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A Cellulose Synthase-Like Protein Involved in Hyphal Tip Growth and Morphological Differentiation in *S. e ce* ▽

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Cellulose synthase and cellulose synthase-like proteins, responsible for synthesizing β-glucan-containing polysaccharides, play a fundamental role in cellular architectures, such as plant cell and tissue morphogenesis, bacterial bio lm formation, and fruiting-body development. However, the roles of the proteins involved in the developmental process are not well understood. Here, we report that a cellulose synthase-like protein ($CslA_{Sc}$) in *Streptomyces* has a function in hyphal tip growth and morphological differentiation. The $cslA_{Sc}$ replacement mutant showed pleiotropic defects, including the severe delay of aerial-hyphal formation and altered cell wall morphology. Calco uor white uorescence analysis demonstrated that polysaccharide synthesis at hyphal tips was dependent on $CslA_{Sc}$. $cslA_{Sc}$ was constitutively transcribed, and an enhanced green uorescent protein-$CslA_{Sc}$ fusion protein was mostly located at the hyphal tips. An extract enriched in morphogenetic chaplin proteins promoted formation of aerial hyphae by the mutant. Furthermore, a two-hybrid experiment indicated that the glycosyltransferase domain of $CslA_{Sc}$ interacted with the tropomyosin-like polarity-determining DivIVA protein, suggesting that the tip-located DivIVA governed tip recruitment of the $CslA_{Sc}$ membrane protein. These results imply that the cellulose synthase-like protein couples extracellular and cytoskeletal components functioning in tip growth and cell development.

Cellulose is considered to be the most abundant macromolecule on earth, and cellulose synthase proteins exist in many bacterial species, most groups of algae, the slime mold *D c e* , plants, and tunicates. In plants, cellulose synthases and cellulose synthase-like proteins are responsible for cell wall synthesis and are essential for growth and tissue development. In *A ab d a a a*, the 10-member cellulose synthase A (*Ce A*) gene family (*Ce A1* to *Ce A10*) is involved in primary and secondary cell wall synthesis (28). Furthermore, there are another six families of cellulose synthase-like genes (*C*) in *A ab d* , *C A*, *C B*, *C C*, *C D*, *C E*, and *C G* (28), whose functions are still unknown. It is thought that they may be involved in tissue-specific expression and/or response to environmental stresses. For instance, the *C* gene KOJAK/AtCSLD3 is expressed in hair cells of the epidermis and is involved in the biosynthesis of β-glucan-containing polysaccharides that are required during root hair elongation (14).

The gram-negative bacterium *G c ace bac e* (formerly *Ace bac e*) has long been the model organism for the study of bacterial cellulose biosynthesis (30). Cellulose in bacteria often acts as the extracellular polysaccharide matrix and is associated with the formation of cell aggregates. Cellulose fibrils are also important during biofilm formation as a component of the extracellular matrices of many bacterial species, including *Sa e a e e ca* serovar Typhimurium and *E c e c a c* (41). In *G.* and enterobacteria, genes determining cellulose biosynthesis are organized in an operon (*bc* , for *b*acterial *c*ellulose ynthesis). The cellulose synthase encoded by *bc A* catalyzes the polymerization of UDP-glucose by forming β-1,4 glucosidic bonds. The activity of BcsA is allosterically regulated by a cyclic di-GMP binding protein encoded by *bc B*. *bc Z* encodes a cellulase (family 8 glucosidase), which is required for cellulose synthesis. The *bc C* product is an unknown protein, but it is needed for cellulose production in vivo (40, 41). The cellulose-producing bacteria mentioned above all contain genes homologous to the enterobacterial *bc ABZC* operon on their chromosomes. Similar gene clusters are also present in other bacterial genomes (for instance, those of *B de a*, *Ra a*, and *A fe*) (29).

Members of the genus *S e ce* are gram-positive soil-dwelling filamentous bacteria that undergo an ordered, complicated colony differentiation process. After spore germination, vegetative hyphae grow from the germ tubes by tip elongation and branching (7, 12, 18). On solid media, vegetative or substrate hyphae intrude into the agar to absorb nutrients for growth. Subsequently, a fluffy aerial mycelium emerges from the colony-air interface (5). In the model species, *S e ce c e c* , this transition requires the *b d* genes and two classes of surface-active molecules, SapB and the chaplins (5, 6, 8, 13, 38, 39). The aerial hyphae finally form long chains of spores. In shaking liquid culture, many *S e ce* species form pellets of tightly tangled hyphae. In standing liquid media, *S. c e c* cultures can show a biofilm-like growth (36).

The *S. c e c* genome has one gene (SCO2836) encoding a cellulose synthase-like protein (1). In this study, we show that this cellulose synthase-like protein is located at hyphal tips, apparently via an interaction with the polarity-determining protein DivIVA, and is involved in the deposition of β-linked glucan at the tips. Elimination of SCO2836 has marked effects on growth and development.

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TABLE 1. Bacterial strains and oligonucleotides

Strain or oligonucleotide	Characteristics	Source
Strains		
E. c		
DH5α	*E44 acU169* (ϕ*80 acZ*ΔM15) *dR17 ecA1 e dA1 g A96 -1 e A1*	31
ET12567/pUZ8002	*da dc dS ca e a e* RP4	21
S. c e c		
M145	SCP1⁻ SCP2⁻	1
XE	M145Δ*c A*$_{Sc}$::Hyg^r cassette	This work
Oligonucleotide primers for PCR amplification		
pcslAsc5	5′ AATGGATCCTCACACTCCCGGTCGGCAGG3′	
pcslAsc6	5′ TCTAGATTACATATGTCATTCCCCCCACACGCGGG3′	
pcslAsc7	5′ ATTAGATCTGGTTCTGGTGGTTCTGGTATGACGTCGACGCCGACGGG3′	
pcslAsc8	5′ TTATGCGGCCGCTTATCATTCCTTACGTCCCCCAAG3′	
pramS1	5′ AGTAGATCTGCCGGTCGTGTCGTAGGTG3′	
pramS2	5′ TCTAGATTACATATGTCCGAGGTGCGGAACGCAC3′	
pdivIVA1	5′ AGTGGATCCGAGGACGTGCGGAACAAGCAG3′	
pdivIVA2	5′ AGTGAATTCCTCGTCGATCAGGAACCCGCG3′	
pcslAsc3	5′ AGTGAATTCTTCCTCACCTCCTTCGTGCCC3′	
pcslAsc4	5′ AGTAGATCTCGAGCCGCCGATCTGCTTGAG3′	

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are listed in Table 1. *S. c e c* strains were cultivated at 30°C on solid R2YE or MS agar or in TSB or YEME liquid medium, as described previously (21). *E. c* DH5α and ET12567 (*da dc dS*) were grown and transformed by standard methods (31). ET12567 was used to propagate unmethylated DNA for introduction into *S. c e c* by transformation or conjugation (25).

