Atomic force microscopy of a *ctpA* mutant in *Rhizobium leguminosarum* reveals surface defects linking CtpA function to biofilm formation

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Atomic force microscopy was used to investigate the surface ultrastructure, adhesive properties and biofilm formation of *Rhizobium leguminosarum* and a *ctpA* mutant strain. The surface ultrastructure of wild-type *R. leguminosarum* consists of tightly packed surface subunits, whereas the *ctpA* mutant has much larger subunits with loose lateral packing. The *ctpA* mutant strain is not capable of developing fully mature biofilms, consistent with its altered surface ultrastructure, greater roughness and stronger adhesion to hydrophilic surfaces. For both strains, surface roughness and adhesive forces increased as a function of calcium ion concentration, and for each, biofilms were thicker at higher calcium concentrations.

INTRODUCTION

The Gram-negative bacterial cell envelope is a complex, multi-component structure that can include a diffuse layer of exopolysaccharides (EPSs) at its exterior, a more intimately attached capsular layer of secreted polysaccharides (CPSs), followed by an outer membrane with a predominantly lipopolysaccharide (LPS) outer leaflet, a peptidoglycan cell wall and finally a cytoplasmic membrane (Lepek & D'Antuono, 2005; Silhavy *et al.*, 2010). The envelope is an important protective barrier preventing the entry of harmful substances into the cell. It is also important in host–bacteria interactions, representing a point of physical contact between the host cells and bacteria (Silhavy *et al.*, 2010). During changing environmental conditions, a cell must maintain envelope function to ensure the selective passage of nutrients into the cell and waste products out of the cell.

Symbiotic bacteria, like rhizobia, are subject to a variety of changing environmental conditions and can be subjected to a variety of environmental and host-derived stresses, including desiccation, temperature fluctuations, plant immune defence response and exposure to toxic compounds secreted by plant roots or competing rhizobacteria (Vlassak & Vanderleyden, 1997; Soto *et al.*, 2009). Therefore, the rhizobial cell envelope must be a dynamic structure capable of adapting to changing environmental conditions.

Rhizobium leguminosarum, a symbiont frequently associated with peas (*Pisum sativum*), is a model organism for studying the rhizobial cell envelope. The outer membrane LPS and EPS of *R. leguminosarum* have been intensely studied (Carlson *et al.*, 1999; Skorupska *et al.*, 2006). Despite this focused research, uncharacterized genes important for envelope assembly and function continue to be reported, emphasizing that much remains to be elucidated regarding cell envelope structure and function in rhizobia. A recent example is the *ctpA* gene from *R. leguminosarum*, which was shown to play an important role in cell envelope function (Gilbert *et al.*, 2007). Mutation of *ctpA* in *R. leguminosarum* decreased cell envelope integrity, altered cell morphology and increased desiccation sensitivity (Gilbert *et al.*, 2007).

The CtpA protein is located in the periplasm (Hoge *et al.*, 2011) and its biochemical function was first described in *Escherichia coli* in which it was shown to act as a C-terminal protease. CtpA substrates are often proteins linked to cell envelope development, for example the penicillin-binding protein 3 (FtsI) from *E. coli* (Hara *et al.*, 1989, 1991) and outer-membrane proteins from *Borrelia burgdorferi* (Pinne *et al.*, 2006). The general function of *ctpA* in cell envelope function can be inferred from our earlier study, but the pleiotropic nature of *ctpA* mutants (Bandara *et al.*, 2005; Gilbert *et al.*, 2007) makes it difficult to define precisely its role in envelope function. Modern cell imaging techniques to describe the cell surface of these mutants may offer new insights into the role of CtpA in envelope function.

Atomic force microscopy (AFM) is the technique of choice to fully characterize the ultrastructure, physical and chemical properties of microbial surfaces (reviewed by

Abbreviations: AFM, atomic force microscopy; CPS, capsular polysaccharide; EPS, extracellular polysaccharide; FS, force spectroscopy; PLL, poly-L-lysine.

Three supplementary figures are available with the online version of this paper.

