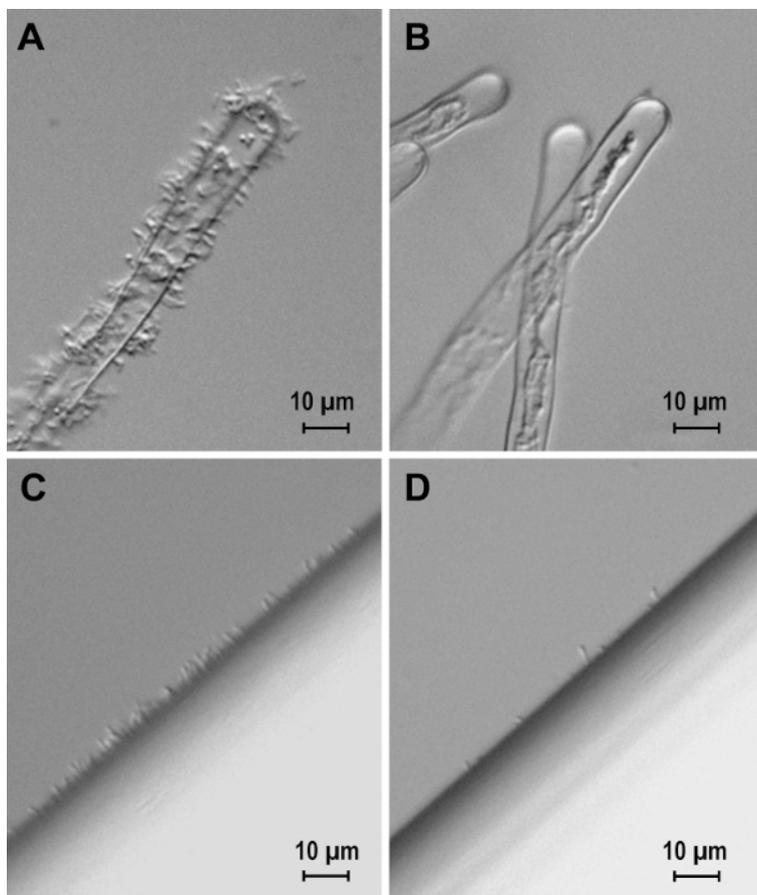


Figure 4: The effect of calcium on the binding of *A. tumefaciens* to tomato root hairs and nylon threads. *A. tumefaciens* was incubated with tomato roots (A and B) or nylon threads (C and D) for 24 h in a 1:10 dilution of MS salts and a 1:20 dilution of AB minimal medium, 0.4 % sucrose (A and C) or in a 1:10 dilution of MS salts and 1:20 dilution of AB minimal medium, 0.4 % sucrose containing 60 mM CaCl_2 (B and D)³¹. The added CaCl_2 inhibited bacterial binding to both roots and nylon threads suggesting that the inhibition was primarily due to an effect on the bacteria rather than on the plant surface. [Please click here to view a larger version of this figure.](#)

Discussion

It is important to be aware of all of the surfaces to which bacteria can adhere during the experiment. Thus bacteria which are capable of binding to glass may be underestimated if viable cell counts are done using glass tubes and pipettes. If plants are grown in agar or soil and some of the agar or soil remains on the plants then the bacteria may bind to the adhering material rather than to the plants. On the other hand, washing the plant surface, particularly in the case of roots, may remove natural surface coatings such as mucous and thus alter the results of adherence tests.

It is important to be certain that the bacteria added to the incubation mixture remain alive during the experiment. Thus viable cell counts of free as well as attached bacteria should routinely be made. Some treatments or bacterial mutations may reduce bacterial growth rate or actually cause the death of a fraction of the bacterial population. Live and dead bacteria may not be distinguishable in the microscope unless special stains are used. There is a useful stain kit for live/dead bacteria which depends on the exclusion of dyes from the living bacteria. However, if a mixed population of bacterial species is present then viable cell counts of the species of interest is likely to be the easiest method to determine whether the incubation has resulted in bacterial death.

Medium composition will influence bacterial survival and growth. Root exudate and materials released from wound and cut sites will provide substrate to support modest bacterial growth. Phosphate, nitrogen, and iron tend to be limiting in these conditions. Divalent cations such as calcium and magnesium may influence adhesion. In some cases carbon source can influence adhesion. pH also matters. In general the pH of the rhizosphere is between 5.5 and 6.5.

It is necessary to be careful in using bacteria marked with antibiotic resistance. The antibiotics most often used are rifampicin and nalidixic acid. Resistance to these antibiotics is generally due to mutations in chromosomal genes (RNA polymerase and gyrase, respectively) and thus cannot easily be transferred to another strain during the incubation. This type of resistance also does not result in the degradation or modification of the antibiotic. Marking bacteria with a plasmid-borne gene marker is not recommended unless the plasmid cannot be transferred to any other bacteria. The antibiotic resistance must not be due to degradation or chemical modification of the antibiotic as antibiotic sensitive bacteria will then be able to grow on antibiotic plates if they are located close to the resistant bacteria.

The methods described in this paper are useful for small sample sizes and/or experiments where the samples need to be contained (for example, experiments involving human pathogens). For large sample sizes (above 100 g of material or more than 50 plants) other methods or drastic modification of these methods would be needed^{10,19,32,19,33,34,35,36}. The presence of large numbers of microorganisms other than the species being studied can also pose significant problems. Possible solutions include the use of bacteria marked with a fluorescent protein or antibiotic resistance as described in steps 6.1.2 and 7.3. However, when the bacteria of interest are rare individuals in a large population of other microorganisms these markers may not be adequate to allow an unambiguous assessment of the numbers of the bacteria being studied.

All of the methods described here are laboratory based methods. Minor modifications would be needed for greenhouse studies. More major modifications are likely to be required for field studies where protozoa, insect and other animal predation and climate variation complicate the provision of defined conditions for the experiments. In the future these methods may be extended to include the interactions of two or more microorganisms on the plant surface.

Disclosures

The author declares that she has no competing financial interests.

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