The *Dickeya dadantii* biofilm matrix consists of cellulose nanofibres, and is an emergent property dependent upon the type III secretion system and the cellulose synthesis operon

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Dickeya dadantii is a plant-pathogenic bacterium that produces cellulose-containing biofilms, called pellicles, at the air–liquid interface of liquid cultures. *D. dadantii* pellicle formation appears to be an emergent property dependent upon at least three gene clusters, including cellulose synthesis, type III secretion system (T3SS) and flagellar genes. The *D. dadantii* cellulose synthesis operon is homologous to that of *Gluconacetobacter xylinus*, which is used for industrial cellulose production, and the cellulose nanofibres produced by *D. dadantii* were similar in diameter and branching pattern to those produced by *G. xylinus*. *Salmonella enterica*, an enterobacterium closely related to *D. dadantii*, encodes a second type of cellulose synthesis operon, and it produced biofilm strands that differed in width and branching pattern from those of *D. dadantii* and *G. xylinus*. Unlike any previously described cellulose fibre, the *D. dadantii* cellulose nanofibres were decorated with bead-like structures. Mutation of the cellulose synthesis operon genes resulted in loss of cellulose synthesis and production of a cellulase-resistant biofilm. Mutation of other genes required for pellicle formation, including those encoding FlhA (a sigma factor that regulates flagella production), HrpL (a sigma factor that regulates the T3SS), and AdrA, a GGDEF protein, affected both biofilm and cell morphology. Mutation of the cellulose synthase *bcsA* or of *bcsC* resulted in decreased accumulation of the T3SS-secreted protein HrpN.

INTRODUCTION

Bacterial cellulose has been successfully used in a wide range of applications, including wound dressings, scaffolds for tissue growth, food additives, and flexible screen displays (Tabuchi, 2007). It is biocompatible and biodegradable, and can be modified by altering bacterial growth conditions or by adding compounds or polymers after it has been synthesized (Brown & Laborie, 2007; Svensson *et al.*, 2005; Wiegand *et al.*, 2006). To this end, there is progress toward incorporating particles, enzymes, growth factors and antibiotics into bacterial cellulose scaffolds, which will improve their utility in tissue regeneration, biosensors and wound dressings (Phelps *et al.*, 1994; Sharp *et al.*, 2008; Tsiptsias & Panayiotou, 2008). Although many bacterial species produce cellulose,

the soil bacterium *Gluconacetobacter xylinum* is the species most commonly used for industrial cellulose production.

Synthesis of bacterial cellulose, which has mainly been examined in *G. xylinus*, remains poorly understood. This bacterium produces cellulose fibres 3–8 nm thick from a linear array of pores along the sides of the bacterial cells (Bielecki *et al.*, 2002). The array of pores secretes strands of cellulose that are crystallized into nanofibres upon secretion from the cell. *G. xylinus* cellulose production requires two polycistronic operons. One operon encodes a cellulose synthase subunit with a 3′-5′-cyclic diguanylic acid (c-di-GMP) binding domain (*bcsA*), the beta subunit of the synthase (*bcsB*), and two genes (*bcsC* and *bcsD*) implicated in the assembly of the glucan chains (Saxena *et al.*, 1990, 1991, 1994). A second operon encodes a cellulase and a gene of unknown function (Standal *et al.*, 1994). Homologous genes are found in some enterobacteria, but the operon has greatly diverged from that of *G. xylinus*, and an additional operon, *bcsEFG*, is required for cellulose synthesis in species with the second type of cellulose synthesis gene cluster (Solano *et al.*, 2002). Recently, *bcsQ* (*yhjQ*), which is encoded upstream of *bcsA*, has been shown to be required for cellulose synthesis, and BscQ is located at the cell pole in *Escherichia coli* (Le Quéré & Ghigo, 2009).

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Abbreviations: c-di-GMP, 3′-5′-cyclic diguanylic acid; SAL biofilm, surface–air–liquid interface biofilm; SEM, scanning electron microscopy; T3SS, type III secretion system.

The signal molecule c-di-GMP plays a role in cellulose synthesis. In enterobacteria, AdrA and/or YedQ, which are both predicted to be diguanylate cyclases, signal the cellulose synthesis machinery through an as-yet-undescribed mechanism (Da Re & Ghigo, 2006; García *et al.*, 2004; Kader *et al.*, 2006). In some enterobacteria, such as *Salmonella enterica*, the curli regulator AgfD also regulates cellulose synthesis (Römling *et al.*, 2000). However, this regulator is not widespread within the enterobacteria, and AgfD does not regulate cellulose in some enterobacterial strains that encode this gene (Da Re & Ghigo, 2006).

The enterobacterial plant pathogen *Dickeya dadantii* forms two types of biofilm in culture: a surface-air-liquid interface biofilm (SAL biofilm) and a biofilm at the air-liquid interface (pellicle) (Yap *et al.*, 2005). The SAL biofilm and the pellicle are genetically and chemically distinct. Only the pellicle contains cellulose, and the type III secretion system (T3SS) and flagella are required only for the formation of a pellicle, and not a SAL biofilm (Jahn *et al.*, 2008; Yap *et al.*, 2005). *D. dadantii* is a broad-host-range pathogen that causes wilt and rot diseases on many vegetable and ornamental crops. There are multiple stages in the *D. dadantii* life cycle in which biofilms could play a role, including pathogen survival in waterways and on plant debris, and during colonization of plant xylem vessels. *D. dadantii* is also an insect pathogen, and it is feasible that biofilms could play a role in its interactions with insects (Grenier *et al.*, 2006).

