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Differences in optical trapping prompt investigations of *Agrobacterium* surface characteristics

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Comparison of the optical trapping efficiency of *Agrobacterium rhizogenes* and *A. tumefaciens* strains indicates the *A. rhizogenes* strain, ATCC 11325, is significantly less efficiently trapped than *A. rhizogenes* A4, ATCC 15834, and the *A. tumefaciens* strain LBA4404. Differences were also found in capsule generation, growth media viscosity, and transmission electron microscopy negative staining. These observations imply a difference in surface structure exists. Calcofluor fluorescence suggests the difference involves an exopolysaccharide.

Keywords: *Agrobacterium rhizogenes*; *Agrobacterium tumefaciens*; capsule; calcofluor; exopolysaccharide; optical tweezers; rheometry

Introduction

Agrobacterium rhizogenes and *A. tumefaciens* are naturally occurring 'genetic engineers' of plants [23] and have been exploited for plant transformation [5]. However, *Agrobacterium* plant transformation is not completely understood [23] and highly efficient transformation events remain elusive. Bacterial cell walls or cellulose fibril generation may be important in initial bacterial binding to plant cells before T-DNA transfer [14]. Acidic polysaccharides, lipopolysaccharides, and capsular polysaccharides may also assist plant–agrobacteria interactions [16,17]. Many bacterial plant pathogens and nitrogen-fixing species produce extracellular products, such as exopolysaccharides [5,13], that contribute to virulence [18] or may protect against reactive oxygen species [12]. Extracellular bacterial polysaccharides are also of increasing interest to industry in polymer applications. Nonenterobacterial species, in particular, require further characterization [20].

Disagreements exist over the taxonomic placement of *Agrobacterium* species [19]. Some suggest that *A. rhizogenes* strain ATCC 11325 should be placed with *A. tumefaciens* due to nopaline production in transformed plants [15,16]. Others have determined differential spermidine composition in the same strain [9]. *A. rhizogenes* ATCC 11325 was ranked less virulent than *A. rhizogenes* strains ATCC 15834 and A4 [16]. However, strain ATCC 11325 was successfully used to transform gymnosperm species [7,10].

As with mutants, differences between strains can be compared to help elucidate unknown properties between organisms. Addressing these questions concerning the mechanics, biochemistry, and taxonomy of agrobacteria not only advances fundamental knowledge of biological processes, but also may lead to improved protocols for the genetic manipulation of agronomically important plants.

While conducting optical trapping experiments [3], we discovered a significant difference between the trapping efficiencies of the ATCC 11325 strain of *A. rhizogenes*, other *A. rhizogenes* strains (ATCC 15834 and A4), and an *A. tumefaciens* strain (LBA4404). Preliminary investigations involving calcofluor fluorescence indicate this difference involves an exopolysaccharide.

Materials and methods

Bacterial strains and culture media

A. rhizogenes strains (A4, ATCC 11325, ATCC 15834) and *A. tumefaciens* (LBA4404) were inoculated from frozen glycerol stocks and incubated on rotary shakers at 200 rpm and 25°C in 10 ml liquid YMB medium [11] unless otherwise noted. Media for strain LBA4404 had 50 μg ml^{-1} rifampicin added after autoclaving.

Optical trapping

The optical system [3] included a pulsed (30 Hz, 100 μs duration, long-pulse mode) Nd:YAG laser (Model GCR 170, Spectra Physics, Mountain View, CA, USA) which produced a stationary trapping beam at wavelength $\lambda = 1.06$ μm. The beam was focused with a 100× Neofluar (numerical aperture 1.3) oil immersion microscope objective (Carl Ziess, Thronwood, NY, USA) to a nearly diffraction-limited spot ($\cong\lambda$ in diameter). The bacteria were placed in a CoverWell culture chamber (PCI-0.5, Grace Bio-Labs, Sunrise, OR, USA). A No. $1\frac{1}{2}$ cover slip (25×25 mm) sealed the top of the CoverWell and allowed light transmission. The bacteria were diluted in a solution of 0.45 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris pH 7.2 and 0.01% calcofluor. To determine the trapping strength of a bacterium, the trapping chamber was accelerated until the viscous drag forces became strong enough to displace the bacterium from the trap. A computer-controlled actuator (StepperMike, Model 18510, Oriel Inc, Stratford, CT,

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USA) provided an acceleration, $a = 1\ \mu m\ s^{-2}$, allowing a linearly increasing velocity with time. The velocities at which viscous drag overcame the trapping force were recorded and used to determine the average trapping force using Stokes' Law, $F = 6\pi\mu R_p v$; where F is the force on the bacterium; $\mu = 1.43 \times 10^{-3}$ Ns m^{-2} [2], the kinematic viscosity of the surrounding solution; $R_p \cong 0.8\ \mu m$, the average radius of the particle; and v, the velocity. The trapping efficiency, $Q = Fc/n_0P$ [1], may be determined from the average trapping force; where P is the average trapping power; $n_0 = 1.33$ [2] is the refractive index of the surrounding medium; and c is the speed of light in a vacuum. The statistical significance of the calculated values of the trapping efficiency was determined by multiple regression analysis using SAS.

