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Transgenic expression of *Gluconacetobacter xylinus* strain ATCC 53582 cellulose synthase genes in the cyanobacterium *Synechococcus leopoliensis* strain UTCC 100

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Abstract We report the transfer of cellulose synthesis genes (*acsAB*Δ*C*) from the heterotropic alpha proteobacterium, *Gluconacetobacter xylinus* strain ATCC 53582 to a photosynthetic microbe (*Synechococcus leopoliensis* strain UTCC 100). These genes were functionally expressed in this cyanobacterium, resulting in the production of non-crystalline cellulose. Although the cellulose lacks the structural integrity of the product synthesized by *G. xylinus*, the non-crystalline nature of the cyanobacterial cellulose makes it an ideal potential feedstock for biofuel production.

Keywords Cyanobacteria · Cellulose · Biofuels · *Gluconacetobacter xylinus* · Functional expression · Transgenic · Cellulose synthase

Introduction

Cellulose, the most abundant biopolymer, is synthesized by bacteria, protists, and animals (Brown 1985; Ross et al. 1991; Blanton et al. 2000; Kimura et al. 2001). However, the photosynthetic conversion of CO_2 to biomass in the cell walls of plants and algae is the primary source of approximately 10^{11} tons of cellulose created and destroyed annually (Lynd et al. 2002; Hess et al. 1928). The abundance of plant-derived cellulose makes it a significant sink for CO_2 and an important industrial biopolymer for applications such as textiles, paper, construction materials, cardboard, rayon, cellophane, coatings, laminates, and optical films. Thus, cellulose biosynthesis has a significant impact on the environment and human economy.

Cellulose is widely considered the most viable feedstock for the large scale, renewable production of ethanol (US DOE 2006). For biofuel production, cellulose must be separated from other cell wall constituents. However, the cellulose of plant cell walls is intimately associated with lignin and hemicelluloses, forming a complex polymer composite that is exceptionally recalcitrant to mechanical and biological degradation (Himmel et al. 2007). Cellulose purification is both energetically expensive and environmentally unfriendly (Bajpai 2004). Additionally, the cultivation of cellulose crops, e.g. cotton for textiles or switchgrass for biofuels, entails the extensive use of arable land, fertilizers and pesticides as well as the consumption of fresh water for irrigation.

Microbial cellulose stands as a promising possible alternative to traditional plant sources. The α proteobacterium *Gluconacetobacter xylinus* is the most

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prolific of the cellulose producing microbes. The ATCC 53582 strain (Brown and Lin 1990) is capable of converting 50% of glucose supplied in the medium into an extracellular cellulosic pellicle (R. Malcolm Brown Jr., unpublished results). Although it possesses the same molecular formula as cellulose derived from plant sources, microbial cellulose has a number of distinctive properties that make it attractive for diverse applications. The cellulose synthesized by *G. xylinus* is "spun" into the growth medium as highly crystalline ribbons with exceptional purity, free from the contaminating polysaccharides and lignin found in most plant cell walls (Brown et al. 1976). The resulting membrane, or pellicle, is composed of cellulose with a high degree of polymerization (2,000–8,000) and crystallinity (60–90%) (Klemm et al. 2005). Contaminating cells are easily removed, and relatively little processing is required to prepare membranes for use. In its never-dried state, the cellulose membrane displays exceptional strength and is highly absorbent, holding hundreds of times its weight in water (White and Brown 1989). *Gluconacetobacter xylinus* cellulose is well suited as a reinforcing agent for paper and diverse specialty products (Shah and Brown 2005; Tabuchi et al. 2005; Czaja et al. 2006; Helenius et al. 2006). However, *G. xylinus* is heterotrophic. Therefore, its use for the production of low cost products and biofuels is not feasible.

The ideal cellulose producing organism would be able to synthesize cellulose of the quality and in the quantities observed in *G. xylinus*, have a photoautotrophic lifestyle, and possess the ability to grow with a minimum use of natural resources in environments unsuitable for agriculture. Cyanobacteria are capable of utilizing low photon flux densities for carbon fixation, withstanding hypersaline environments, tolerating desiccation, and surviving high levels of uv irradiation (Vincent 2000; Wynn-Williams 2000). Additionally, many species are diazotrophic (e.g. nitrogen-fixing) (Castenholz and Waterbury 1989). This combination of exceptional adaptive characteristics has made mass cultivation of cyanobacteria attractive for production of nutritional biomass, fatty acids, bioactive compounds, and polysaccharides (Cogne et al. 2005; Moreno et al. 2003; Kim et al. 2005).