We used microscopy and mutagenesis to examine *D. dadantii* cellulose synthesis, and found that *D. dadantii* produces a cellulose nanofibre decorated with regularly spaced beads, unlike any previously described biofilm matrix. To further explore how genes known to contribute to *D. dadantii* pellicle formation affected the structure of the cellulose nanofibres, we used microscopy and immunoassays to examine the pellicle matrix and composition in wild-type cells and in strains carrying mutations in gene clusters known to affect *D. dadantii* pellicle formation, including cellulose biosynthesis, the type III secretion system (T3SS), and flagella genes, as well as an orthologue of a gene known to regulate cellulose biosynthesis in *S. enterica*, *adrA*. Through these experiments, we found that mutation of the cellulose synthase *bcsA* and of *bcsC* reduces accumulation of the T3SS-secretion protein HrpN. In turn, mutation of HrpL, the sigma factor that controls the T3SS, abolishes synthesis of decorated cellulose nanofibres. Mutation of *adrA* did not eliminate cellulose synthesis, but did alter pellicle properties. We also found that beads of the reactive oxygen-scavenging secondary metabolite indigoidine produced by *D. dadantii* become embedded in the cellulose matrix.

METHODS

Bacterial strains and growth conditions. Wild-type *D. dadantii* and derivatives and *S. enterica* were grown in Luria-Bertani (LB) medium at 30 or 37 °C for DNA isolation, for mutagenesis, and for starter cultures for biofilm experiments. SOBG was used to induce

biofilm formation in *D. dadantii* (Yap *et al.*, 2005). Bacterial growth in SOBG was measured both by monitoring the optical density of the cultures and by dilution plating from SOBG cultures. *G. xylinus* was grown in buffered S&H medium (per litre: 5 g peptone, 5 g yeast extract, 2.7 g Na₂HPO₄, 1.15 g citric acid and 40 ml 50 % filter-sterilized glucose, which was added after autoclaving the other medium components). *S. enterica* biofilm cultures were grown in LBNS (LB no salt) medium (per litre: 10 g tryptone, 5 g yeast extract). For pellicle assays, bacterial cultures were suspended in SOBG (*D. dadantii*), buffered S&H medium or SOBG (*G. xylinus*), or LBNS (*S. enterica*) to approximately 10⁶ c.f.u. ml⁻¹, and the cultures were incubated at 28 °C in stationary 13 or 20 mm glass tubes for up to 3 days. When required, antibiotics were used at the following concentrations (in mg ml⁻¹): nalidixic acid (50), ampicillin (100), chloramphenicol (50) and kanamycin (50). Strains used in this study are described in Table 1.

Mutant and plasmid construction. Primers were obtained from Integrated DNA Technologies (Table 2). To disrupt *adrA*, a chloramphenicol-resistance cassette was ligated into the unique restriction site *Bgl*II in pGEM-T Easy::*adrA*. Similarly, to disrupt *celY*, a kanamycin-resistance cassette was ligated into the unique restriction site *Xcm*I of pGEM-T Easy::*celY*. The complete *bcsC* coding region was removed with crossover PCR, and a fragment containing only the *bcsC* flanking regions was inserted into pGEM-T Easy. Primers P0808 and P0809 amplify the region 1.5 kb upstream of *bcsC*, while P0810 and P0811 amplify the region 1.5 kb downstream of *bcsC*. Primers P0809 and P0810 have 19 bp of overlapping nucleotide sequence; thus, crossover PCR with primers P0808 and P0811, with the flanking regions amplified by P0808/P0809 and P0810/P0811 as template, amplified a fragment with *bcsB* adjacent to *bcsD*. This 3.0 kb crossover product was ligated into pGEM-T Easy. A chloramphenicol-resistance gene cassette was then ligated into a *Hind*III site engineered between *bcsB* and *bcsD* to construct pTΔ*bcsC*::Cm. These plasmids were electrotransformed into *D. dadantii* strains for allelic exchange following the methods described by Ried & Collmer (1987). All mutations were confirmed by PCR and Southern blot analysis. Transformation, restriction endonuclease digestion and other DNA manipulations were performed essentially as described in Sambrook & Russell (2001).

Cellulase assays. Cellulase assays were performed essentially as described in Yap *et al.* (2005), except that the incubation time was increased from 40 min to overnight. Pellicles were removed from 3 day-old biofilm-inducing cultures by pouring the culture into a Petri plate. The pellicles were washed twice with 10 ml sterile water and then placed in 5 ml 50 mM potassium phosphate (pH 6.0) containing no cellulase or 0.1 % (w/v) cellulysin cellulase (Calbiochem-Novabiochem). The pellicles were incubated on a rotating shaker (30 r.p.m.) at room temperature overnight. At least three pellicles were used for each treatment, and the experiments were repeated at least three times.