DNA manipulations. *S e ce* genomic DNA was isolated according to a protocol described previously (21). Plasmid preparation, digestion, and ligation followed standard methods (31). For Southern analysis, digoxigenin labeling of DNA probes, hybridization, and detection were performed according to the protocols provided by the manufacturer (Roche). Restriction enzymes and other molecular biology reagents were from commercial sources.

Plasmid constructs for gene replacement, gene complementation, gene fusions, and two-hybrid analysis. The *c A*$_{Sc}$ gene replacement plasmid pHL151 was constructed as follows. A 3.8-kb BglII-BglII fragment from *S. c e c* cosmid SCE20 containing *c A*$_{Sc}$ was amplified and subcloned into pOJIJ1 between the BglII and BamHI sites (21), yielding pBBE20 (data not published). pBBE20 was digested with BamHI and ligated with the BamHI *g* cassette from pHP45 Ω *g* (3), giving pHL151, in which a 756-bp internal BamHI fragment encoding amino acids (aa) 189 to 439 of the CslA$_{Sc}$ protein was replaced by the *g* cassette encoding hygromycin resistance. The 2.9-kb PvuII-BglII fragment from pBBE20 was also inserted between the BamHI and EcoRV sites of pSET152 (21) to yield pHL155 for complementation of the *c A*$_{Sc}$ mutant. pHL155 has the ΦC31 phage integration function region and can therefore integrate into the *S. c e c* chromosome.

To translationally fuse *egf* to the 5′ end of *c A*$_{Sc}$, the 551-bp promoter region of *c A*$_{Sc}$ was amplified from genome DNA using the primers pcslAsc5 and pcslAsc6 and then digested with BamHI and NdeI and cloned between the BglII and NdeI sites of pHL117, which has the ΦC31 phage integration function region and can integrate into the *S. c e c* chromosome (23), generating pHL177. Then, the NdeI-NotI *egf* gene fragment excised from pHL167 (data not published) was ligated between the NdeI and NotI sites of pHL177, generating pHL178. The whole *c A*$_{Sc}$ gene was amplified from genome DNA using the primers pcslAsc7 and pcslAsc8, digested with BglII and NotI, and cloned between the BglII and NotI sites of pHL178, generating pHL179, in which the enhanced green fluorescence protein (EGFP) was translationally fused to the N terminus of CslAsc in frame and a linker (GSGGSG) was added between EGFP and the CslA$_{Sc}$ protein.

To construct a transcriptional fusion of the *a* gene cluster promoter to *egf*, the PCR product generated using the primers pramS1 and pramS2 was digested with BglII and NdeI and cloned between the BglII and NdeI sites of pHL117 (23). This generated pHL171, which contains the major promoter of the *a* gene cluster, Pi *a* (20).

pKF59 (a gift from Klas Flardh) is an integrative plasmid containing a *d IVA-egf* translational fusion gene (15). It was digested with StuI and cloned at the SmaI site of pOJ260 (2). This generated pHL175, which has an RP4-derived origin of transfer (*T*) region to facilitate *E. c -S e ce* conjugation.

Plasmids for bacterial two-hybrid analysis were constructed as follows. The *d IVA* gene PCR product generated using the primers pdivIVA1 and pdivIVA2 was digested with BamHI and EcoRI and cloned between the BamHI and EcoRI sites of the "target" plasmid, pTRG, generating plasmid pHL172. The glycosyltransferase-encoding fragment of *c A*$_{Sc}$ was amplified by PCR using the primers pcslAsc3 and pcslAsc4. The product was digested with BamHI and EcoRI and cloned between the same sites of the "bait" plasmid, pBT, giving pHL173.

***cslA*$_{Sc}$ gene replacement and genetic complementation.** pHL151 was introduced into M145 from ET12567/pUZ8002 by *E. c -S e ce* intergeneric conjugation as described previously (21). Apr^r Hyg^r exconjugants were transferred to selective agar medium containing nalidixic acid and hygromycin, allowed to sporulate, and then transferred to MS agar without antibiotic selection. Spores from the MS agar were then plated on MS agar containing hygromycin to obtain single colonies, which were replicated on agar media containing only hygromycin and both apramycin and hygromycin. Five Apr^s Hyg^r colonies were obtained, all with the same phenotype on MS, MM, and R2YE agars. To confirm gene replacement in these isolates, Southern blots were performed with a digoxigenin-labeled 3.8-kb BglII/PvuII fragment containing *c A*$_{Sc}$ as a probe. Complementation of the mutant was achieved by introducing pHL155 by conjugation. The vector pSET152 (21) was also introduced into the mutant and M145 to provide negative controls.

Microscopy. Live hyphae were examined by light microscopy as described previously (17, 21). For fluorescence microscopy, the samples were prepared as described above and the hyphae were mounted in 50% glycerol before observation. For staining hyphae or DNA, samples of cells were fixed three times with methanol and then washed with water and covered with poly-L-lysine solution (21). The cells were stained with propidium iodide (PI) or calcofluor work solution for about 10 or 5 min, respectively, and then washed with water and mounted with 50% glycerol before observation under the Olympus BX51 fluorescence microscope (camera, Pixera penguin 150CL).

For transmission electron microscopy (TEM) analysis, the mycelium and spores were harvested from R2YE agar. The cells were fixed with freshly made 2% glutaraldehyde in phosphate buffer for 2 hours and washed with PBS three times; then, the samples were fixed with 1% osmium tetroxide and washed with PBS three times. The cells were embedded in Spuurr resin after dehydration with ethanol. Sections (ca. 50 to 60 nm thick) were examined with an H7650/Hitachi-H-7000 FA transmission electron microscope (21).

For scanning electron microscopy (SEM), about 5- by 5- or 10- by 10-mm pieces made from coverslips were laid flat on R2YE agar before the agar solidified. A little medium was added to the edges of the coverslips, and spores were inoculated at the edges. After sporulation, the samples were fixed with 1% osmium tetroxide for 3 hours. The samples were then examined with the JSM-6390 scanning electron microscope after being sputter coated with gold.