Many species of cyanobacteria are capable of cellulose biosynthesis (Nobles et al. 2001). However, production levels are generally low and cyanobacterial cellulose often exists as part of a complex extracellular matrix (Nobles et al. 2001) making purification and digestion problematic. In this study, we sought to combine the prodigious cellulose biosynthetic capacity of *G. xylinus* with the photosynthetic ability of cyanobacteria. Toward this end, we report the functional expression of a partial cellulose synthase operon (*acsABΔC*) of *G. xylinus* in the unicellular cyanobacterium, *Synechococcus leopoliensis* strain UTCC 100 (synonym, *Synechococcus elongatus* strain PCC 7942).

Materials and methods

Bacterial strains and culturing conditions

The relevant bacterial strains and plasmids used in this study are listed in Table 1. *Synechococcus elongatus* strain PCC 7942 was obtained from the University of Toronto Culture Collection as *Synechococcus leopoliensis* strain UTCC 100. Cyanobacterial cultures were maintained in BGll medium (Bustos and Golden 1992) at 24–28 °C with 12 h light/dark cycles on a rotary shaker or on agar as previously described (Golden et al. 1987). When appropriate the growth medium of *S. leopoliensis* was supplemented with chloramphenicol at a concentration of 7.5 μg/mL. *E. coli* strains were grown in LB broth or agar with antibiotics at the following concentrations: ampicillin (50 μg/mL); chloramphenicol (25 μg/mL); and, tetracycline (12.5 μg/mL). *Gluconacetobacter xylinus* strains AY201 and ATCC 53582 were grown in SH medium as previously described (Shram and Hestrin 1954).

DNA manipulations

Genomic DNA was isolated from *S. leopoliensis* essentially as described previously (Golden et al. 1987), with the exception that DNA was ethanol precipitated rather than purified using glass fines. Plasmids were isolated using Qiagen miniprep kits. Restriction enzymes and T4 DNA ligase were purchased from Promega and used following the manufacturer's instructions. Agarose gels were routinely prepared and examined as previously described (Mantiatis et al. 1982). When more delicate handling

Table 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Escherichia coli		
S17-1	*recA pro hsdR RP4-2-Tc::Mu-Km::Tn7*; mobilizer strain	Simon et al. (1983)
DH5αMCR	F2 *mcrA* D(*mrr-hsdRMS-mcrBC*) f80d*lacZ*DM15 D(*lacZYA- argF*)U169 *deoR recA1 endA1 supE44* l2 *thi-1 gyrA96 relA1*	Bethesda Research Laboratories
XL10 Gold Kan^R	Tetr Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*q*ZΔM15* Tn*10* (Tetr) Tn5 (Kanr) Amy].	Stratagene, La Jolla, CA
Synechococcus leopoliensis strain UTCC 100	Coidentities, *Synechococcus elongatus* PCC 7942, *Anacystis nidulans* R2	The University of Toronto Culture Collection
NS::P_{lac}-acsABΔC	Transgenic strain of *S. leopliensis* with the *acsABΔC* from *Gluconacetobacter xylinus* strain ATCC 53582 inserted in neutral site II. *acsABΔC* is fused with a *lac* promoter.	This study
NS::cat	*S. leopoliensis* with a chloramphenicol acetyltransferase cassette incorporated in Neutral Site II.	This study
NS::abΔc7S	Sub-isolate of ::P_{lac}-*acsABΔC*	This study
Gluconacetobacter xylinus AY201	Derivative of *Gluconacetobacter. xylinus* ATCC 23769	Laboratory Stock
Gluconacetobacter xylinus strain ATCC 53582	Coidentity, *Gluconacetobacter xylinus* strain NQ5	Laboratory Stock
pUC19	Amp^r; cloning vector	Norander et al. (1983)
pIS311-9	Tet^r; HinDIII-BamHI *acsABΔC* fragment from *G. xylinus* strain ATCC 53582 cloned in pRK311	Inder Saxena, This Laboratory
pAM1573	Amp^r , Cam^r; NSII cargo vector, mobilizable by conjugation, for homologous recombination into the chromosome of *S. elongatus* PCC 7942	Susan Golden Texas A & M University
pSAB1	Amp^r; HindIII-BamHI fragment from pIS311-9 cloned in pUC19	This study
pSAB2	Amp^r, Cam^r; PvuII fragment from pSAB1 cloned in pAM1573	This study
pDS4101	Amp^r; ColK derived helper plasmid for mobilization	Finnegan and Sherratt (1982)

of DNA was required, visualization of bands was accomplished via agarose gels supplemented with 40 µL of 2 mg/mL crystal violet (CV) per 50 mL of 0.6% agarose. When using CV gels, DNA samples were run in loading buffer composed of 30% glycerol, 20mM EDTA, and 100 µg/mL CV. Using this procedure allowed direct visualization of DNA, thus eliminating exposure of DNA ethidium bromide and ultraviolet light. Unless otherwise noted, the transformation of chemically competent *E. coli* strains was performed as described previously (Chung and Miller 1993).