Virulence assays. One-week-old *Nicotiana benthamiana* seedlings were transplanted into 10 cm pots. Seedlings were placed in a growth chamber operating at 25 °C with 12 h continuous light per 24 h period. Light was provided by 40 W cool white fluorescent bulbs at 240 μmol photons m⁻² s⁻¹. Plants were watered once daily and fertilized once per week with a 50 % solution of Miracle Gro (Scotts). Plant assays were performed essentially as described by Hirano *et al.* (1997). Four- to six-week-old *N. benthamiana* leaves were infiltrated with a bacterial suspension of 10⁴ c.f.u. ml⁻¹. Inoculated plants were placed in plastic bags and incubated in a growth chamber at 25 °C throughout the course of the assay. At each sampling time, whole leaves were homogenized using a Polytron equipped with a model PTA 20 TS probe (Brinkmann Instruments) in 20 ml sterile water, and the suspension was dilution-plated on LB medium containing appropriate antibiotics. The data were analysed using a Student's *t* test with a 95 % confidence interval.

Table 1. Strains and plasmids used in this study

Abbreviations: Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Km^R, kanamycin resistance; Nx^R, nalidixic acid resistance.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>E. coli</i> DH5α	<i>supE44 ΔlacU169 (Δ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Clontech
<i>S. enterica</i> serovar Enteritidis		
27655-3b	Clinical isolate	Collinson <i>et al.</i> (1991)
<i>bcsA</i> mutant	In-frame deletion removing nucleotides encoding amino acids 165–828 in BcsA	White <i>et al.</i> (2003)
<i>G. xylinus</i>	ATCC 53524, stable agitation strain	Kojima <i>et al.</i> (1997)
<i>D. dadantii</i>		
3937	Wild-type, <i>Saintpaulia ionantha</i> (African violet) isolate	Lemattre & Narcy (1972)
Nx3937	3937 Nx ^R derivative	Yap <i>et al.</i> (2008)
WPP472	Cm ^R , <i>adrA::cat</i>	This work
WPP473	Km ^R Cm ^R , chromosomal insertion <i>lacY-adrA-kan-prt</i> in WPP472	This work
WPP101	Nx ^R Cm ^R , <i>bcsA::cat</i>	Yap <i>et al.</i> (2005)
WPP474	Nx ^R Cm ^R , <i>ΔbcsC::cat</i>	This work
WPP475	Cm ^R , <i>celY::cat</i>	This work
WPP97	Cm ^R , <i>ΔhrpL::cat</i>	This work
WPP381	Km ^R , <i>fliA::kan</i>	Jahn <i>et al.</i> (2008)
A1524	Cm ^R , <i>pecS::MudIIPR13</i>	Reverchon <i>et al.</i> (1994)
Plasmids		
pGEM-T Easy	Ap ^R , <i>lacZ'</i> , cloning vector	Promega
pBluescript SK(+/–)	Ap ^R , <i>lacZ'</i> , cloning vector	Stratagene
pKD4	Km ^R , template plasmid carrying <i>kan</i> cassette	Datsenko & Wanner (2000)
pKD3	Cm ^R , template plasmid carrying <i>cat</i> cassette	Datsenko & Wanner (2000)
p <i>TadrA::cat</i>	Ap ^R Cm ^R , 2.7 kb fragment containing <i>adrA::cat</i> in pGEM-T Easy	This work
p <i>ΔbcsC::cat</i>	Ap ^R Cm ^R , 4.0 kb fragment containing flanking regions of <i>bcsC</i> , cut with <i>HindIII</i> and ligated to <i>cat</i> in pGEM-T Easy	This work
p <i>TcelY::kan</i>	Ap ^R Km ^R , 5.1 kb fragment containing <i>celY::kan</i> in pGEM-T Easy	This work
p <i>ThrpYJcat</i>	Ap ^R Cm ^R , 5.2 kb <i>hrpL</i> -deleted <i>hrpY-hrpJ</i> region on pGEM-T Easy; complete <i>hrpL</i> coding region removed with <i>SmaI</i> and <i>HindIII</i> , blunt-ended and ligated to <i>cat</i>	This work
p <i>AdrA</i>	Ap ^R , 2.2 kb <i>ardA</i> gene and promoter in pGEM-T Easy	This work

Table 2. Oligonucleotides used in this study

Primer	Sequence (5'→3')	Restriction sites*	Amplified region
P0198	<u>CTGCAGGATCC</u> GTAGGCTGGAGCTGCTTC	<u>PstI</u> , BamHI	1 kb <i>cat</i> or 1.6 kb <i>kan</i>
P0199	<u>CTGCAGGATCCC</u> ATATGAATATCCTCCTTA	<u>PstI</u> , BamHI	
P0804	TCGTCAGCCATTCAAAA GGATCCC CGGAAATC	<i>BamHI</i>	2.7 kb <i>adrA</i> vs spanning region
P0805	GAACGGGTCGGGG GGATCCC TGATGCATAA	<i>BamHI</i>	
P0806	GCTACCTGGAGCTGGGCGAT	—	2.2 kb <i>adrA</i> spanning region
P0807	TCAGATGCTGGATCGGCGCGACGT	—	
P0808	AAGAGCGGCATGGTGTTTGG	—	1.5 kb <i>bcsC</i> flanking region
P0809	<i>CGTACTCTGCGAAGCTT</i> CCCGAGATAAAAAATAAGCGAGCCACCT	<i>HindIII</i>	
P0810	GGAAGCTT CGCAGAGTACGGCATGAGTGACCTGCAACAGCA	<i>HindIII</i>	1.5 kb <i>bcsC</i> flanking region
P0811	TCGCCATGTGTACCGCAAGAAATAAGG	—	
P0812	AATATCGATCAGGTTACGGTTGATTACACAGGG	—	3.5 kb <i>bcsD</i> and <i>celY</i> spanning region
P0813	AAGAGGAAGTCCCCAGTACGATCAGC	—	