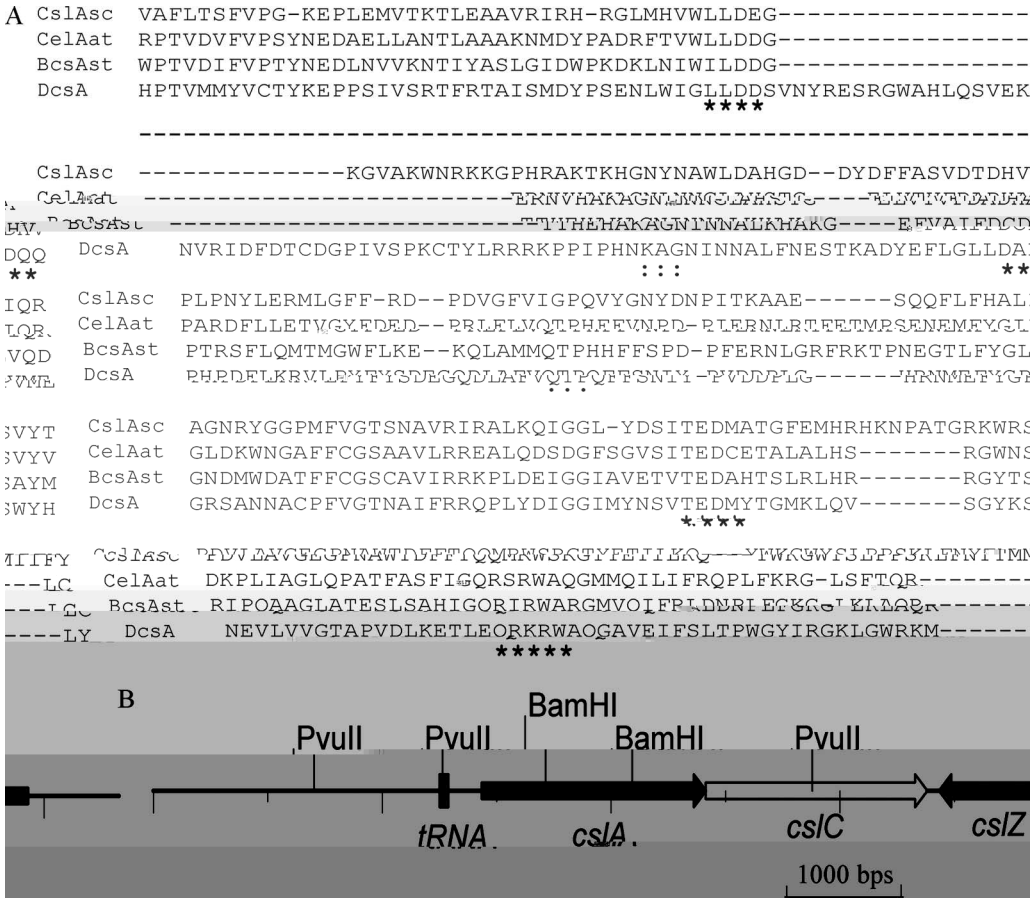

FIG. 1. The *c A*$_{Sc}$ gene and comparison of its product with related proteins. (A) Alignment of the highly conserved U_1, U_2, U_3, and U_4 regions in the central cytoplasmic loop of the predicted *S. c e c* cellulose synthase-like protein with cellulose synthase protein sequences from *Ag bac e efac e* (CelA$_{At}$; NP_533806), *S. e e ca* serovar Typhimurium (BcsA; CAC86199), and *D c e d c de* (DcsA; AAF00200). The D, D, and D_{35}QXXRW motifs; KAG motif; and QTP motif are indicated by asterisks, colons, and dots, respectively. The dashed line between the first and second blocks indicates that some regions that do not contain conserved motifs are omitted. (B) The *c* genes in *S. c e c* . Compared with the operon of *S. e e ca* serovar Typhimurium/*E. c* , *S. e ce* does not have the *bc B* gene. The *c* genes also exist in *S. a e* .

Two-hybrid bacterial analysis. The BacterioMatch II two-hybrid system (Stratagene) was used to detect protein-protein interactions. The plasmid pairs were used to cotransform the XL1-Blue MRF′ reporter strain on M9 salt agar without 3-amino-1,2,4-triazole (3-AT). Colonies were then restreaked on M9 salt agar containing 5 mM 3-AT at 37°C for 24 h for the first detection of interactions. For confirmation, the colonies were cultured on dual-selective medium containing 5 mM 3-AT and streptomycin as described in the manual.

Puri cation of chaplin and rodlin proteins. For purification of chaplin and rodlin proteins, the wild-type strain M145 was grown on cellophane discs on the surface of MS agar medium. After 4 days of growth, the hyphae were harvested and extracted using trifluoroacetic acid as described previously (8, 13).

RESULTS

Bioinformatic analysis of the "cellulose synthase-like (*cslA*$_{Sc}$) gene in *S. coelicolor*. The *S. c e c* genome contains only one gene (SCO2836) encoding a cellulose synthase-like protein. This protein has weak similarity to the catalytic subunits of cellulose synthases from bacteria and plants. We therefore named SCO2836 *c A*$_{Sc}$ (*c*ellulose ynthase- ike gene of *S. c e c*). Orthologs of *c A*$_{Sc}$ were also found in *S. e ce a e* MA-4680 (19) and *S. e ce a c a ge* (35). Structure prediction suggested that the protein has seven transmembrane domains, with the N terminus being cytoplasmic and the C terminus outside the membrane. The large cytoplasmic domain in the middle region contains domains that match entries pfam03552 (cellulose synthase) (E value, 2e−9), from aa 338 to 451, and PF00535 (glycosyltransferase family 2) (E value, 1e−7), from aa 166 to 342 (26). The central active site of cellulose synthases is thought to comprise four particularly conserved subregions (U_1 through U_4) that contain the conserved Asp (D) residues (in U_1 to U_3) and the motif QXXRW (in U_4) (32). These are considered essential for substrate binding and catalysis (32), and they are present in CslA$_{Sc}$ (Fig. 1A). The A residue of a normally conserved KAG motif is replaced by H and a QTP motif is not conserved in CslA$_{Sc}$, but the functions of these two motifs in cellulose synthase are not yet established.