Transmission Electron Microscopy (TEM)

Overnight bacterial cultures were concentrated by centrifugation, spread on 2% (w/v) agar, allowed to dry, coated with Formvar (0.5% w/v in ethylene dichloride, Electron Microscopy Sciences, Ft Washington, PA, USA), floated onto 2% phosphotungstic acid (pH 5.0 with KOH), placed on copper grids, and viewed using a JEOL-120 transmission electron microscope (Japan Electron Optics Lab, Tokyo, Japan). Micrographs were exposed for 4 s on Kodak electron microscope film (Type 4489).

Viscosity measurements

Media from overnight cultures were tested rheologically with bacterial cells, after centrifugation, and following cell removal by filtration (0.2 μm, low protein binding Acrodisk, Gelman Scientific, Ann Arbor, MI, USA). Rheometry was performed using a Bohlin rheometer (Model DSR-F, Cranberry, NJ, USA) with a 40-mm cone at a 4° angle. The plate and spindle had a 0.15-mm gap. Data were recorded with the constant shear rate module of Bohlin's software package.

Calcofluor fluorescence

Bacteria were grown for 3 days on solid (1.5% (w/v) agar) YMB plus 200 $\mu g\ ml^{-1}$ calcofluor [4], added after autoclaving. The cultures were photographed under long-wave UV light on Kodak Gold film, ASA 200 and digitized in black and white. Controls were grown without calcofluor.

Capsule determination

Presence or absence of bacterial capsules was detected by India ink following Duguid's method [8]. The bacteria were visualized at 2000× using the optical setup for trapping. Bacteria with capsules show up as refractile rods and those individuals lacking capsules are invisible. Bacteria were stained with crystal violet or India ink and videotaped.

Results and discussion

The force was plotted against the laser power to ascertain a regression line slope (Table 1) to determine the differences in trapping efficiency between the various strains and species. The *A. rhizogenes* ATCC 11325 efficiency was half that of the other strains (highly significant, $P<0.0001$). Table 1 indicates the efficiencies of the various strains of *Agrobacterium* calculated from the plotted slopes.

Trapping differences are related to size or optical characteristics of a trapped particle [1]. We found the sizes of the bacteria were equal to within 10% as determined by crystal violet stain and light microscopy (Table 1). However, a putative capsule increases the effective radius of *A. rhizogenes* A4 and ATCC 11325 by 23 and 30%, respectively. Although the capsule increased the hydrodynamic radius, and thus the drag force, it does not account for the entire 50% decrease in trapping efficiency.

Variations in chemical makeup of the capsule or cell may account for additional trapping differences due to absorption or refraction differences. For example, *A. rhizogenes* ATCC 11325 absorbed phosphotungstic acid by TEM (Table 1), suggesting a cell surface difference that may scatter light and affect the trapping efficiency. We also believe the polysaccharide slime layer may induce light scattering [6] contributing to the decreased trapping efficiency of the bacterium.

Growth media viscosity differences between the different strains were also observed (Figure 1). *A. rhizogenes* ATCC

Table 1 Summary of differences between *Agrobacterium rhizogenes* and *A. tumefaciens* strains

Strains	Regression line slope (Force (N)/Laser Power (W)) × 10^{-11}	Trapping efficiency (Q, unitless) × 10^{-3}	Average radius[a] (μm) by stains		PTA[b] absorption	Capsule[c]	Doubling time (h)
A. rhizogenes strains			*India ink*	*Crystal violet*			
A4	1.77	4.00	1.02 ± 0.01	0.83 ± 0.01	–	+[d]	1.1
ATCC 11325	0.93	2.10[e]	0.91 ± 0.02	0.70 ± 0.01	+	+	3.0
ATCC 15834	2.02	4.56	—	0.75 ± 0.01	–	–	1.1
A. tumefaciens							
LBA4404	1.74	3.92	—	0.73 ± 0.01	n.t.	–	n.t.

[a]Determined by (*length* + *width*)/4.
[b]Phosphotungstic acid.
[c]Determined by India ink stain.
[d]Requires >2 days to become prevalent.
[e]Highly significant ($P<0.0001$).
n.t., not tested.

