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 Flia (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; Tsioptsias & Panayiotou, 2008). Although many bacterial species produce cellulose, the soil bacterium *Gluconacetobacter xylinus* 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 polycistrionic 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, bscQ (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 Quévé & Ghigo, 2009).
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 diluting 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 NaHPO₄, 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 10⁷ c.f.u. ml⁻¹, and the cultures were incubated at 28 °C in stationary or 20 mm glass tubes for up to 3 days. When required, antibiotics were used at the following concentrations (in mg ml⁻¹): naldixic 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 BglII in pGEM-T Easy::adrA. Similarly, to disrupt celY, a kanamycin-resistance cassette was ligated into the unique restriction site XcmI in 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 HindIII site engineered between bcsB and bcsD to construct pTAbcsC::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 10⁶ 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\textsuperscript{R}, ampicillin resistance; Cm\textsuperscript{R}, chloramphenical resistance; Km\textsuperscript{R}, kanamycin resistance; Nx\textsuperscript{R}, nalidixic acid resistance.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
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<td></td>
</tr>
<tr>
<td>E. coli DH5\textalpha</td>
<td>supE44 ΔlacU169 (Δ80lacZΔM15) hisD17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Clontech</td>
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<tr>
<td><strong>Enteritidis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27655-3b</td>
<td>Clinical isolate</td>
<td>Collinson et al. (1991)</td>
</tr>
<tr>
<td>bcsA mutant</td>
<td>In-frame deletion removing nucleotides encoding amino acids 165–828 in BcsA</td>
<td>White et al. (2003)</td>
</tr>
<tr>
<td><strong>S. enterica serovar Enteritidis</strong></td>
<td>S. enterica serovar Enteritidis</td>
<td>Collinson et al. (1991)</td>
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<tr>
<td><strong>G. xylinus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 53524</td>
<td>Stable agitation strain</td>
<td>Kojima et al. (1997)</td>
</tr>
<tr>
<td><strong>D. dadantii</strong></td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td>pGEM-T Easy</td>
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<td>Promega</td>
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<td>pBluescript SK(+/−)</td>
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<td>Stratagene</td>
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<td>pKD4</td>
<td>Km\textsuperscript{R}, template plasmid carrying kan cassette</td>
<td>Datsenko &amp; Wanner (2000)</td>
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<td>pKD3</td>
<td>Km\textsuperscript{R}, template plasmid carrying cat cassette</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pTadrA::cat</td>
<td>Ap\textsuperscript{R} Cm\textsuperscript{R}, 2.7 kb fragment containing adrA::cat in pGEM-T Easy</td>
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<tr>
<td>pTbcsC::cat</td>
<td>Ap\textsuperscript{R} Cm\textsuperscript{R}, 4.0 kb fragment containing flanking regions of bcsC, cut with HindIII and ligated to cat in pGEM-T Easy</td>
<td>This work</td>
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<tr>
<td>pTcefY::kan</td>
<td>Ap\textsuperscript{R} Km\textsuperscript{R}, 5.1 kb fragment containing cefY::kan in pGEM-T Easy</td>
<td>This work</td>
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<td>pThrYJcat</td>
<td>Ap\textsuperscript{R} Cm\textsuperscript{R}, 5.2 kb hrpL-deleted hrpY–hrpJ region on pGEM-T Easy; complete hrpL coding region removed with SmaI and HindIII, blunt-ended and ligated to cat</td>
<td>This work</td>
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<td>pAdrA</td>
<td>Ap\textsuperscript{R}, 2.2 kb adrA gene and promoter in pGEM-T Easy</td>
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Table 2. Oligonucleotides used in this study

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<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>Restriction sites*</th>
<th>Amplified region</th>
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<tr>
<td>P0198</td>
<td>CTGCAAGATCCGTAGGCTGGAGCTGCTTCC</td>
<td>PstI, BamHI</td>
<td>1 kb cat or 1.6 kb kan</td>
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<tr>
<td>P0199</td>
<td>CTGCAAGATCCGTAGGCTGGAGCTGCTCTTAA</td>
<td>PstI, BamHI</td>
<td>1 kb cat or 1.6 kb kan</td>
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<tr>
<td>P0804</td>
<td>TCGTACGACCATCAAAAAGAGATCCGGCGAATT</td>
<td>BamHI</td>
<td>2.7 kb adrA vspanning region</td>
</tr>
<tr>
<td>P0805</td>
<td>GAACGGGTCGGGGATGCTGCTTCAAA</td>
<td>BamHI</td>
<td>2.7 kb adrA vspanning region</td>
</tr>
<tr>
<td>P0806</td>
<td>GCACCTGAGGCTGGGCGAT</td>
<td>−</td>
<td>2.2 kb adrA spanning region</td>
</tr>
<tr>
<td>P0807</td>
<td>TCAGATGCTGAGGCGGCGACGTT</td>
<td>−</td>
<td>2.2 kb adrA spanning region</td>
</tr>
<tr>
<td>P0808</td>
<td>AAGAGCGGCGATGCTGTTTG</td>
<td>−</td>
<td>1.5 kb bcsC flanking region</td>
</tr>
<tr>
<td>P0809</td>
<td>GCCTTCTGGAAGCTTCCCGATGAATATAACCGAGCCACCT</td>
<td>HindIII</td>
<td>1.5 kb bcsC flanking region</td>
</tr>
<tr>
<td>P0810</td>
<td>GGAAAGCTTCCCGAGATCGGAGCATGAGCTGCTCAGACG</td>
<td>HindIII</td>
<td>1.5 kb bcsC flanking region</td>
</tr>
<tr>
<td>P0811</td>
<td>TCGGATCTATGCTGACGAGTGAAGAATTAA</td>
<td>−</td>
<td>1.5 kb bcsC flanking region</td>
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<tr>
<td>P0812</td>
<td>AATATCGATCGTTAGGTTGATCCAGGG</td>
<td>−</td>
<td>3.5 kb bcsD and celY spanning region</td>
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<tr>
<td>P0813</td>
<td>AAGAGGAGGCTGCCAGTACGACGC</td>
<td>−</td>
<td>3.5 kb bcsD and celY spanning region</td>
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</table>

*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 (Epigence), 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 milliliter 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 a SDS 4–20% polyacrylamide gel by electrophoresis (NuSep). Proteins were subsequently analyzed 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) according to the manufacturers’ instructions. The stained 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* SCRI1043 *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 pellets were transferred from culture medium to water. *S. enterica* cells do not stream from pellicles when the pellets 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 bscC 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 microscopically, 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 had been reported earlier, that the *S. enterica* pellicle is composed primarily of cellulose-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 cellulose (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
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.
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 HprN protein.

**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 μm, (d, e) 2 μm. 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 μm. (h) Light micrograph of the pellicle produced by a pecS mutant; bar, 10 μm. 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.
**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 cellulose 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.

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**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 immuno-assay 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 elicitation of the FliA 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 producingdecorating 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 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 producingdecorating 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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