Construction of cargo plasmid pSAB2

A 5.2 kb BamHI-HindIII fragment from pIS311-9 containing a partial cellulose synthase operon (*acsABΔC*) from *G. xylinus* strain ATCC 53582 was ligated into the BamHI-HindIII sites of pUC19 to create pSAB1. A 7.9 kb PvuII fragment from pSAB1 containing the lac operon promoter/operator with a P_{lacZ}-*acsABΔC* fusion was ligated into the unique SmaI site of pAM1573 to create pSAB2.

Conjugation procedures

The cargo plasmid pSAB2 was mobilized to *S. leopoliensis* UTCC 100 via biparental matings with the *E. coli*, strain S17. Cultures of *S. leopoliensis* were grown to an OD_{750} of 0.4–0.6. A total of 1.5 mL aliquots were centrifuged (3 min, 8,000 rpm, RT), and the pellets were resuspended in 200 µL BGll growth medium. *E. coli* strains S17-1, S17-1(pAM1573) and S17-1(pSAB2) were grown

overnight at 37 °C in LB broth with chloramphenicol selection in plasmid-bearing strains. *E. coli* strains were harvested by centrifugation in an Eppendorf 5,415 C microcentrifuge (2 min, 5,000 rpm, RT). The pellets were washed twice with 1 mL of LB followed by gentle resuspension in H_2O. The resuspended *S. leopoliensis* cells were serially diluted from 10^{-1} to 10^{-5} in BG11 growth medium. The final volume of each serial dilution was 200 µL. Hundred microliters of aliquots from each *E. coli* strain were combined with each *S. leopoliensis* dilution. A total of 200 µL of *E. coli/S. leopoliensis* matings were spread on BGll plates supplemented with 5% LB. The plates were incubated without selection overnight, under the growth conditions described above. The plates were then underlaid with chloramphenicol as previously described (Golden et al. 1987). Putative exconjugate colonies were restreaked on BGll with chloramphenicol selection in order to obtain *S. leopoliensis* colonies free of *E. coli*. Since BGll growth medium has no fixed carbon source, restreaking on medium without supplementation results in the elimination of *E. coli* by starvation. However, in order to insure that exconjugates were free of contaminating *E. coli*, the *S. leopoliensis* colonies were restreaked on LB plates followed by overnight incubation at 37 °C and examination for the presence of *E. coli* colonies.

PCR screens for acsAB

DNA from *S. leopoliensis* colonies was prepared as follows: Individual colonies were transferred from plates to the wall of a 200 µL PCR tube with a sterile toothpick or pipette tip. The colonies were suspended in 100 µL of TE, pH 8.0 supplemented with 1% Triton and homogenized by vortexing. The cell suspension was incubated at 95 °C for 3.5 min and cooled to 4 °C. The suspension was extracted twice with equal volumes of chloroform. Phases were separated by centrifugation (5 min, 14,000 rpm, RT). The aqueous phase was collected for subsequent PCR screening.

A 1,084 bp fragment spanning the acsAB genes was amplified using the primers acsAB-Forward2—TGGCG TGGTGTCTATGAACTGTCTTT and acsAB-Reverse2—CGGATATACTGCTCGTTCAGCGTCAT. PCR screens were performed with Herculase Hotstart DNA polymerase (Stratagene): 1× Herculase reaction buffer (Stratagene), 200 µM each dNTP, 0.25 µM of each primer, 2.5 U 50 μL^{-1} Herculase Hotstart polymerase (Stratagene), and 4% DMSO. Templates were added to 5 µL reactions as follows: 1 uL of prepared colony solution, and 0.25 µl of strain ATCC 53582 genomic or plasmid DNA ($\sim$10 ng). Reaction conditions were set up according to the manufacturer's instructions for high GC targets.

RNA isolation and RT-PCR

RNA was isolated essentially as previously described (Boison et al. 2000). Briefly, 20 mL of cells were harvested by centrifugation (10 min, 1,744*g*, RT), washed with 1 mL of BG11 medium, collected by centrifugation in a microcentrifuge (5 min, 14,000 rpm, RT). After discarding the supernatant, the cell pellets were frozen in liquid N_2 and stored at −80 °C. Cell pellets were thawed briefly on ice followed by resuspension in 100 µL of TE, pH 8.0 and 350 µL of RLT buffer from Qiagen's RNeasy Kit (supplemented with β-mercaptoethanol). Cells were shaken intensely on a vortex for 2 min followed by incubation with pre-warmed acidic phenol (10 min, 65 °C) and vortexing again for 2 min. This was followed by three extractions with chloroform/isoamyl alcohol (24:1), brief vortexing, and centrifugation as above. The resulting aqueous layer was removed by pipetting and mixed with 0.71 volumes of ethanol. RNA was purified using the Qiagen RNeasy kit following the manufacturer's protocol for on column DNAse digestion.