Dorobantu & Gray, 2010). The ability to simultaneously probe and image viable specimens at high resolution offers a distinct advantage over scanning electron microscopy (Kaminskyj & Dahms, 2008). Since AFM is based solely on the interaction of a probe tip with the sample surface, its resolution is only dependent on tip size and shape rather than the diffraction limit inherent to optical microscopy. AFM imaging is capable of producing a topographic surface map with height and lateral resolution at the Å–nm scale, while force spectroscopy (FS; another AFM mode) can probe whole cell turgor, envelope viscoelasticity and adhesion with pN sensitivity. AFM and FS have been applied to studying host–microbe interactions (Reis *et al.*, 2006), microbial biofilms (Volle *et al.*, 2008; Díaz *et al.*, 2011), the nano-mechanical properties of whole cells (Hu *et al.*, 2009; Vadillo-Rodriguez *et al.*, 2009), cellular structures such as pili (Miller *et al.*, 2006), and finer details such as specific molecular distributions on microbial cell surfaces or ligand–receptor interaction forces (Alsteens *et al.*, 2009; Heinisch *et al.*, 2010). Despite the wide applicability of these techniques to microbiology, their application to rhizobia is limited, with only one study describing the interaction of soybean agglutinin with *Bradyrhizobium japonicum* (Chen *et al.*, 2006).

We used AFM to demonstrate that the surface ultrastructure and roughness, and FS to show that relative surface chemistry, of a *ctpA* mutant are altered compared with the wild-type. The aberrant surface roughness of the *ctpA* mutant led to a further investigation of its ability to form biofilms. We used confocal microscopy and standard biofilm quantification assays to show that the *ctpA* mutant does not form wild-type biofilms. This report successfully demonstrates the ability of AFM and FS to fully characterize the surface structure and properties of *R. leguminosarum*, and to identify the importance of the *ctpA* gene in rhizobial biofilm formation.

METHODS

Bacterial strains, plasmids and media. The wild-type strain used in the study was *R. leguminosarum* biovar viciae 3841, a spontaneous streptomycin-resistant derivative of strain *R. leguminosarum* bv. viciae strain 300 (Johnston & Beringer, 1975). The *ctpA* mutant, strain 3845, and the complemented mutant strain, 3845pKG62, were described by Gilbert *et al.* (2007). *R. leguminosarum* strains were cultured using tryptone–yeast (TY) broth (Beringer, 1974) with 1.8, 3.4 (standard) or 3.6 mM CaCl₂, or Vincent's minimal medium (VMM; Vincent, 1970) with 10 mM mannitol as a carbon and energy source. Media was supplemented as required with the following concentrations of antibiotics (µg ml⁻¹): streptomycin, 500; neomycin, 100; tetracycline, 5.

Biofilm cultivation and crystal violet staining for biomass. Biofilms were grown in micro-titre dishes according to the method described by Vanderlinde *et al.* (2009, 2010). Briefly, bacterial strains from VMM solid medium were resuspended in sterile water to an approximate OD₆₀₀ of 1.0. The starting inoculum was then diluted 1 in 15 into the appropriate TY medium with 1.8, 3.4 or 3.6 mM CaCl₂, and 150 µl was added to each well of a cell-culture-treated, polystyrene microtitre plate. Plates were then sealed with Parafilm and incubated at 30 °C for 48, 96 and 144 h with shaking at 100 r.p.m.

Following incubation, planktonic cells and media were removed, and biofilms were washed once with 200 µl sterile distilled water. For quantification of adherent biomass, biofilms were stained with crystal violet according to the method described by O'Toole & Kolter (1998). Briefly, a 0.4 % crystal violet solution was added to each rinsed well, and plates were incubated at room temperature for 20 min. Wells were then rinsed three times with 200 µl sterile distilled H₂O. To measure the amount of adherent biomass, 200 µl 95 % ethanol was added, and the absorbance of the solubilized crystal violet was measured at 550 nm in a BioTek Synergy HT microplate reader, with Gen5 analysis software (BioTek Instruments).

Biofilm visualization and analysis. Biofilm formation was tested on different substrates, including coverslips made of glass, poly-L-lysine (PLL)-coated glass and plastic, mica and dialysis tubing to facilitate AFM imaging. Glass coverslips (22 × 22 mm) were cleaned in 1 M HCl for 1 min, followed by immersion in piranha solution [3:1 (v/v), H₂SO₄:H₂O₂] for 1 h. After sequential rinsing with deionized water, methanol and acetone, coverslips were air-dried and autoclaved for 20 min. For coated glass, clean glass coverslips were immersed in 0.1 % PLL solution for 10 min and air-dried overnight, rinsed with sterile deionized water and air-dried. Mica, freshly cleaved with adhesive tape, and dialysis tubing were cut to size (11 × 22 mm). Plastic coverslips, mica and dialysis tubing were all sterilized in 70 % ethanol prior to use. *R. leguminosarum* 3841 and 3845 were cultured in TY broth with CaCl₂ (1.8, 3.4 or 3.6 mM), to OD₆₀₀ ~0.4. Subsequently, 20 ml of culture was transferred to sterile Petri-dishes, each containing biofilm substrates for incubation at 30 °C. Biofilms were prepared with and without shaking in an incubator or shaking incubator (Lab line; 50 r.p.m.). Substrates were removed from the Petri dishes after 6, 12, 18, 24, 48 and 72 h, and rinsed with VMM buffer (5.74 mM K₂HPO₄, 7.35 mM KH₂PO₄, 5.94 mM KNO₃, 1.01 mM MgSO₄·7H₂O, 0.5 mM CaCl₂·6H₂O; mimicking VMM ionic strength) to remove planktonic cells.