In addition, *c A*$_{Sc}$ and its downstream genes are organized in a way similar, but not identical, to the *bc ABZC* cellulose biosynthesis operons in enteric bacteria (41), which encode a cellulose synthase catalytic subunit (*bc A*), a cellulose synthase regulatory subunit (*bc B*), an *e d* -1,4-β-glucanase (*bc Z*), and an oxidoreductase (*bc C*) (41). In *S. c e c* , the immediately downstream gene SCO2837 encodes a secreted copper metal-

loenzyme that in vitro oxidizes simple alcohols to aldehydes and reduces dioxygen to hydrogen peroxide (37), while the next gene (SCO2838, which converges on SCO2836 and SCO2837) encodes a putative *e d* -1,4-β-glucanase (Fig. 1B). There is no homolog of *bc B*, which encodes a regulatory subunit proposed to stimulate/activate BcsA upon binding of cyclic di-GMP (30).

Inactivation of *cslA*$_{Sc}$ affects mycelial differentiation. To address the function of *c A*$_{Sc}$, a mutant was constructed by replacing a BamHI fragment internal to the gene with a hygromycin resistance cassette. The mutant (named XE) produced a very sparse aerial mycelium only after 8 days on R2YE medium, while the wild type produced abundant aerial mycelium after 4 days (Fig. 2A). However, unlike many aerial-mycelium-deficient (*b d*) mutants, XE retained the ability to produce pigmented antibiotics under the conditions tested. In liquid culture, such as TSB or YEME, the mutant did not form the large aggregates (pellets) typical of the wild type (Fig. 2B), though phase-contrast microscopy confirmed that apparently normal branching mycelial material was abundant.

Light microscopy and SEM showed that sporulating hyphae branched from the supporting hyphae at unusually close intervals compared with the wild-type M145 (Fig. 3A). Fluorescence microscopy of samples stained with PI showed that the spores of the mutant all contained normal amounts of DNA (Fig. 3B). This indicated that DNA was replicated and segregated normally, although multiple sporulating hyphae developed.

Further analysis by TEM revealed that the mutant had subtle changes in its cell wall and septum structure and spore shape (Fig. 4). The laminated appearance of wild-type septa and lateral walls was not seen in the mutant (Fig. 4B), and the walls of XE spores showed many small deformations compared with the smooth oval contour of the wild-type cell wall (Fig. 4C). Thus, the *c A*$_{Sc}$ gene indeed contributes, directly or indirectly, to normal cell wall structure.

To complement the mutant, we constructed pHL155, in which a 2.9-kb fragment containing the intact *c A*$_{Sc}$ open reading frame, along with 0.5-kb upstream and 0.2-kb downstream sequences, was cloned into pSET152. The introduction of pHL155 into XE restored a wild-type appearance to colonies on solid media (Fig. 2C) and pellet formation in liquid culture, confirming that the mutant phenotype was the direct result of *c A*$_{Sc}$ inactivation and ruling out phenotypically significant polarity effects of the disruption cassette on the expression of the possibly cotranscribed downstream gene SCO2837.

***cslA*$_{Sc}$ is involved in the accumulation of β-glucan at mycelial tips.** Group 2 glycosyltransferase and cellulose synthase-like proteins catalyze the biosynthesis of various β-linked polysaccharides, such as cellulose, chitin, and curdlan (4, 10). To examine the distribution of β-glucan in *S. c e c* and the XE mutant, we used calcofluor white, a fluorescent dye that specifically binds β-1,4 polysaccharides, such as chitin and cellulose. The vegetative hyphae were stained with calcofluor white after growth on MS solid medium for 16 h. The mycelium of the wild type was weakly fluorescently stained by the dye, but nearly all hyphal tips showed much more intense fluorescence (Fig. 4A). In the XE mutant, the tip-located brighter loci were abolished (Fig. 4A), although some small fluorescent foci were observed along the hyphae. These results showed that *c A*$_{Sc}$ is involved in the preferential biosynthesis of certain β-1,4-linked polysaccharides at hyphal tips.

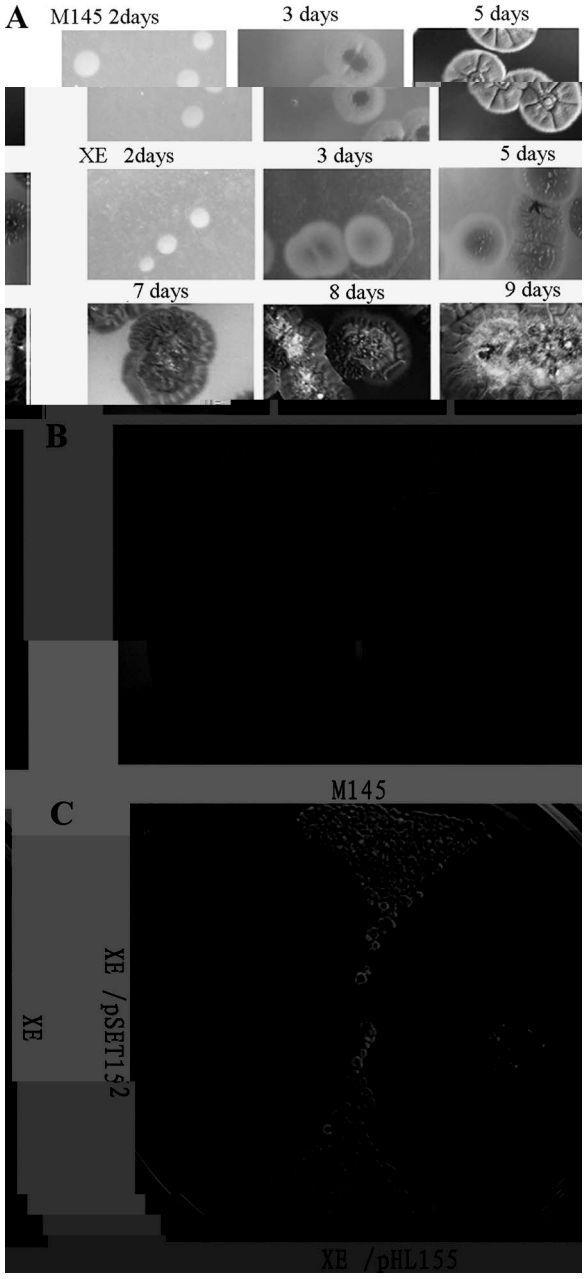

FIG. 2. Disrupting *c A*$_{Sc}$ in *S. c e c* . (A) Aerial-mycelium formation by the mutant was severely delayed on solid R2YE medium compared with that of the wild-type M145. (B) Clumping, and resulting sedimentation, of vegetative hyphae in liquid culture (TSB; 24 h) was less pronounced in the *c A* mutant than in the wild-type M145. Mycelial clumps of M145 sedimented to the bottom quickly when the bottle was allowed to stand (right). (C) Genetic complementation of the *c A* mutant. The mutant containing pHL155 formed normal aerial hyphae on solid R2YE medium, but the mutant containing the empty plasmid pSET152 did not. The wild-type M145, XE, and XE/pSET152 were used as controls.