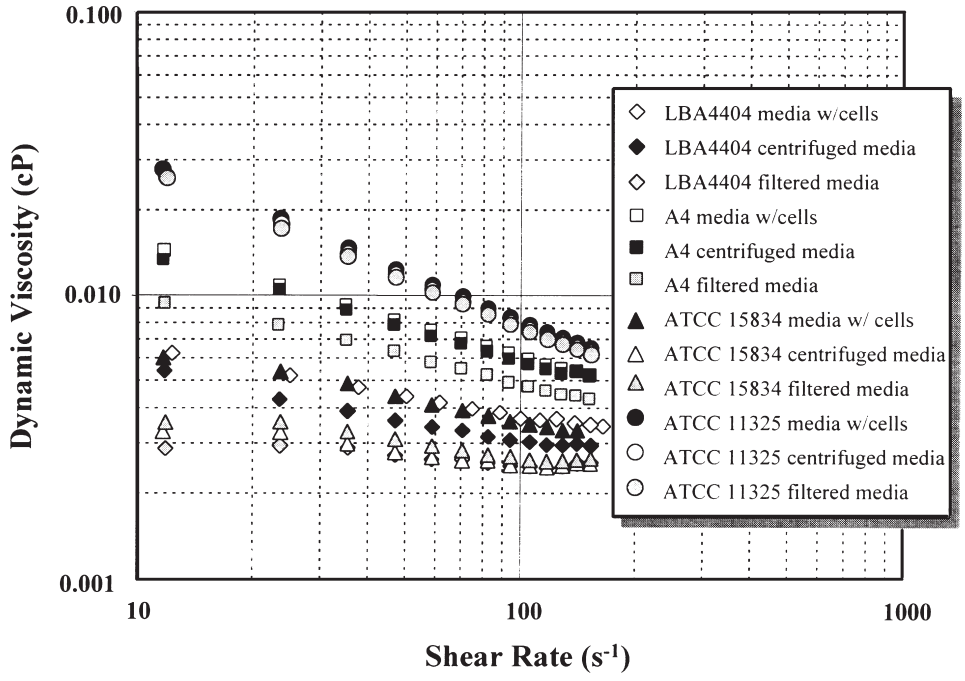

Figure 1 Differences in media viscosity between *Agrobacterium rhizogenes* and *A. tumefaciens* strains.

11325 becomes nearly isopycnic following overnight growth. Extended centrifugation (30 min at $6000 \times g$), compared to the other strains (10 min at $6000 \times g$), does not clear the media of all cells. *A rhizogenes* strain A4 also approches isopycnic conditions, but the process requires >2 days growth. The viscosity of media in which *A. rhizogenes* ATCC 11325 was grown changes little after centrifugation or filtration, indicating the difference may remain in the media. Centrifugation of the A4 strain changes the viscosity slightly, while filtration changes the viscosity more, indicating that the molecule may be large or that the filtration process sheared the viscous agent. Viscosity of the *A. rhizogenes* ATCC 15834 and *A. tumefaciens* LBA4404 media changed more dramatically after centrifugation or filtration. The viscosity of culture media has been used as an indicator of the relative amount of extracellular polysaccharide produced by bacteria [12].

A. rhizogenes ATCC 11325 grew about three times slower during log phase compared to the other *A. rhizogenes* strains (Table 1). *A. tumefaciens* LBA4404 was not tested, but growth appeared comparable to *A. rhizogenes* strains A4 and ATCC 15834 based on the volume of the cell pellet after centrifugation.

A. rhizogenes ATCC 11325 grown on medium with calcofluor exhibits considerably less fluorescence under long-wave UV light, suggesting a difference in exopolysaccharide production (Figure 2). When mutant strains of *A. tumefaciens* were compared by Cangelosi *et al* [4], a β-1,2-glucan fluoresced when calcofluor was added to the growth media. Calcofluor also fluoresces in the presence of cellulose, chitin, callose, other β-1,4-glucose polysaccharides [21], and β-(1→3)(1→4)-d-glucan [22] commonly found in plants. Cellulose, chitin, and callose are structural polysaccharides, not particularly common to prokaryotes. Agrobacteria species produce cellulose fibrils involved in attachment to host plants, but generally formation occurs after invasion [14].

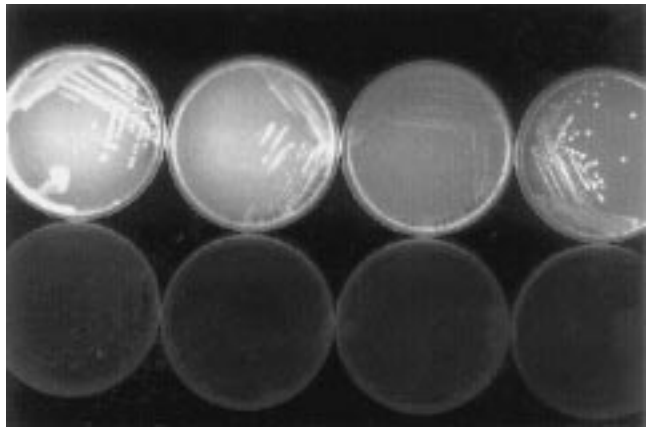

Figure 2 Calcofluor fluorescence differences between *Agrobacterium* strains (top row, L to R): *A. rhizogenes* A4, *A. tumefaciens* LBA4404, *A. rhizogenes* ATCC 11325, and ATCC 15834. The bottom row is the correponding controls without calcofluor. Fluorescence is bluish during excitation with long-wave UV light. Petri dishes are 9 cm.

To our knowledge, this is the first time an optical trap has been used to compare different bacterial strains and species. Without optical trapping, the dissimilarities between these *Agrobacterium* species and strains may have gone unnoticed. Further characterization of the associated, putative exopolysaccharide difference is necessary to determine what chemical differences may exist, whether these differences affect virulence, if the differences would constitute a phylogenetic marker, or if the polymer would be of value to industry.

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