Samples examined using a light microscope were stained with 0.1 % Toluidine Blue O (10 min) and those for confocal microscopy imaging were incubated with calcofluor white (1 µg ml⁻¹) in 5 % KOH solution (1 min), each followed by rinsing with VMM buffer to remove planktonic cells. Coverslips used for confocal imaging were inverted over a clean glass slide and the edge was sealed with nail polish to prevent evaporation.

Biofilms established on different substrate surfaces were evaluated using a light microscope (Olympus BX51) at 400 × magnification, and images were recorded with a digital camera (Olympus DP70). Live biofilms were observed using inverted laser scanning confocal microscopes (Leica SP5 and Zeiss LSM 700).

Sample preparation for AFM. Biofilms formed on glass coverslips over various time periods were rinsed with VMM buffer, fixed (3.7 % formaldehyde and 0.2 % Triton X-100 in VMM buffer; 20 min, 30 °C), rinsed with deionized water to remove excess fixation solution and then air-dried. Individual *R. leguminosarum* and those located at the edge of biofilms were imaged.

Live *R. leguminosarum* grown on dialysis tubing (prepared as above) were cultured for 24 h, rinsed with VMM buffer to remove non-adhering microbes, and attached to a glass coverslip with double-sided tape so one end was sealed and the other left open. Filter paper, cut to a width slightly smaller than the dialysis tubing, was inserted under the open end of the dialysis tubing, attached to the coverslip with adhesive tape (Ma *et al.*, 2005) and transferred onto the AFM sample mount.

AFM. A commercial Explorer 2000 AFM (TopoMetrix, Bruker Instruments) equipped with a dry AFM scanner (model 400006) was used for all measurements. Topography and lateral force images

were acquired in contact mode using V-shaped silicon nitride tips (MLCT-EXMT-A1, Bruker; nominal spring constant $k=0.05 \text{ N m}^{-1}$) in contact mode. The same tips were used to collect force curves. Fixed specimens, either individual cells or biofilms thereof, were imaged in air, but for live cell imaging TY broth was supplied to *R. leguminosarum* through the dialysis tubing with drop-wise addition ($\sim 50 \mu\text{l h}^{-1}$) to the underlying filter paper (Ma *et al.*, 2005).

FS. The force of adhesion between the fixed rhizobium and the hydrophilic AFM probe was quantified from force versus distance curves. The spring constant of the cantilever was determined directly prior to force curve acquisition according to Cleveland *et al.* (1993). The AFM tip was allowed to approach the sample ($10 \mu\text{m s}^{-1}$) until it reached a pre-set position and was then retracted. Surface adhesion values (nN) were measured from the last segment (e–f) of the retraction cycle (Fig. 1d, h).

Data processing. Light and microscope image contrast was adjusted using Adobe Photoshop. Confocal and AFM images were processed using FV1000 Viewer (version 02.1c) and SPMLab (version 5.01), respectively. Boundaries for measuring biofilm thickness were determined according to fluorescence intensity and excluding any planktonic cells. AFM images were levelled to remove image curvature or background tilt, and a histogram of Z data was used to adjust image contrast. Average cell surface roughness was calculated using the area analysis function, using the entire cell surface of 2–3 individual bacteria, each from a minimum of two different samples. Roughness was calculated based on the following equation:

$$R_a = \frac{1}{n} \sum_{i=0}^n |Z_i - \bar{Z}|$$

where Z is the average height of surface features deviating from the levelling plane, and Z_i is the height of single surface features.