Fluorescently tagged CslA$_{Sc}$ protein accumulates at hyphal tips. To examine the temporal and spatial pattern of CslA$_{Sc}$ abundance, the EGFP gene was fused in frame to the 5′ end of

c A$_{Sc}$. A linker coding sequence was added between EGFP and *c A*$_{Sc}$, and the promoter region of the *c A*$_{Sc}$ gene was cloned before the *egf* gene. The generated reporter plasmid, pHL179, integrated into the chromosome by site-specific re-

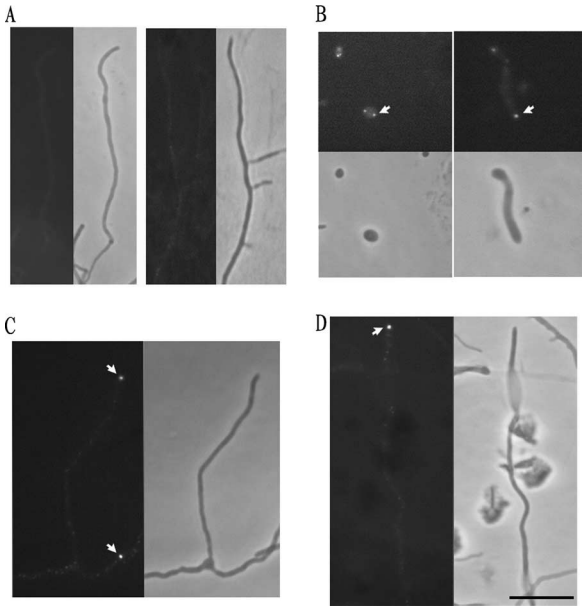

FIG. 5. Localization of $CslA_{Sc}$-EGFP fusion protein. The $CslA_{Sc}$-EGFP fusion protein was localized at hyphal tips. (A) Controls; aerial hypha (left) and vegetative hyphae (right) of wild-type M145 without an *egf* fusion. (B, C, and D) M145/pHL179. (B) Left, spore germination; right, germ tube elongation. (C) Vegetative hyphae. (D) Aerial hyphae. (A, C, and D) Left, fluorescent images; right, phase-contrast images. (B) Top, fluorescent images; bottom, phase-contrast images. The arrows indicate the fluorescent foci at tips. The strains were grown on MS medium. Bar, 10 μm.

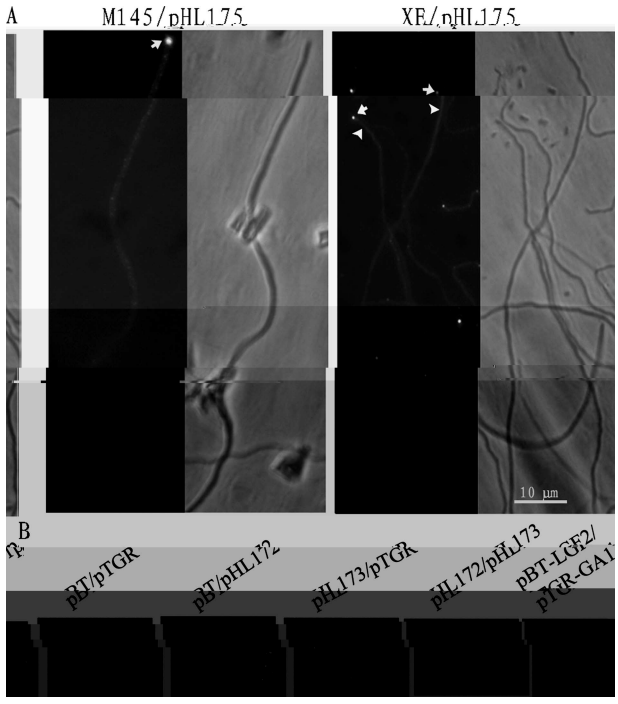

FIG. 6. Interaction between $DivIVA_{Sc}$ and $CslA_{Sc}$. (A) Localization of $DivIVA_{Sc}$-EGFP protein in wild-type M145 and XE. The $DivIVA_{Sc}$-EGFP protein was mostly at hyphal tips, with some smaller foci along the hyphae. In the XE mutant, the foci were also mostly at the tips (arrows), but in some aerial hyphae, the fluorescence was dispersed through the cell (arrowheads). (B) A bacterial two-hybrid experiment showed that DivIVA interacted with the glycosyltransferase domain of the $CslA_{Sc}$ protein. The reporter strain XL1-Blue MRF′ with different plasmid pairs was grown on double-selective indicator plates containing 3-AT and streptomycin. Experimental construct, pHL172/pHL173; positive control, pBT-LGF2/pRGT-GAL11P; negative controls, pBT/pRGT, pHL173/pTRG, and pBT/pHL172.

plasmid, pTRG, to yield pHL172. As shown in Fig. 6B, the strain with pHL173 and pHL172 (XL1-Blue MRF′/pHL173/pHL172) and the positive control strain (XL1-Blue MRF′/pBT-LGF2/pRGT-GAL11P) grew on the double-selective indicator plate containing 5 mM 3-AT and streptomycin, while the negative control strains (XL1-Blue MRF′/pBT/pRGT, XL1-Blue MRF′/pHL173/pTRG, and XL1-Blue MRF′/pBT/pHL172) did not grow, suggesting that the glycosyltransferase domain of the $CslA_{Sc}$ protein interacted with the DivIVA protein directly.