Reverse transcription reactions were carried out with Superscript II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions, using 1 µL of DNAse-treated RNA. Subsequent PCR was carried out as described above using 0.4 µL of the reverse transcription products of both wild-type and NS::abΔc7S. Controls reactions were prepared using purified RNA from both strains.

Membrane preparations

Synechococcus leopoliensis cultures were grown in 1L volumes to an OD_{750} of 0.4–0.6. Cells were harvested by centrifugation (10 min, 3,470*g*, 4 °C). Pellets were resuspended in 5 mL of 20 mM K_2PO_4, pH 7.8 supplemented with 3% PMSF. Crude membranes were prepared as previously described (Norling et al. 1998). *Gluconacetobacter xylinus*

(AY201) was grown at 28 °C for 2 days in 200 mL of SH supplemented with 0.25% Celluclast (Novozyme, Inc, obtained as Sigma, C2730). Cells were collected by centrifugation (10 min, 3,470*g*, 4 °C), resuspended in 2 mL TME, and stored at −80 °C. Frozen cells were resuspended to a volume of 20 mL in TE and passed four times through a prechilled French pressure cell at 1,200 psi. Immediately following passage through the French Press, 20 µl of 3% PMSF was added to the lysate. The lysate was centrifuged (10 min, 3,470*g*, 4 °C) in order to remove cell debris. The supernatant was collected and centrifuged (30 min, 103,000*g* 4 °C). The resulting pelleted crude membranes were resuspended in 200 µL TME and frozen at −80 °C. Protein concentrations of membrane fractions were determined using the BioRad DC kit following the manufacturer's instructions.

Western analyses

Polyacrylamide gel electrophoresis was conducted as previously described (Laemmli 1970). For Western blots, protein samples were transferred from the gels to nitrocellulose (manufacturer) overnight at a constant current of 150 mA using a Bio-Rad Semi-Dry Transfer Cell. Western blots were performed using enhanced chemiluminescence (ECL) detection (Amersham, manufacturer's protocol). Anti-93 serum (Chen and Brown 1996) was used a 1:30,000 dilution. The goat-anti-rabbit serum was used at a dilution of 1:10,000.

Microscopy

Wildtype and mutant cells were collected in aliquots from liquid culture or as aqueous suspensions from plates. For TEM preparations, CBHI-gold labeling was performed essentially as described previously (Okuda et al. 1993) with the following exceptions: (a) 10 nm gold was used for the CBHI-gold complex; (b) rather than floating grids, 6 µl drops of enzyme complex were added to Formvar grids; and, (c) the enzyme complex and product were incubated for 1−3 min at RT. Grids were negatively stained with 2% uranyl acetate.

Cyanobacterial cellulose hydrolysis

Celluclast was diluted 1:1 in 20 mM Sodium Acetate Buffer, pH 5.2 and sterilized by passage through a 0.2 µm filter (Pall Life Sciences PN 4433). A total of 50 mL cultures of NS::cat and NS::abΔc7S were grown to stationary phase under the conditions described above. The OD_{750} of each culture was recorded. 40 mL of each culture was centrifuged (10 min, RT, 1,744*g*) in an IEC clinical centrifuge. The supernatants were discarded, wet weights recorded, and the pellets resuspended in 10 mM Sodium Acetate Buffer, pH 5.2. For buffer-only samples, 250 µL aliquots were transferred to 1.5 mL Eppendorf tubes. For Celluclast digestions, 247.5 µL of resuspended cells and 2.5 µL of sterilized Celluclast were combined in 1.5 µL eppendorf tubes. Enzyme blanks containing only Celluclast and buffer were also prepared. The tubes were placed on a rotisserie and incubated overnight at 30 °C with constant illumination.

Glucose assays

After overnight incubation, cells were pelleted by centrifugation (5 min, RT, 14,000 rpm) in a microcentrifuge. The supernatant was carefully pipetted off the cell pellet and retained for the glucose assay. Glucose concentration was measured using the hexokinase/glucose 6-phosphate dehydrogenase enzymatic assay (Sigma G3293). Assays were performed with 50–100 µl of supernatant per reaction following the manufacturer's instructions.