A paired Student's *t*-test was used to assess differences in cell size and surface subunit size between the wild-type and *ctpA* mutant strains (InStat3, GraphPad). Differences in roughness, adhesion and biofilm thickness for the wild-type and *ctpA* mutant strains under normal, Ca^{2+} supplemented and limited conditions were assessed using one-way ANOVA and a paired Student's *t*-test (InStat3, GraphPad).

RESULTS

R. leguminosarum morphology and surface characteristics

Fixed *R. leguminosarum* wild-type and *ctpA* mutant immobilized on PLL-coated glass coverslips were imaged by AFM to determine their gross morphologies and surface ultrastructures (Fig. 1a, b, e, f). The *ctpA* mutant strain of *R. leguminosarum* (3845) does not grow on TY agar (Gilbert *et al.*, 2007). When grown in TY broth, the wild-type and *R. leguminosarum ctpA* mutant grow as rods of identical size, but the mutant can also take on a spherical morphology (Table 1). The wild-type *R. leguminosarum* surface has

Fig. 1. AFM images of fixed *R. leguminosarum* wild-type (a–d) and *ctpA* mutant (e–h). Shown are fixed whole cells (a, e) and corresponding surface ultrastructure (b, f) (600×600 nm) at high resolution compared with those of the live cell surface (c, g), and corresponding force curves (d, h). Bars, 100 nm (c, g). Force curves show the approach (labels i–iii, solid line) and retraction (labels iii–vii, dashed line) cycles.

Table 1. Morphometry of fixed wild-type *R. leguminosarum* 3841 and the *ctpA* mutant strain 3845Data shown are mean $\pm$ SD. NA, Not applicable.

<i>R. leguminosarum</i> strain	Cell size (μm)		Subunit size* (nm)
	Length	Width	
Wild-type 3841	1.50 $\pm$ 0.20	1.00 $\pm$ 0.06	21 $\pm$ 3†
<i>ctpA</i> 3845 rod-shaped‡	2.2 $\pm$ 0.8	1.2 $\pm$ 0.4	31 $\pm$ 9§
<i>ctpA</i> 3845 spherical‡	1.6 $\pm$ 0.2	NA	40 $\pm$ 10§

*Diameter of smallest dimension.

†The wild-type *R. leguminosarum* had rod-shaped subunits, and the *ctpA* mutant had irregularly shaped subunits.‡The *ctpA* mutant had two distinct morphologies.§Differences in surface subunit size between the *ctpA* mutant and the wild-type *R. leguminosarum* were statistically significant with a paired Student's *t*-test ($P < 0.05$).

densely packed, elongated surface subunits, composed of small aggregates of surface molecules (Fig. 1), while those of the *ctpA* mutant are round but statistically larger ($P < 0.0001$) than the smallest dimension of that of the wild-type and are less well defined, regardless of cell morphology (Table 1). Live specimens show the same results, but with subunits that appear larger than expected (Ma *et al.*, 2005) and with a less even surface distribution (Fig. 1c, g).

Surface roughness is a quantitative assessment of surface texture, defined as the average of the vertical deviations from the nominal surface over a specified surface area. Wild-type *R. leguminosarum* is statistically rougher ($P < 0.05$) compared with the *ctpA* mutant strain (Table 2). Depressions apparent on the *ctpA* mutant cell surface (19 ± 4 nm deep) were statistically larger ($P < 0.0001$) than those observed in the wild-type (4 ± 0.4 nm deep), indicating variations below or within the cell envelope (Fig. 1a–c, e–g).

The adhesion force between the bacterial surface and the hydrophilic AFM probe was measured using FS (Fig. 1d, h, Table 2). The adhesion forces measured between the hydrophilic AFM probe and the bacteria somewhat mimic that measured between bacteria and the hydrophilic glass coverslips. Approach speed (1, 2, 5 or $10 \mu\text{m s}^{-1}$) did not affect absolute adhesion values, so the fastest rate ($10 \mu\text{m s}^{-1}$) was chosen to reduce lateral contact time with the sample, thus sacrificing the nonlinear region of the force curve. Adhesion forces of the *ctpA* mutant strain were statistically larger ($P < 0.05$) than those of the wild-type strain.

Effect of Ca^{2+} on *R. leguminosarum* surface characteristics

AFM images revealed that the surface ultrastructures of both wild-type and *ctpA* mutant *R. leguminosarum* are altered in

Table 2. Surface subunit, surface roughness and adhesion of fixed wild-type *R. leguminosarum* 3841 and *ctpA* mutant 3845Data shown are mean $\pm$ SD. WT, Wild-type.