Examination of the expression of amphipathic aerial-hyphal surface proteins in the mutant. The aerial hyphae of *S. c e - c* are covered by different surface-active proteins and peptides (chaplins, rodlins, and SapB) that facilitate aerial growth (8, 13, 38). The addition of such molecules to many kinds of *b d* mutants can result in partial suppression of the morphological defect. We determined whether the XE mutant could be phenotypically suppressed in this way. We first extracted the chaplin and rodlin proteins from wild-type M145 cultured on MS medium. The extract was expected to include both chaplin and rodlin proteins (8, 9, 13), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis verified that proteins of the expected electrophoretic mobility were present. The XE mutant was cultured for a day on R2YE solid medium, and then the extract was added. After 2 days, the mycelium formed aerial hyphae (Fig. 7A), suggesting that the chaplin and/or rodlin protein could restore aerial growth to the XE mutant. We then investigated the expression of genes for chaplins, rodlins, and SapB production in the mutant, using transcriptional fusions of the *egf* reporter gene to the structural genes *c C*, *c H*, *d A* (8), and P1 *a* (pHL171). The P1 promoter is the principal promoter of the *a CSAB* operon in *S e ce da* (20), and *a S* encodes the precursor of SapB (22). Fluorescence microscopy revealed that *c C*, *c H*, *d A*, and P1 *a* were comparably expressed in the aerial hyphae of both strains (Fig. 7B). The fluorescence of *c H* and *d A* was strong, and that of *c C* was weak, which matched the previous observations (8, 9). These data implied that replacement of the *c* A_{Sc} gene did not interfere with the aerial-mycelium-specific expression of these genes.

DISCUSSION

The growth of *S e ce* aerial hyphae from the vegetative mycelium requires the solution of several problems, two of which have been particularly studied: growth away from water and the maintenance of apical dominance to permit rapid and efficient growth into the air. The first gene shown to be very important for tip growth, *d IVA* (15), encodes a tropomyosin-like protein that can form an alpha-helical coiled-coil structure (34). *d IVA* could not be deleted unless another copy of the gene was present, and ectopic overexpression of DivIVA caused hyphal tips to appear swollen (15). The targeting of

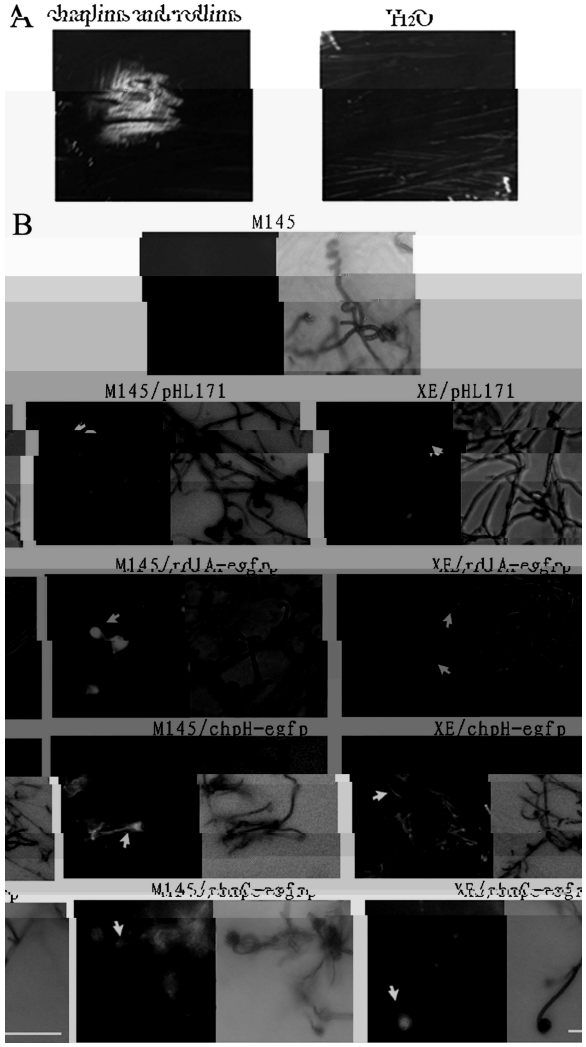

FIG. 7. Extracellular complementation of the XE mutant by a chaplin/rodlin extract and analysis of the expression of chaplin and rodlin hydrophobins in XE by EGFP fusions. (A) Extracellular chaplins and rodlins promoted the XE mutant to form aerial mycelium. The extracted chaplin and rodlin material or control material (water) was spotted on the lawns after 1 day of mycelial growth on R2YE solid medium. The extract was seen to promote the mutant to form aerial hyphae 1.5 days after addition. Water had no effect. The photographs were captured with a Fuji FinePix S602 2 days after addition. (B) EGFP fluorescence expressed from pHL171 derivatives carrying the P1 *a -egf* , *d A-egf* , *c H-egf* , and *c C-egf* fusions in aerial hyphae of both the wild type and the XE mutant. EGFP expression was observed in aerial hyphae of both strains containing the P1 *a -egf* , *d A-egf* , *c H-egf* , and *c C-egf* reporters. The spores were inoculated on solid R2YE medium, and aerial hyphae attached to the surfaces of coverslips were analyzed 2 days after inoculation in the cases of M145 and its derivatives and after 7 days in the cases of XE and its derivatives. Wild-type M145 without EGFP was set up as the control. Bars, 10 μm.