*Restriction sites are indicated in the primer sequence by underlined or bold type. Italic type indicates the overhang used in crossover PCR.

Immunoblot analysis. Strains were grown at 28 °C in SOBG medium for 2 days, at which point the wild-type *D. dadantii* pellicle had just completely covered the surface of the culture. Cultures were run in duplicate to obtain soluble and whole-cell lysate fractions. The pellicle was decanted for the preparation of whole-cell lysate and soluble proteins. For mutants that did not form pellicles, the SAL biofilm was harvested. Pellicles and SAL biofilms were centrifuged to harvest bacterial cells, and the pellets were resuspended in 200 µl EasyLyse (Epicentre), then incubated for 10 min at room temperature. Soluble protein samples were briefly centrifuged and the supernatant was transferred to a fresh microcentrifuge tube. The protein concentration of both soluble and whole-cell lysate fractions was determined with a Bradford assay (Bio-Rad Laboratories). One milligram per millilitre of total protein from each sample was suspended in a modified Laemmli buffer (Hammer *et al.*, 2007), incubated at 100 °C for 10 min, and separated in an SDS 4–20% polyacrylamide gel by electrophoresis (NuSep). Proteins were subsequently analysed by immunoblotting with HrpN-specific polyclonal antiserum (a gift from A. Collmer, Cornell University) with an ImmunoStar AP goat anti-rabbit IgG detection kit (Bio-Rad Laboratories), following the manufacturer's instructions. The immunoblot experiment was repeated three times.

Epifluorescence microscopy. Epifluorescence microscopy was performed essentially as previously reported (Yap *et al.*, 2005). Briefly, pellicle and SAL biofilm fragments were heat-fixed to glass slides and stained with the DNA stain propidium iodide (Invitrogen) and the β -glucan stain calcofluor (Becton, Dickinson and Co.) according to the manufacturers' instructions. The stained *D. dadantii* biofilms were observed by epifluorescence microscopy with a BX60 epifluorescence microscope (Olympus America). Separate filters were used to observe cells stained with propidium iodide (excitation, 556 nm; emission, 592 nm) and β -glucans stained with calcofluor (excitation, 360 nm; emission, 450 nm). Images of the propidium iodide-stained cells and the calcofluor-stained extracellular matrix of *D. dadantii* were captured separately as monochrome images with a Magnafire camera (Optronics) and Image Pro Plus software (MediaCybernetics). The images were false-coloured and merged with Photoshop CS2 (Adobe Systems).

Scanning electron microscopy (SEM). Pellicles were removed from 3 day-old cultures by pouring the culture into 15 ml conical tubes. The pellicles were washed twice for 3 min with 10 ml 0.1 M phosphate buffer (pH 7.4). Sections were fixed for 2 h with 1.5% glutaraldehyde and 1% tannic acid in 0.1 M phosphate buffer. The samples were rinsed twice in 0.1 M phosphate buffer for 3 min and then dehydrated by successive ethanol treatment (30% for 10 min, 50% for 10 min, 70% for 10 min, 95% for 10 min, and three washes of 100% for 10 min). Final desiccation was accomplished by critical-point drying (Tousimis). Specimens were mounted on aluminium stubs and sputter-coated with gold/palladium alloy. Samples were observed in a scanning electron microscope (Hitachi S5700) in the high-vacuum mode at 10 kV. The images were processed for display using Adobe Photoshop version CS2 (Adobe Systems).

RESULTS

D. dadantii encodes one of the two types of cellulose synthesis gene cluster present in the *Enterobacteriaceae*

Examination of sequenced *Enterobacteriaceae* genomes showed that two distinct cellulose synthesis gene clusters are encoded by the *Enterobacteriaceae* (Fig. 1). One type

(group A) is homologous to the cellulose synthesis cluster encoded by *G. xylinus*, while the other (group B) has mainly been characterized in *S. enterica* and *E. coli*. Based on sequence similarity and operon organization, *D. dadantii* encodes a group A cellulose synthesis gene cluster, while the *Enterobacteriaceae* plant pathogen *Pectobacterium atrosepticum* and the animal pathogens *S. enterica* and *E. coli* encode group B clusters. The *Enterobacteriaceae* group A cellulose synthesis BcsA and BcsB proteins are encoded by two genes, whereas in *G. xylinus*, this is a single protein. The *D. dadantii*, *P. atrosepticum*, *E. coli* and *S. enterica* cellulose synthesis gene clusters are all located in an otherwise collinear region of the chromosome. Some *Enterobacteriaceae*, such as *Klebsiella pneumoniae*, encode both types of gene cluster in this locus, while others, such as *Erwinia tasmaniensis* (not shown), encode both gene clusters in a different region of the chromosome. These gene clusters are present in other Gram-negative bacteria. For example, group B gene clusters are present in *Pseudomonas*, *Ralstonia* and *Burkholderia* species.