CaCl ₂ in TY broth (mM)	Subunit size* (nm)		Surface roughness (nm)		Adhesion (nN)	
	WT	<i>ctpA</i>	WT	<i>ctpA</i>	WT	<i>ctpA</i>
1.8	27 $\pm$ 7†	25 $\pm$ 3‡	2.3 $\pm$ 0.5†	1.5 $\pm$ 0.1‡	8.5 $\pm$ 2.9	5.6 $\pm$ 2.5†
3.4	21 $\pm$ 3	31 $\pm$ 9§	3.2 $\pm$ 0.4	2.0 $\pm$ 0.3§	7.7 $\pm$ 5.1	11.2 $\pm$ 4.6§
3.6	48 $\pm$ 11	45 $\pm$ 9‡	4.0 $\pm$ 1.3	2.6 $\pm$ 0.6‡	19.7 $\pm$ 7.2	17.8 $\pm$ 8.8‡

*Diameter of smallest dimension.

†Differences in wild-type surface subunit size and surface roughness between calcium-limited and standard conditions were statistically significant with a paired Student's *t*-test ($P < 0.05$).‡Differences in the *ctpA* mutant surface subunit size, surface roughness and adhesion force between the three different calcium conditions were statistically significant with a paired Student's *t*-test ($P < 0.05$).§Differences in surface subunit size, roughness, adhesion and biofilm thickness of the *ctpA* mutant were statistically significant compared with the wild-type under standard conditions with a paired Student's *t*-test ($P < 0.05$).||Differences in wild-type surface subunit size and adhesion force between calcium standard and supplemented conditions are statistically significant with a paired Student's *t*-test ($P < 0.05$).

response to varying Ca^{2+} ion concentrations (Figs 1 and 2). Subunit sizes of the wild-type and *ctpA* mutant were statistically different under standard conditions, but were statistically identical in size when grown with decreased (1.8 mM) or increased (3.6 mM) calcium chloride concentrations (Table 2). Both the wild-type and *R. leguminosarum* *ctpA* mutant grown in TY broth with increased calcium chloride (3.6 mM; Fig. 2a, c) had statistically larger ($P < 0.05$) surface subunits than each strain grown in standard TY broth (Table 2, Fig. 1). The normally elongated surface subunits of the wild-type *R. leguminosarum* grown in standard TY broth were round and statistically identical to those of the *ctpA* mutant strain growth in increased Ca^{2+} conditions (Table 2).

Ca^{2+} concentration also had a strong effect on adhesion, for which ranges were similar for wild-type (~7–20 nN) and the *ctpA* mutant (~5–18 nN). In general, the surface roughness and adhesion of both strains increased as a function of Ca^{2+} concentration, with the exception of wild-type *R. leguminosarum*, which had statistically identical adhesion values when grown with 1.8 and 3.4 mM calcium chloride (Table 2). Two distinct force curves were observed, and notably the force curve of the *ctpA* mutant strain grown in the TY broth with 3.6 mM calcium chloride was the only one with a stepwise return to zero deflection (data not shown), indicative of multiple pull-off events detected by the AFM cantilever. In this case, the events likely represent stretching of polymers on the bacterial surface, such as EPS or CPS as the AFM tip was retracted.

Biofilm formation

Biofilm formation was initiated by the adherence of individual cells to the substrate, and reached a maximum

at 24 h. Adhesion was independent of substrate type, although wild-type biofilm accumulation was less apparent on mica (Supplementary Fig. S1, available with the online version of this paper). Adhesion (*ctpA* mutant) and biofilm formation (wild-type) increased with incubation time (Supplementary Fig. S2). Shaking, a standard practice for biofilms studies, resulted in initially fewer *R. leguminosarum* cells attached to the glass surface, but gradually gave rise to similar biofilms after 24 h (data not shown).

Confocal microscopy showed that the *ctpA* mutant biofilm (~7 μm , 3–4 bacteria deep) has large areas of continuous cell aggregates lacking detailed internal structures, while the wild-type was capable of forming mature biofilms (~13 μm deep) with channels throughout (Fig. 3). After 72 h incubation, both the wild-type and *ctpA* mutant cells in the biofilm began to disperse (Supplementary Fig. S2). The lamellar continuous biofilms of the wild-type strain and *ctpA* mutant strain supplemented with mannitol–TY broth were released from the glass surface, but the biofilm of the *ctpA* mutant strain lacking mannitol supplement appeared as tiny floccs (data not shown). The *ctpA* mutant biofilms grown under standard conditions were much more homogeneous than wild-type and were surrounded by a thin transparent layer (data not shown), which stained more efficiently with Coomassie blue (protein) than with Toluidine blue O (carbohydrate). Wild-type biofilms had a greater number of planktonic cells at their surface (Fig. 3).