DivIVA is a typical feature of alpha-helical coiled-coil proteins (11). This suggests that DivIVA can act as a scaffold for the recruiting of related proteins to target sites, for example, to the growing tips or division sites (15, 16). Protein-protein interaction between $CslA_{Sc}$ and DivIVA suggested that the tip location of $CslA_{Sc}$ might be governed by the tip-located DivIVA protein. This might be particularly important during the emergence of aerial hyphae. Given the diverse organization of cellulose synthase complexes ("rosette terminal complexes") in different organisms and developmental contexts (33), it will be of interest to investigate the assembly of such complexes during vegetative and aerial growth of *S. c e c* and their effects on the pattern of assembly of β-glucan fibrils. The hyphal tip location of $CslA_{Sc}$ and the β-glucan accumulated under its direction, the association of $CslA_{Sc}$ with DivIVA, and the severe delay of aerial-hyphal formation in the *c* A_{Sc} mutant all indicate a role for $CslA_{Sc}$ in hyphal tip growth. As a working model, we propose that the multiple transmembrane regions of $CslA_{Sc}$ enable its integration into the cell membrane and that the interaction of its cytoplasmic segment with DivIVA precisely locates it in the tip position for the correctly positioned synthesis of β-glucan-containing polysaccharide. Why does *S e ce* need β-glucan-containing polysaccharides, which are not a component of the gram-positive bacterial cell wall? Perhaps the β-glucan fibers act as a "bandage" to wrap the hyphal tips, which are vulnerable to loss of integrity because of the continuous remodeling of the growing cell wall. The proposed bandage might help to maintain cell integrity, flexibility, and rigidity under different circumstances, notably including growth into the air. During aerial hyphal development, the hyphae must emerge from an aqueous environment and achieve cell wall growth in the air, using chaplins, rodlins, and SapB as hydrophobins (8, 9, 13, 39). The delayed aerial-hyphal formation of the XE mutant was phenotypically suppressed by adding an extract highly enriched for chaplins and rodlins, yet genes for each class of molecule showed the normal pattern of spatially specific expression in aerial hyphae. This suggests that the defect in aerial growth of the mutant is at least partly a quantitative deficiency in the hydrophobin-like molecules at the time of initial emergence. The hydrophobic β-glucan fibers may help to catalyze the correct assembly of the aerial-hyphal surface components in an (indirectly) self-inducing loop, since it appears that emergence into the air is a signal for increased chaplin and rodlin gene expression. In plants, cellulose synthase and cellulose synthase-like proteins cooperate with the cytoskeleton to control cell shape and to shape growth (24). Movement of the cellulose synthase complex is also coincident with the cortical microtubules (24, 27). The colocation of $CslA_{Sc}$ and DivIVA, and the protein-protein interaction between them, is to some extent a prokaryotic equivalent of the plant cellulose synthase-cytoskeleton correlation, maintaining apical dominance and guiding β-glucan synthesis and deposition.

In addition to its slow aerial-mycelium development, the *c* A_{Sc} mutant appeared to have abnormalities in the lateral and cross walls of vegetative hyphae and in its spore walls. These abnormalities may largely be manifestations of partially uncoordinated synthesis and maturation of the cell wall at tips lacking the β-glucan bandage. The structure of the lateral walls may provide a template for the precise structure of cross walls, accounting for the abnormal cross walls of the mutant.

Finally, the more dispersed growth of the mutant in liquid media may indicate that the β-glucan fibers play a role in the aggregation of clumps. This could be of interest in industrial fermentations of *S e ce* species for antibiotics and other

valuable secondary metabolites, in which the degree of clumping has profound rheological effects.