Results

Colony screening

DNA from exconjugate colonies determined to be free of *E. coli* was used in PCR screens for genomic integration of *acsAB*. Integration of the *G. xylinus acsABΔC* sequence into the neutral site (NSII) (Clerico et al. 2007) of the genome of *S. leopoliensis* is clearly demonstrated by PCR using primers which span segments of *ascAB* (Fig. 1). Colonies with positive PCR results were restreaked and examined for variations from the smooth colony morphology characteristic of wild type *S. leopoliensis*. Both rough and smooth colonies were isolated from transgenic colonies (Fig. 2). The presence of rough colonies is associated with cellulose biosynthesis in a number of bacterial strains (Römling and Lünsdorf 2004).

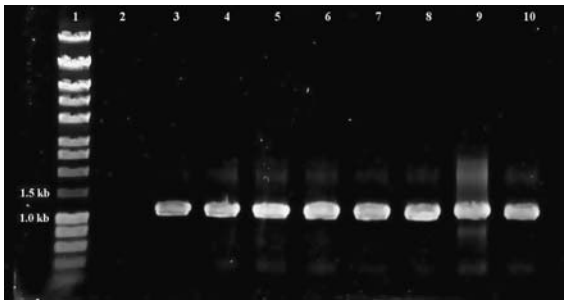

Fig. 1 Colony PCR screen of exconjugate *Synechococcus leopoliensis* UTCC 100. From left to right: Lane 1: DNA Ladder, Lane 2: wild type *S. leopoliensis*, Lanes 3–6: exconjugate colonies, Lane 9: strain ATCC 53582 DNA, and Lane 10: pSAB2 plasmid DNA

However, initial assays for cellulose production showed that the smooth colony transgenic isolates produced equal or greater amounts of cellulose than rough colonies. Rough and smooth colony clones were also screened for differences in liquid culture characteristics—*S. leopoliensis* cultures are homogenous when grown with moderate shaking. Rough colony clones grew as homogenous cultures and demonstrated a decreased growth rate as compared with the wild type. Smooth colony clones formed aggregates and exhibited growth rates similar to wild type *S. leopoliensis*. The formation of aggregates in liquid culture has been associated with polysaccharide production in bacteria (Deinema and Zevenhuizen 1971). Since they showed a higher growth rate and no apparent difference in cellulose production from the most productive rough colonies, smooth colony clones were used for later cellulose assays.

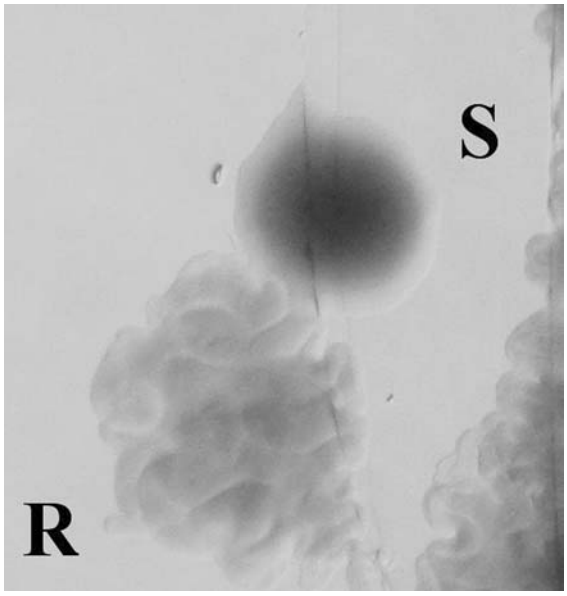

Fig. 2 Rough (**R**) and smooth (**S**) colonies of NS::abΔc7S grown on BG11 agar plates viewed through a dissecting microscope

Expression analyses

The *acsABΔC* fragment is under the expressional control of the *lac* promoter from *E. coli*. We predicted that this would result in low level constitutive expression of the *acsAB* coding region. Transcription of *acsAB* was verified by RT-PCR (Fig. 3). Western blots using an anti-AcsB antibody demonstrates the presence of a faint 93 kD band in both the AY201 lanes and *NS*::P_{lac}-*acsABΔC* lanes with no band of this size present in the UTCC100 wild type lane was observed (Fig. 4). However, there are multiple bands present in both wild type and mutant lanes. The *NS*::P_{lac}-*acsABΔC* lanes show two prominent bands of 45 and 42 kD. The 45 kD band is also present in the wild type. Since searches against the genomic database of *S. elongatus* PCC 7942 (http://genome.jgi-psf.org/finished_microbes/synel/synel.home.html) yield no sequences with significant similarity to AcsB, this likely represents nonspecific binding of the antibody. However, the 42 kD band is