The two types of cellulose synthesis gene clusters encode some homologous genes, and some genes unique to each type of cluster. Bacteria encoding the group B cluster require *bcsEFG* for cellulose synthesis, an operon that is not present in *D. dadantii* or *G. xylinus*, and the gene *bcsD*, for example, is only present in group A clusters. All of the cellulose synthesis clusters encode an endoglucanase homologue (*celY* or *bcsZ*), but they are located in different places relative to the other cellulose synthesis genes in group A and B gene cluster operons.

D. dadantii biofilms are more similar in structure to *G. xylinus* biofilms than to *S. enterica* biofilms

We hypothesized that there were differences in the biofilm matrix among species encoding different types of cellulose synthesis gene clusters, so we examined biofilms from three different species which encode the two different types of *bcs* cluster. Since biofilm formation is dependent on medium components, and inducing media differ among species, we used media previously reported to induce biofilm formation for each species. We also tested *G. xylinus* in SOBG medium, a medium developed for *D. dadantii* biofilm studies (Yap *et al.*, 2005). SEM images showed significant differences in pellicle structure among these three species (Fig. 2). *S. enterica* cells were completely enmeshed within a matrix of smooth, highly branched strands with cells located in crypts, which may have formed as a result of bacterial digestion of the surrounding matrix (Fig. 2a, b). In contrast, *G. xylinus* formed thick cables of cellulose, no crypts were present, and cells were sparse in the biofilm (Fig. 2c, d). The *D. dadantii* and *G. xylinus* matrices closely resembled each other, with thicker strands and fewer branches than the *S. enterica* matrix (Fig. 2e, f). However, the *D. dadantii* matrix fibres had the appearance of strands of beads; thus, we refer to these

Fig. 1. Organization of the group A and group B cellulose synthesis operons. There are two classes of cellulose biosynthesis gene cluster: group A is indicated with grey arrows and group B with white arrows. Members of the *Enterobacteriaceae* may encode one or both of these gene clusters, typically in an otherwise collinear region of the chromosome. Numbers above the arrows indicate the amino acid identity of selected Bcs proteins compared with *S. enterica* LT2; numbers below the arrows indicate the amino acid identity of these proteins compared with *D. dadantii* 3937. Gene clusters encoding homologous genes are shown from the *Enterobacteriaceae* species *E. coli* EDL933, *P. atrosepticum* SCRI1043, *K. pneumoniae* MGH78578 and *D. dadantii* 3937, and from *G. xylinus* ATCC 23769 and *Pseudomonas syringae* DC3000. The *P. atrosepticum* SCRI 1043 *bcsA* and *E. coli* EDL933 *bcsQ* genes have nonsense mutations near the 5' ends, so amino acid identity was not included for these genes. *Pseudomonas* cellulose operon nomenclature differs from that of other bacteria (*bcsQ*=*wssA*, *bcsA*=*wssB*, *bcsB*=*wssC*, *bcsZ/celY*=*wssD* and *bcsC*=*wssE*).

strands as decorated cellulose nanofibres. The pellicles of both *D. dadantii* and *S. enterica* appeared to have a greater cell density than that of *G. xylinus*. Because the *S. enterica* cells were larger than the pore size in the biofilm lattice, the lattice would need to be digested before the cells could escape. In contrast, the *D. dadantii* cells were smaller than the holes in the cellulose lattice, and we observed *D. dadantii* cells streaming from pellicles when the pellicles were transferred from culture medium to water. *S. enterica* cells do not stream from pellicles when the pellicles are transferred to water.

***D. dadantii* *bcsA*, *bcsC* and *adrA* mutant pellicles are resistant to cellulase**

We previously found that the cellulose synthesis mutant *bcsA* still forms pellicles, but that these pellicles are thinner and more fragile than those formed by wild-type cells (Yap *et al.*, 2005). Mutation of *bcsC* showed that pellicles formed by a *bcsC* mutant are identical to those formed by the *bcsA* mutant (not shown). We hypothesized that the biofilm matrices formed by *bcsA* and *bcsC* are not composed of cellulose, and we found that the fragile pellicles produced by these strains were not degraded by cellulase, unlike pellicles formed by *D. dadantii* wild-type cultures (Fig. 3).

AdrA is required for biofilm formation by some *Enterobacteriaceae*. The pellicle formed by the *D. dadantii* *adrA* mutant appeared similar to that formed by wild-type cells macroscopically, but it was only partially degraded by cellulase (Fig. 3). Expression of *adrA* from a plasmid complemented this phenotype (not shown). Thus, the matrix was composed of cellulose and an additional cellulase-resistant polymer.

The pellicle formed by a *celY* mutant, which in all respects resembled that of the wild-type, degraded when cellulase was added, as did the *G. xylinus* pellicle. Notably, the *S. enterica* pellicle did not degrade when cellulase was added. Thus, although the cellulose synthase homologue *bcsA* contributes to *S. enterica* pellicle formation, we found, as had been reported earlier, that the *S. enterica* pellicle is composed primarily of cellulase-resistant polymers (Solano *et al.*, 2002).