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REFERENCES

1. **Bentley, S. D., K. F. Chater, A. M. Cerdeno-Tarraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C. H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O'Neil, E. Rabbinowitsch, M. A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, S. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill, and D. A. Hopwood.** 2002. Complete genome sequence of the model actinomycete *S e ce c e c* A3(2). Nature **417:**141–147.
2. **Bierman, M., R. Logan, K. O'Brien, E. T. Seno, R. Nagaraja Rao, and B. E. Schoner.** 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *E c e c a c* to *S e ce* spp. Gene **116:**43–49.
3. **Blondelet-Rouault, M.-H., J. Weiser, A. Lebrihi, P. Branny, and J.-L. Pernodet.** 1997. Antibiotic resistance gene cassettes derived from the [pi] interposon for use in *E. c* and *S e ce* . Gene **190:**315–317.
4. **Campbell, J. A., G. J. Davies, V. Bulone, and B. Henrissat.** 1997. A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. Biochem. J. **326:**929–939.
5. **Chater, K. F.** 1993. Genetics of differentiation in *S e ce* . Annu. Rev. Microbiol. **47:**685–713.
6. **Chater, K. F.** 1989. Multilevel regulation of *S e ce* differentiation. Trends Genet. **5:**372–377.
7. **Chater, K. F., and R. Losick.** 1997. Mycelial life style of *S e ce c e - c* A3(2) and its relatives, p. 149–182. *I* J. A. Shapiro and M. Dworkin (ed.), Bacteria as multicellular organisms. Oxford University Press, New York, NY.
8. **Claessen, D., R. Rink, W. de Jong, J. Siebring, P. de Vreugd, F. G. Boersma, L. Dijkhuizen, and H. A. Wosten.** 2003. A novel class of secreted hydrophobic proteins is involved in aerial hyphae formation in *S e ce c e c* by forming amyloid-like fibrils. Genes Dev. **17:**1714–1726.
9. **Claessen, D., H. A. B. Wosten, G. van Keulen, O. G. Faber, A. M. C. R. Alves, W. G. Meijer, and L. Dijkhuizen.** 2002. Two novel homologous proteins of *S e ce c e c* and *S e ce da* are involved in the formation of the rodlet layer and mediate attachment to a hydrophobic surface. Mol. Microbiol. **44:**1483–1492.
10. **Coutinho, P. M., E. Deleury, G. J. Davies, and B. Henrissat.** 2003. An evolving hierarchical family classification for glycosyltransferases. J. Mol. Biol. **328:**307–317.
11. **Edwards, D. H., H. B. Thomaides, and J. Errington.** 2000. Promiscuous targeting of *Bac b* cell division protein DivIVA to division sites in *E c e c a c* and fission yeast. EMBO J. **19:**2719–2727.
12. **Elliot, M. A., M. J. Buttner, and J. R. Nodwell.** 2007. Multicellular development in *S e ce* , p. 419–438. *I* D. E. Whitworth (ed.), Myxobacteria: multicellularity and differentiation. ASM Press, Washington, DC.
13. **Elliot, M. A., N. Karoonuthaisiri, J. Huang, M. J. Bibb, S. N. Cohen, C. M. Kao, and M. J. Buttner.** 2003. The chaplins: a family of hydrophobic cell-surface proteins involved in aerial mycelium formation in *S e ce c e - c* . Genes Dev. **17:**1727–1740.
14. **Favery, B., E. Ryan, J. Foreman, P. Linstead, K. Boudonck, M. Steer, P. Shaw, and L. Dolan.** 2001. KOJAK encodes a cellulose synthase-like protein required for root hair cell morphogenesis in *A ab d* . Genes Dev. **15:**79–89.
15. **Flardh, K.** 2003. Essential role of DivIVA in polar growth and morphogenesis in *S e ce c e c* A3(2). Mol. Microbiol. **49:**1523–1536.
16. **Flardh, K.** 2003. Growth polarity and cell division in *S e ce* . Curr. Opin. Microbiol. **6:**564–571.
17. **Flardh, K., K. C. Findlay, and K. F. Chater.** 1999. Association of early sporulation genes with suggested developmental decision points in *S e - ce c e c* A3(2). Microbiology **145:**2229–2243.
18. **Hopwood, D. A.** 1999. Forty years of genetics with *S e ce* : from through to *c* . Microbiology **145:**2183–2202.
19. **Ikeda, H., J. Ishikawa, A. Hanamoto, M. Shinose, H. Kikuchi, T. Shiba, Y. Sakaki, M. Hattori, and S. Omura.** 2003. Complete genome sequence and comparative analysis of the industrial microorganism *S e ce a e* - . Nat. Biotechnol. **21:**526–531.
20. **Keijser, B. J. F., G. P. van Wezel, G. W. Canters, and E. Vijgenboom.** 2002. Developmental regulation of the *S e ce da a* genes: involvement of RamR in regulation of the *a CSAB* operon. J. Bacteriol. **184:**4420–4429.
21. **Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood.** 2000. Practical *S e ce* genetics. John Innes Foundation, Norwich, United Kingdom.
22. **Kodani, S., M. E. Hudson, M. C. Durrant, M. J. Buttner, J. R. Nodwell, and J. M. Willey.** 2004. The SapB morphogen is a lantibiotic-like peptide derived from the product of the developmental gene *a S* in *S e ce c e c* . Proc. Natl. Acad. Sci. USA **101:**11448–11453.
23. **Li, W., X. Ying, Y. Guo, Z. Yu, X. Zhou, Z. Deng, H. Kieser, K. F. Chater, and M. Tao.** 2006. Identification of a gene negatively affecting antibiotic production and morphological differentiation in *S e ce c e c* A3(2). J. Bacteriol. **188:**8368–8375.
24. **Lloyd, C., and J. Chan.** 2004. Microtubules and the shape of plants to come. Nat. Rev. Mol. Cell Biol. **5:**13–22.
25. **MacNeil, D. J., J. L. Occi, K. M. Gewain, T. MacNeil, P. H. Gibbons, C. L. Ruby, and S. J. Danis.** 1992. Complex organization of the *S e ce a e* genes encoding the avermectin polyketide synthase. Gene **115:** 119–125.
26. **Marchler-Bauer, A., J. B. Anderson, P. F. Cherukuri, C. DeWeese-Scott, L. Y. Geer, M. Gwadz, S. He, D. I. Hurwitz, J. D. Jackson, Z. Ke, C. J. Lanczycki, C. A. Liebert, C. Liu, F. Lu, G. H. Marchler, M. Mullokandov, B. A. Shoemaker, V. Simonyan, J. S. Song, P. A. Thiessen, R. A. Yamashita, J. J. Yin, D. Zhang, and S. H. Bryant.** 2005. CDD: a Conserved Domain Database for protein classification. Nucleic Acids Res. **33:**D192–D196.
27. **Paredez, A. R., C. R. Somerville, and D. W. Ehrhardt.** 2006. Visualization of cellulose synthase demonstrates functional association with microtubules. Science **312:**1491–1495.
28. **Richmond, T. A., and C. R. Somerville.** 2000. The cellulose synthase superfamily. Plant Physiol. **124:**495–498.
29. **Romling, U.** 2002. Molecular biology of cellulose production in bacteria. Res. Microbiol. **153:**205–212.
30. **Ross, P., H. Weinhouse, Y. Aloni, D. Michaeli, P. Weinberger-Ohana, R. Mayer, S. Braun, E. de Vroom, G. A. van der Marel, J. H. van Boom, and M. Benziman.** 1987. Regulation of cellulose synthesis in *Ace bac e* by cyclic diguanylic acid. Nature **325:**279–281.
31. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York, NY.
32. **Saxena, I. M., R. M. Brown, Jr., M. Fevre, R. A. Geremia, and B. Henrissat.** 1995. Multidomain architecture of beta-glycosyltransferases: implications for mechanism of action. J. Bacteriol. **177:**1419–1424.
33. **Saxena, I. M., and R. M. Brown, Jr.** 2005. Cellulose biosynthesis: current views and evolving concepts. Ann. Bot. **96:**9–21.
34. **Stahlberg, H., E. Kutejova, K. Muchova, M. Gregorini, A. Lustig, S. A. Muller, V. Olivieri, A. Engel, A. J. Wilkinson, and I. Barak.** 2004. Oligomeric structure of the *Bac b* cell division protein DivIVA determined by transmission electron microscopy. Mol. Microbiol. **52:**1281–1290.
35. **Sun, Y., X. Zhou, H. Dong, G. Tu, M. Wang, B. Wang, and Z. Deng.** 2003. A complete gene cluster from *S e ce a c a ge* NS3226 encoding biosynthesis of the polyether ionophore nanchangmycin. Chem. Biol. **10:** 431–441.
36. **van Keulen, G., H. M. Jonkers, D. Claessen, L. Dijkhuizen, and H. A. B. Wosten.** 2003. Differentiation and anaerobiosis in standing liquid cultures of *S e ce c e c* . J. Bacteriol. **185:**1455–1458.
37. **Whittaker, M. M., and J. W. Whittaker.** 2006. *S e ce c e c* oxidase (SCO2837p): a new free radical metalloenzyme secreted by *S e ce c e c* A3(2). Arch. Biochem. Biophys. **452:**108–118.
38. **Willey, J., J. Schwedock, and R. Losick.** 1993. Multiple extracellular signals govern the production of a morphogenetic protein involved in aerial mycelium formation by *S e ce c e c* . Genes Dev. **7:**895–903.
39. **Willey, J. M., A. Willems, S. Kodani, and J. R. Nodwell.** 2006. Morphogenetic surfactants and their role in the formation of aerial hyphae in *S e ce c e c* . Mol. Microbiol. **59:**731–742.
40. **Wong, H. C., A. L. Fear, R. D. Calhoon, G. H. Eichinger, R. Mayer, D. Amikam, M. Benziman, D. H. Gelfand, J. H. Meade, A. W. Emerick, et al.** 1990. Genetic organization of the cellulose synthase operon in *Ace bac e* . Proc. Natl. Acad. Sci. USA **87:**8130–8134.
41. **Zogaj, X., M. Nimtz, M. Rohde, W. Bokranz, and U. Romling.** 2001. The multicellular morphotypes of *Sa e a* and *E c e c a c* produce cellulose as the second component of the extracellular matrix. Mol. Microbiol. **39:**