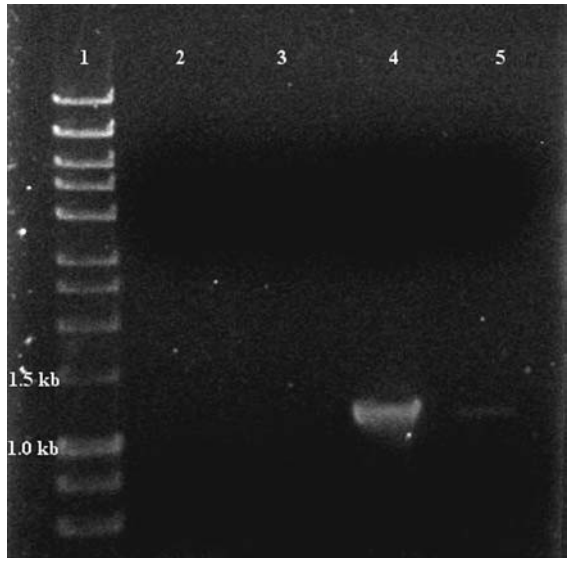

Fig. 3 Reverse Transcriptase PCR for the presence of *acsAB* transcripts in NS::cat and NS::abΔc7S. From left to right: Lane 1: DNA Ladder, Lane 2: UTCC100 with transcriptase, Lane 3: UTCC100 without transcriptase, Lane 4: NS::abΔc7S with transcriptase, and Lane 5: NS::abΔc7S without transcriptase

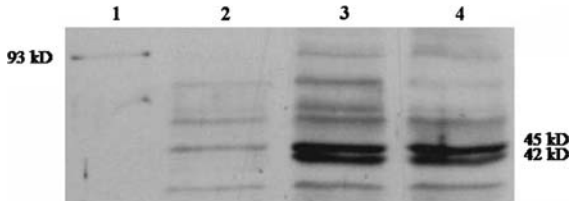

Fig. 4 Western Blot with an anti-AcsB antibody. From left to right membrane proteins from: Lane 1: *G. xylinus* AY201 and Lanes 2 and 3: *NS*::P_{lac}-*acsABΔC*

present only in transgenic *S. leopoliensis* lanes and may indicate products of protein degradation or processing. These data provide firm evidence that the AcsAB proteins of *G. xylinus* are expressed in the *S. leopoliensis* host cell.

TEM examination

Wild-type and transgenic strains of *S. leopoliensis* were labeled with CBHI-gold and negatively stained. Cell aggregates connected by copious amounts of an extracellular matrix were common in the NS::abΔc7S transgenic strain in contrast with wild type *S. leopoliensis* for which little extracellular material was detected (Fig. 5). The extracellular material secreted by NS::abΔc7S is labeled with CBHI-gold (Fig. 6) which positively identifies cellulose as a component of the matrix. All naturally occurring forms of cellulose identified to date are crystalline in nature. However, the lack of any fibrillar structure in the cellulosic material observed in NS::abΔc7S precludes crystallinity (Fig. 6).

Glucose assays

Data comparing the release of glucose from NS::cat and NS::abΔc7S by Celluclast is shown in Table 2. These data show that NS::abΔc7S produces moderately more background glucose than NS::cat, as indicated by the Sodium Acetate-only samples. However, NS::abΔc7S produces a significantly larger amount of cellulosic material than NS::cat.

Discussion

The cellulose synthase operon of *G. xylinus* was first characterized in 1994 (Saxena et al. 1994). Given this time frame, surprisingly little knowledge of the