Both FliA, a sigma factor required for flagella production, and HrpL, a sigma factor that controls the T3SS, are required for pellicle formation, but not SAL biofilm formation, in *D. dadantii* (Jahn *et al.*, 2008; Yap *et al.*, 2005). The biofilm ring formed by the *hrpL* mutant was not degraded by cellulase (Yap *et al.*, 2005), whereas the SAL biofilm formed by the *fliA* mutant was degraded (Fig. 3), suggesting that the two biofilm matrices differ.

To confirm that the lack of pellicle formation by the T3SS, *fliA* and *bcs* mutants was not due to the inability of these strains to grow in pellicle-inducing medium, bacterial growth of *hrpX*, *hrpY*, *hrpL*, *hrcJ*, *bcsA*, *bcsC*, *adrA* and *fliA* mutants was measured in SOBG medium for 24 h. The growth of the mutants in SOBG did not differ significantly

from each other or from that of wild-type cells (not shown).

We stained the pellicles and SAL biofilms with calcofluor, a fluorescent dye that stains β -glucans, including cellulose, chitin and chitosan. As previously reported, the *bcsA* mutant did not produce strands stained by calcofluor; nor did a *hrpL* mutant, which is consistent with the resistance of these aggregates to cellulase (Yap *et al.*, 2005). The *adrA* mutant produced a matrix that was stained by calcofluor, indicating that this mutant still produced cellulose, which is consistent with the partial degradation of this pellicle by cellulase (not shown).

***bcsA* and *bcsC* are required by *D. dadantii* to produce decorated cellulose nanofibres, whereas *adrA* and *celY* are not**

We predicted that the pellicle matrix structures of the wild-type and *bcs* mutants would differ. SEM images of wild-type and *celY* mutant pellicles were indistinguishable (Figs 2e, f and 4a). In contrast, the *bcsA* and *bcsC* mutant pellicle matrix strands did not resemble the wild-type in decoration, branching pattern or strand width (Fig. 4c, d). Compared with the wild-type, the strands of the *bcsA* and *bcsC* mutants were more highly branched and irregular in width, and were not coated with uniformly spaced beads. The *D. dadantii* *adrA* mutant pellicle appeared to be intermediate in structure between the wild-type and the *bcs* mutants, with beaded strands, highly branched strands, and bare strands (Fig. 4b).

Although both the *fliA* mutant and the *hrpL* mutant are able to produce a SAL biofilm, the structure of the biofilm differs

Although the T3SS sigma factor HrpL and the flagellar sigma factor FliA are required for pellicle formation across liquid surfaces, *hrpL* and *fliA* mutants still form SAL biofilms (Yap *et al.*, 2005). SEM images of the cellulose-resistant biofilm ring formed by a *D. dadantii* *hrpL* mutant (Fig. 5a) resembled those of the *bcsA* and *bcsC* mutants (Fig. 4c, d). Unlike the biofilm formed by T3SS mutants, the *fliA* mutant biofilm is easily dislodged from the culture tube surface (Jahn *et al.*, 2008), and it can be degraded by cellulase (Fig. 3). The matrix formed by the *fliA* mutant resembled that formed by wild-type cells (Fig. 5b, c, d). However, the *fliA* mutant cells were greatly elongated compared with the wild-type (Fig. 5d). Cells of the *fliA* mutant were harvested from culture media that do not induce biofilm formation, such as LB broth, and were found to resemble the wild-type (Fig. 5f, g).

D. dadantii wild-type and pellicle-deficient mutant strains sporadically produced the blue pigment indigoidine in SOBG after pellicles had formed. Indigoidine production is regulated by PecS, and mutation of *pecS* results in high levels of pigment production and reduced bacterial growth in culture (Reverchon *et al.*, 1994). Indigoidine is produced

Fig. 2. Scanning electron micrographs of *S. enterica* (a, b), *G. xylinus* (c, d) and *D. dadantii* (e, f) biofilms. The images are representative of the observations from two experiments with two replicates each. *S. enterica* cells were grown in LBNS, *G. xylinus* cells were grown in S&H medium (c) or SOB (d), and *D. dadantii* cells were grown in SOB. Bars: (a, c, e) 2 μm, (b, d, f) 1 μm.

by both Gram-positive and Gram-negative species, and in *D. dadantii* is thought to absorb reactive oxygen during pathogenesis (Reverchon *et al.*, 2002; Takahashi *et al.*, 2007). The pigment accumulated as beads that were up to twice the diameter of the bacterial cells (Fig. 5e, h).

The *D. dadantii* *bcsA* mutant is reduced in HrpN accumulation

HrpN is secreted via the T3SS, binds to the surface of planktonic *D. dadantii* cells and contributes to pellicle formation in *D. dadantii* (Yap *et al.*, 2006). To determine

Fig. 3. Cellulase digestion of bacterial pellicles. Pellicles were removed from test tubes, placed in Petri plates containing buffer with and without cellulase, and incubated overnight. The images are representative of the observations from two to five experiments with at least two pellicles used per treatment per experiment.