R. leguminosarum grown in TY broth with 3.4 (standard), 1.8 or 3.6 mM calcium chloride was used to assess whether Ca^{2+} ions had an effect on adhesion (Table 2) or biofilm formation (Table 3, Fig. 3). At 3.6 mM calcium chloride, a greater number of cells adhered to glass coverslips for both

Fig. 2. AFM images of fixed wild-type *R. leguminosarum* (a, b) and the *ctpA* mutant (c, d), showing the surface ultrastructure at 3.6 mM (a, c) and 1.8 mM (b, d) CaCl_2 .

Fig. 3. Confocal microscopy images of live wild-type *R. leguminosarum* (a, c, e, g, i) and the *ctpA* mutant (b, d, f, h, j) biofilms formed on glass coverslips after 48 h incubation. Average biofilm depth is shown for conditions with 1.8 mM (e, f), 3.4 mM (g, h, standard) and 3.6 mM (i, j) CaCl_2 for each strain. The double-headed white arrow shows a typical thickness measurement, excluding planktonic cells. Biofilm thickness (e–j) was significantly different between the *ctpA* mutant and wild-type under all conditions ($P < 0.05$; Student's *t*-test). A higher contrast version of Fig. 3 is available with the online version of this paper.

types of *R. leguminosarum* compared with the adhesion in standard TY broth (Supplementary Fig. S3). Wild-type *R. leguminosarum* biofilms were thicker when grown with 1.8 and 3.6 mM calcium chloride compared with standard conditions (Fig. 3e, g, i), the former being statistically identical ($P > 0.05$). On the other hand, biofilms of the *ctpA* mutant (Fig. 3f, h, j) grown with 1.8 or 3.4 mM calcium chloride were statistically identical ($P > 0.05$), but thinner than those grown with 3.6 mM (Table 2). Mannitol (10 mM) supplementation in TY broth restored the *ctpA* mutant strain biofilm to wild-type levels (Table 3).

At the highest Ca^{2+} concentrations, deeper biofilms were observed for both wild-type and *ctpA* mutant strains of *R. leguminosarum* compared with those formed with standard TY broth (Table 2, Fig. 3), which appeared to have large continuous regions that stained with both Toluidine blue O (Supplementary Fig. S3) and Calcofluor white (Fig. 3), but which did not contain bacteria. To estimate the difference in adherent biomass between the wild-type and *ctpA* mutant stains, we used crystal violet staining (Table 3). There was a roughly threefold increase in the amount of crystal violet bound to biofilms formed by the wild-type *R.*

Table 3. Crystal violet staining of adherent biomass under different growth conditions

Crystal violet staining was measured by eluting bound dye with ethanol and reading the A_{550} (see Methods). Data shown are mean $\pm$ SD.

Growth condition		Strain		
Medium	Time (days)	Wild-type (3841)	<i>ctpA</i> mutant (3845)	Complement (3845pKG62)
TY + 3.4 mM CaCl_2	2	1.047 ± 0.188	$0.368 \pm 0.089^*$	1.130 ± 0.238
TY + 3.4 mM CaCl_2 /1 mM mannitol	2	1.623 ± 0.549	1.348 ± 0.356	1.730 ± 0.425
TY + 1.8 mM CaCl_2	2	1.795 ± 0.296	$0.428 \pm 0.271^*$	1.196 ± 0.241
TY + 3.6 mM CaCl_2	2	1.379 ± 0.188	$0.541 \pm 0.234^*$	0.891 ± 0.322
TY + 3.4 mM CaCl_2	4	0.804 ± 0.235	0.605 ± 0.118	0.842 ± 0.235
TY + 3.4 mM CaCl_2	6	0.508 ± 0.105	0.647 ± 0.046	0.649 ± 0.098

*Difference in A_{550} between wild-type and the *ctpA* mutant was statistically significant with a paired Student's *t*-test ($P < 0.05$).

leguminosarum compared with those of the *ctpA* mutant. However, the Ca^{2+} concentration in TY broth did not have a substantial effect on the amount of stain bound to either the wild-type or mutant strain (Table 3).