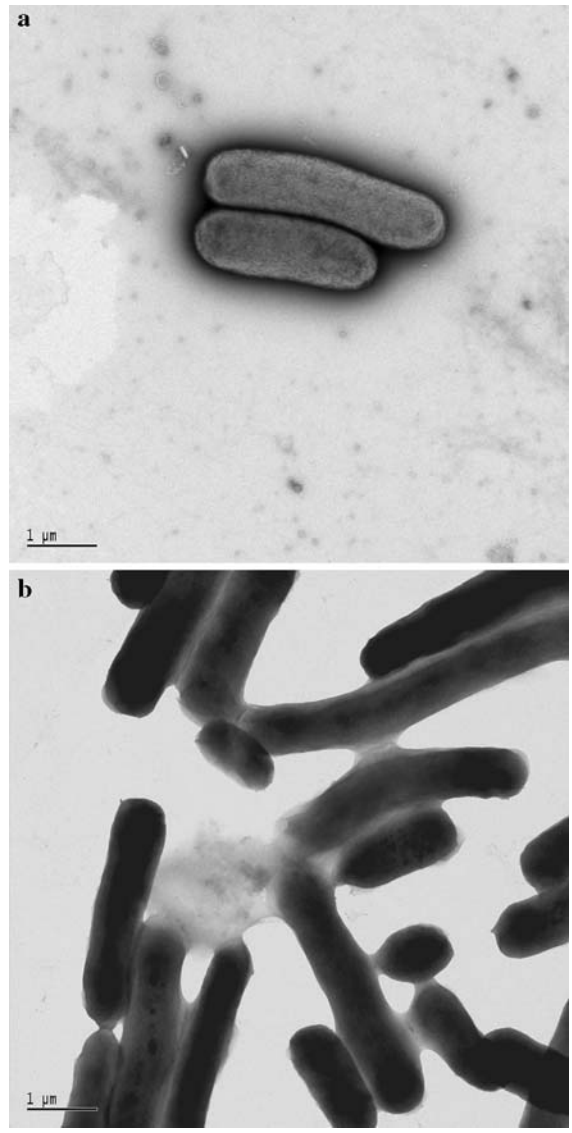

Fig. 5 Negatively stained grids showing extracellular materials in (**a**) wild type *S. leopoliensis* and (**b**) NS::abΔc7S

molecular mechanisms of microbial cellulose biosynthesis has been gained. A positive allosteric activator of cellulose biosynthesis, cyclic diguanylic acid (c-di-GMP) has been identified, as have the enzymes responsible for regulating its concentration—a diguanylate cyclase and its cognate phosphodiesterase (Ross et al. 1987; Tal et al. 1998). Of the four proteins encoded by the *acsABCD* operon, only AcsA (the catalytic subunit) has an experimentally demonstrated function (Lin and Brown 1989; Römling et al. 2005; Amikam and

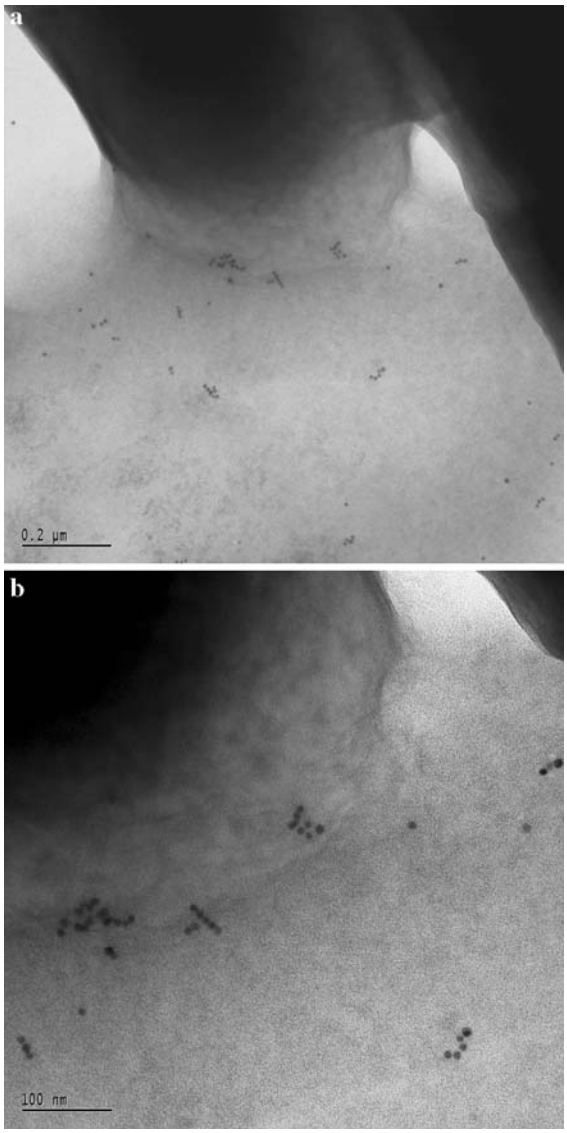

Fig. 6 Negatively stained specimen labeled with CBHI-gold. (**a**) A lower magnification micrograph demonstrating the decoration of the extracellular material of NS::abΔc7S with CBHI-gold. (**b**) A higher magnification image of the region shown in (**a**) demonstrating the lack of fibrillar structure of the extracellular material confirming its non-crystalline nature

Galperin 2006). While AcsC, AcsD, and an endoglucanase seem to be necessary for normal synthesis of cellulose I microfibrils, their precise function in this process remains a mystery (Saxena et al. 1994).