Fig. 4. Scanning electron micrographs of *D. dadantii* wild-type and cellulose gene cluster mutant strains. Biofilms of the *celY* mutant (a), *adrA* mutant (b), *bcsA* mutant (c) and *bcsC* mutant (d) strains. Arrows indicate examples of bare fibres produced by the *bcsA* and *adrA* mutants. The images are representative of the observations from two experiments with two replicates each. Bars, 1 µm.

Fig. 5. Scanning electron micrographs of *D. dadantii* *hrpL* and *fliA* SAL biofilms. Biofilms of *hrpL* mutant (a, e), *fliA* mutant (b, d) and wild-type (c) biofilm matrices. Bars: (a–c) 1 μ m, (d, e) 2 μ m. Bacterial cells of the wild-type (f) and *fliA* mutant (g) cells from LB broth cultures placed on slides and stained with crystal violet; bars, 10 μ m. (h) Light micrograph of the pellicle produced by a *pecS* mutant; bar, 10 μ m. In wild-type cells, PecS suppresses accumulation of indigoidine. Arrows indicate indigoidine beads. The images are representative of the observations from two experiments with two replicates each.

whether HrpN accumulation was affected in mutant strains with altered pellicle structures, total protein was harvested from pellicle and SAL biofilms, and HrpN accumulation was evaluated with immunoblots. The *bcsA* mutant

accumulated less HrpN protein than the wild-type, while the *adrA* mutant resembled the wild-type (Fig. 6). Thus, it is unlikely that the biofilm matrix of the *bcs* mutants is made of HrpN protein.

***D. dadantii* cellulose is not required for bacterial growth in leaves**

D. dadantii is an effective pathogen of *N. benthamiana* and causes multiple types of symptoms in this host, including wilting, maceration and leaf curling (Jahn *et al.*, 2008). No difference in symptom development or the growth of *bcsA* or *adrA* mutant strains compared with the wild-type in *N. benthamiana* leaves was found. Thus, neither cellulose synthesis nor AdrA was required for growth of bacterial cells in *N. benthamiana* leaves (data not shown).

DISCUSSION

We found that *D. dadantii* cells produce decorated cellulose nanofibres unlike any previously reported cellulose fibre, and that both the T3SS and the cellulose synthesis operon are required for production of these fibres. Three gene clusters (T3SS, flagellar and cellulose synthesis) that produce extracellular proteins or polysaccharides contribute to *D. dadantii* biofilm formation (Jahn *et al.*, 2008; Yap *et al.*, 2005). The biofilm phenotypes of strains with mutations in these gene clusters are somewhat enigmatic. For example, although the *D. dadantii* pellicle can be completely degraded by cellulase, mutation of the cellulose synthesis operon does not eliminate pellicle formation, but rather results in the formation of a cellulase-resistant pellicle. In contrast, deletion of T3SS regulatory or structural genes eliminates both pellicle formation and cellulose synthesis (Yap *et al.*, 2005). Deletion of *fliA*, which controls flagellar synthesis, also eliminates pellicle formation, but not cellulose synthesis, since the SAL biofilm that forms can be degraded by cellulase.

D. dadantii encodes one of two cellulose synthesis operons found in the *Enterobacteriaceae*. The variation in cellulose synthesis genes is reminiscent of large gene clusters encoding other secreted macromolecules, such as O antigen, which are highly variable among strains or species, and which are present amidst otherwise conserved genes. Determining which, if either, group of cellulose synthesis

gene clusters is part of the ancestral enterobacterial genome or horizontally acquired will be complicated, since both gene clusters are present in some *Enterobacteriaceae*, as well as being widespread in other Gram-negative families, and it is possible that there has been recombination between these two groups of cellulose synthesis gene clusters. Regardless of the origin, the presence of solely the group A cellulose synthesis gene cluster in *D. dadantii* has resulted in a pathogen that produces a biofilm structure that differs from the enterobacterial structure typified by *S. enterica* biofilms, and which is more similar to biofilms formed by *G. xylinus*. The most striking structural difference between the *D. dadantii* and *S. enterica* biofilm matrices is that *S. enterica* cells are much larger than the biofilm matrix pores and are thus entrapped by the matrix, while the *D. dadantii* cells are small enough to escape their biofilm matrix. Curiously, the soft rot pathogen *Pectobacterium*, which is closely related to *Dickeya*, encodes an *S. enterica*-like cellulose synthesis gene cluster.

Mutation of *bcsA* resulted in the formation of a fragile, but cellulase-resistant, pellicle. We were unable to consistently complement this mutation, so we constructed a *bcsC* mutation in order to have another strain with a mutation in a gene homologous to a *G. xylinus* gene required for cellulose synthesis. The *bcsC* mutant had the same phenotype as the *bcsA* mutant, supporting our hypothesis that the *D. dadantii* cellulose synthesis gene homologues are required for synthesis of a cellulose-containing pellicle, as in *G. xylinus*.