DISCUSSION

CtpA is considered to be a periplasmic protease that is crucial to the maturation of proteins important for cell envelope structure and function. Here we used AFM and PS to further understand the implications of a *ctpA* mutation on envelope structure and to continue characterization of CtpA function in alphaproteobacteria, in which its function remains poorly understood.

Our previous work using AFM to study fungal microbes has offered valuable insight into the relationship between wall function, and its ultrastructure and physical properties (Ma *et al.*, 2005, 2006; Kaminskyj & Dahms, 2008; Paul *et al.*, 2011), and we have applied a similar strategy to the study of *R. leguminosarum*. Images of fixed samples were used to interpret *R. leguminosarum* surface ultrastructure (Fig. 1, Tables 1 and 2). The surface features of the chemically cross-linked fixed samples are more rigidly defined, offering better image contrast than those of live samples that deform under the AFM probe (Ma *et al.*, 2005); nonetheless surface profiles were similar for fixed and live *R. leguminosarum* (Fig. 1). The AFM tip probes the outer surface of the specimen, making surface features ($d_{\text{min}} \sim 20\text{--}40$ nm; Fig. 1) attributable to the outermost layer, in this case EPS and/or CPS.

The EPS of *R. leguminosarum* is similar between strains, consisting of identical conserved octasaccharide backbones containing glucose, glucuronic acid and galactose in a 5:2:1 molar ratio (Robertsen *et al.*, 1981; Skorupska *et al.*, 2006) with branches at the same β -1,6-linked glucosyl residue. However, patterns of acetyl, pyruvyl and hydroxybutanoyl decoration are strain-dependent (O'Neill *et al.*, 1991; Skorupska *et al.*, 2006). The acidic CPS of each *R. leguminosarum* strain has a similar or identical structure to its EPS. Strains deficient in EPS and/or CPS synthesis have a pleiotropic effect resulting in a significant increase in the synthesis and secretion of cyclic β -1,2-glucans (Robertsen *et al.*, 1981). Each surface component contributes to the net negative surface charge of this Gram-negative bacterium. EPS was not produced at sufficiently high concentrations to measure for either the wild-type or *ctpA* mutant grown in TY broth (data not shown), consistent with the repression of EPS production in this growth medium (Borthakur *et al.*, 1986), but this does not necessarily rule out a small contribution of EPS to the surface features observed by AFM. The size of the surface features (20–40 nm) compared with the average diameter of a sugar chain (~ 1 nm) must therefore reflect backbone branching, and implies that subunits are composed of a higher-order structure of multiple sugar backbones.

Surface roughness effectively quantifies the variance of surface feature height. The wild-type strain is consistently

50 % rougher ($P < 0.05$) than the *ctpA* mutant strain under standard and lower calcium levels (Table 2), suggesting that EPS and/or CPS is arranged differently on the surface of the *ctpA* mutant, consistent with the larger-sized surface features. With decreased lateral packing, a greater number of polar groups (i.e. hydroxyl group of glucans, other polysaccharides or hydrophilic amino acids of proteins) would be exposed at the cell surface, consistent with *R. leguminosarum* *ctpA* cells having a greater tendency ($P < 0.05$) for hydrophilic surface adhesion (Table 2).

Calcium is a key component in cell wall organization, stability and permeability (Humphrey & Vincent, 1962), and is an important factor for maintaining the integrity, stability and function of the outer membrane and cell wall in *R. leguminosarum* (de Maagd *et al.*, 1989a, b). Ca^{2+} helps to maintain the outer membrane by shielding the O antigenic portion of LPS (de Maagd *et al.* 1989a). Furthermore, adhesin, a Ca^{2+} binding protein, is considered to be the key protein present on the cell surface of *R. leguminosarum* and is responsible for its preliminary attachment to the leguminous root hairs (Smit *et al.*, 1987; Dardanelli *et al.*, 2003; Mongiardini *et al.*, 2008). Adhesins are extracellular proteins released into the media under Ca^{2+} -limited conditions, but become anchored to the rhizobial surface at higher Ca^{2+} concentrations. Ca^{2+} depletion also promotes the release of Omp22 protein in *Sinorhizobium* (Kim *et al.*, 2005) and can cause the release of LPS (de Maagd *et al.*, 1989a). We therefore expected a rearrangement of the rhizobial surface structure and altered surface characteristics in response to varying Ca^{2+} levels.