We hypothesize that there are regulatory ties between the *D. dadantii* T3SS and cellulose synthesis, and our evidence supports this hypothesis, although the mechanism remains elusive. For example, the SAL biofilm produced by the *hrpL* mutant does not contain cellulose, and *bcsA* mutant cells are reduced in HrpN accumulation. Unfortunately, the *D. dadantii* genome sequence provides no clues to a possible regulatory mechanism linking these two gene clusters, so it remains unknown how the cellulose synthesis operon is integrated into the *D. dadantii* regulatory network. The *bcs* operon promoter contains no apparent binding site for HrpL, the sigma factor that activates the T3SS, nor for HrpY, a two-component system response regulator that is required for activation of HrpL (Yap *et al.*, 2008). No obvious transcriptional regulators are encoded in the *D. dadantii bcs* operon, and AgfD, which regulates cellulose synthesis in some related enterobacteria, is not present in *D. dadantii*.

Recently, regulatory ties between the *D. dadantii* T3SS system and biofilm formation on plastic were found to be mediated by phosphodiesterases containing GGDEF and/or EAL motifs that may affect c-di-GMP turnover (Yi *et al.*, 2010). The GGDEF protein AdrA is required for cellulose synthesis in some *S. enterica* strains (Römling *et al.*, 2000), but we found that mutation of *adrA* in *D. dadantii* does not eliminate cellulose synthesis, although the *adrA* mutant does produce a pellicle that is morphologically different

Fig. 6. Immunodetection of HrpN protein with rabbit polyclonal anti-HrpN. Two-day-old pellicles and SAL biofilms were centrifuged to harvest bacterial cells. Extracts were separated by 20% SDS-PAGE, and the proteins were subsequently analysed by immunoblotting with rabbit polyclonal anti-HrpN followed by conjugation of the membrane with goat anti-rabbit IgG. Lanes: 1, *D. dadantii* 3937 soluble protein; 2, *D. dadantii* 3937 whole-cell lysate; 3, *bcsA* soluble protein; 4, *bcsA* whole-cell lysate; 5, *adrA* soluble protein; 6, *adrA* whole-cell lysate; 7, *hrpL* soluble protein; 8, *hrpL* whole-cell lysate.

from that of the wild-type and more resistant to cellulase. We hypothesized that the cellulase-resistant pellicle produced by the *adrA* mutant might contain an altered ratio of cellulose to T3SS-secreted proteins, but the HrpN immunoassay did not support this hypothesis. However, many *D. dadantii* genes that encode motifs suggesting that they affect c-di-GMP ratios remain unexamined, and some of these may tie together regulation of the T3SS and cellulose.

The *fliA* and *hrpL* mutants both produce only SAL biofilms and not pellicles, and these phenotypes are complemented by expressing *fliA* or *hrpL* in the respective mutant strains (Jahn *et al.*, 2008; Yap *et al.*, 2005). However, the biofilms produced by these two mutants differ in composition, structure and adherence. The *fliA* mutant SAL biofilm is composed of cellulose, adheres poorly to the glass tube surface, and contains filamentous cells. In contrast, mutation of *hrpL* eliminates cellulose synthesis, although the cells are similar to the wild-type in size and the biofilm adheres strongly to the glass. To date, the composition of the SAL biofilm in the *hrpL* mutant remains unknown, although our results suggest that elucidation of the FlhA regulon may help identify the extracellular components of this SAL biofilm. We do not know the mechanism that causes the *fliA* mutant cells to become filamentous in the SAL biofilm, but there is a precedent for a link between flagellar regulatory genes and cell division. In the Gram-negative species *Caulobacter crescentus*, flagellar regulators affect the final stage of cell separation; thus, filamentous cells accumulate in the culture when regulatory genes are mutated (Muir *et al.*, 2005).

Wild-type *D. dadantii* and all of the pellicle-deficient mutants examined sporadically produce large amounts of indigoidine when grown in pellicle-inducing medium. This insoluble blue pigment, which protects cells from oxidative damage during interactions with host plants (Reverchon *et al.*, 2002), accumulates on the pellicle surface as beads of approximately 1–3 µm in diameter. Thus, in addition to producing decorated cellulose nanofibres, *D. dadantii* can embed secondary metabolites into a cellulose matrix. Bacterial cellulose has been used as a wound dressing, and indigoidine may be useful in this application, since absorbing reactive oxygen has been reported to aid in the healing of chronic wounds (Sibbald & Woo, 2008). Whether indigoidine is produced as insoluble beads during plant–microbe interactions or whether it becomes solubilized when bacteria grow in plants remains unknown.

The *D. dadantii* pellicle matrix is an example of an emergent property derived from two unrelated gene clusters, and it is unique among biofilm matrices and cellulose structures described to date. The decorations on the cellulose nanofibres and the accumulation of large particles of a secondary metabolite in the *D. dadantii* biofilm demonstrate novel ways that biofilm matrices can be manipulated and may lead to new industrial uses for bacterial cellulose. The role of biofilms in *D. dadantii* virulence remains unclear. This broad-host-range pathogen

can attack many host species, and sometimes altered *D. dadantii* phenotypes due to gene mutations are only observed on certain plant varieties (Yang *et al.*, 2002). Very little is known about *D. dadantii* ecology; thus, minimal information is available to develop assays for the role that biofilms may play in the survival of this pathogen in waterways or soil. Thus, much work remains to evaluate the role of biofilm formation in *D. dadantii* virulence.

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