The surface structures of both *R. leguminosarum* strains were altered (Fig. 2) and consequently their surface roughness (Table 2) increased as a function of Ca^{2+} concentration. Limiting Ca^{2+} availability had no significant effect on the hydrophilic surface character of wild-type *R. leguminosarum*, but higher Ca^{2+} levels made its surface more hydrophilic. For the *ctpA* mutant, there was a direct relationship between the surface hydrophilicity and Ca^{2+} concentration. The greater availability of calcium ions could lead to a net positive charge at the cell surface, potentially affecting adhesion between *R. leguminosarum* and the AFM probe (partial negative charge character). However, even a modest difference in calcium ion concentration (0.2 mM) in the media was capable of changing the size and shape of the surface features and enhanced the hydrophilic properties of both the wild-type (1.5-fold) and *ctpA* mutant (2.5-fold) strains.

Adhesins fulfil their role only when activated in the presence of excess Ca^{2+} , and here we show a dramatic change in adhesion force only at elevated Ca^{2+} levels, which were greater than expected for electrostatic interactions between the partial negative charge of the nitrogen lone pairs of the AFM tip and the additional Ca^{2+} ions. Large electrostatic forces (3–9 nN) are observed in ionic crystals, in which ions are in close proximity (0.1–0.2 nm), but typical electrostatic forces between the AFM tip within the Debye length

(1 nm) of the sample surface are much smaller (0.001–0.1 nN) (Butt, 1991a, b). Thus, electrostatic force is unlikely to dominate on this length scale, and changes in adhesion and roughness as a function of Ca^{2+} can be attributed to other molecular forces and alteration of the cell surface structure.

Several studies have demonstrated the importance of a functional cell envelope for proper attachment and biofilm maturation (Fujishige *et al.*, 2006; Russo *et al.*, 2006; Vanderlinde *et al.*, 2009, 2010). Here we provide evidence of a role for CtpA in rhizobial biofilm formation on a hydrophilic surface. We were first interested in determining whether alterations to the cell envelope in a *ctpA* mutant affected its attachment to a variety of solid surfaces, but neither wild-type nor the *ctpA* mutant of *R. leguminosarum* showed a preference for abiotic surface type. Adhesion, generally accepted as the initial step in biofilm formation (Fey & Olson, 2010), was not disturbed in the *ctpA* mutant, meaning that neither morphology nor molecular surface arrangement appear to influence initial non-specific surface attachment. Indeed, while the *ctpA* mutant was able to form biofilms, there was a slight preference for attachment to the hydrophilic surfaces (Supplementary Fig. S3), consistent with lower surface roughness and greater hydrophilic character (Table 2). The *ctpA* gene appears to influence the later stages of biofilm development, maintaining only a thin layer of aggregated rhizobial cells. Mannitol restored the *ctpA* mutant strain to wild-type levels (Table 3), but it did not affect the number of cells in culture (data not shown), indicating an impact on biofilm matrix formation. Calcium affected biofilm depth for both the wild-type and mutant (Fig. 3), and at increased Ca^{2+} concentrations, the mutant biofilm was restored to wild-type depth.

Biofilm formation, measured both qualitatively (Supplementary Figs S1–S3) and quantitatively (Tables 2 and 3, Fig. 3), was reduced in the *ctpA* mutant, demonstrating its importance in biofilm maturation. Once in contact with the surface, the process of permanent attachment is mediated by components of the bacterial cell surface such as LPS, EPS, cell wall anchored proteins, pili, flagella and other outer-membrane proteins (Habimana *et al.*, 2007; Manetti *et al.*, 2007; Vu *et al.*, 2009; Bandara *et al.*, 2010; Crawford *et al.*, 2010; Díaz *et al.*, 2011). The interaction of bacterial surface receptors with an abiotic surface can induce increased expression of surface molecules to facilitate specific, permanent adhesion to surrounding cells and surfaces during biofilm structuring, which is mediated by the quorum sensing mechanism (Otto, 2008). The exact role of CtpA in biofilm formation remains to be determined.

Conclusions

We have characterized the cell surface of *R. leguminosarum*, showing that there are distinct differences between wild-type and *ctpA* mutant strains. The surface features observed by AFM, most likely attributable to higher-order structures of polysaccharides, were remodelled as a function of Ca^{2+} concentration. This study shows that higher surface

roughness, hydrophilic character and activation by calcium all contribute to the adhesion of *R. leguminosarum* to hydrophilic abiotic surfaces and biofilm formation. The inability of the *ctpA* mutant to form mature biofilms compared with wild-type *R. leguminosarum* demonstrates that CtpA function is important for biofilm accumulation and maturation.

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