It is interesting to note, that AcsA and AcsB of *G. xylinus* are necessary but not sufficient for cellulose biosynthesis in their native system (Saxena et al. 1994). Yet, expression of these proteins in *S. leopoliensis* results in the synthesis of cellulose, albeit non-crystalline. How can these proteins function alone in a heterologous system when they are apparently non-functional in their native system? A possible explanation can be found in the completed genome of *S. elongatus* PCC 7942. A similarity search reveals the presence of at least two putative, novel cellulose synthesis operons (Nobles and Brown 2007). It is possible that components of these operons are able to act synergistically with AcsA and AcsB. A previous study failed to identify cellulose production in wild type *S. leopoliensis* UTCC 100 (Nobles et al. 2001). However, the methods used only detected acid-insoluble products. Therefore, if the native product is also low crystallinity or non-crystalline, it would not have been detected.

The characterization of cellulose biosynthesis in other bacteria gives some insight into the minimum requirements for cellulose production. *acsA* and *acsB* are conserved in all known proteobacterial operons encoding proteins for cellulose biosynthesis (Römling 2002). Although these enzymes are necessary for cellulose synthesis in the Enterobacteriaceae, they are not sufficient to this end. It is known that the cellulose synthase operon is constitutively transcribed in *E. coli*, yet cellulose is only produced under specific conditions (Zogaj et al. 2001). Control of this process is tightly controlled by regulatory proteins that contain the conserved GGDEF and EAL motifs associated with diguanylate cyclases and phosphodiesterases (Tal et al. 1998; Römling et al. 2005).

The cellulose produced by *E. coli* and *Salmonella* spp. appears as a noncrystalline aggregation of glucan chains in close association with hydrophobic fimbriae constituting the extracellular matrix of the rdar multicellular morphotype (unpublished observations, this lab). Therefore, in addition to regulatory and catalytic proteins, other yet unidentified components necessary for the production of a crystalline cellulose product must exist. It is likely that the highly regular alignment of pores that make up the terminal complex of the cells of *G. xylinus* is critical for crystallization (Saxena et al. 1994; Zaar 1979). It is important to note that unlike the products observed in *E. coli* and *Salmonella* spp. which encase the cells in a cocoon-like structure (unpublished observations, this laboratory), contact of a *G. xylinus* cell to its product is generally limited to the unilateral secretion sites oriented parallel to the long axis (Brown et al.

Table 2 Glucose assays

	OD_{750}	Wet weight (g)	Glucose mg/mL—Sodium Acetate-only	Total glucose mg/mL—Celluclast	Glucose mg/mL from cellulose
NS::cat	1.00 ± 0.18	0.19 ± 0.08	0.03 ± 0.04	0.08 ± 0.03	0.05 ± 0.03
NS::abΔc7S	1.20 ± 0.19	0.20 ± 0.07	0.09 ± 0.06	0.31 ± 0.012	0.22 ± 0.06

Relative cell concentrations are shown as the optical density at 750 nm (OD_{750}) and cell wet weights. Glucose liberated from cellulose was determined by subtracting the concentration of glucose present in Sodium Acetate-only samples from the total glucose obtained from Celluclast digestions. All ± values represent standard deviations

1976). The fact that *E. coli* and *Salmonella* spp. cells are embedded in their extracellular matrix connotes a randomly dispersed rather than a discrete, orderly, and aligned orientation of secretion sites on the cell surface. It is important to note that even in *acsD* mutants of *G. xylinus* which produce crystalline cellulose II in addition to cellulose I, a linearly arranged row of cellulose synthesizing pores is still observed (Saxena et al. 1994). It is possible that close association of glucan chains upon secretion is necessary for the regular formation of any crystallite.

Based on these observations, it becomes obvious that any successful attempt to achieve synthesis of a cellulose I product in a transgenic system must take into account the intricacies of crystallization as well as the catalysis of the β 1, 4 glucan polymer. Although the ultimate goal of this project was large scale crystalline cellulose production from a cyanobacterium, an obvious first step was the functional expression of genes known to be necessary for catalysis. The creation of transgenic strains of *S. leopoliensis* by integration of P_{lac}-*acsABΔC* into the NSII site of the genome represents the first functional expression of the cellulose synthesizing machinery from the strain ATCC 53582 strain of *G. xylinus* in a heterologous system.

Although the non-crystalline material produced by NS::abΔc7S has no utility as a manufacturing material, it has enormous potential as a feedstock for biofuel production.

The non-crystalline nature of this material allows for more efficient and rapid digestion by cellulases. Preliminary results show that cells regenerate cellulose at a rate allowing for harvest intervals of one week or less. Therefore, assuming a lossless scale-up, at current levels of production, we could expect a yield of approximately 80 gallons of ethanol acre ft^{-1} year^{-1}). This is only about 20% of the current production levels from corn (400 gallons acre^{-1} year^{-1}).

However, NS::abΔc7S possesses several advantageous characteristics which may allow it to be competitive with land-based crops: (a) it possesses a rapid generation time; (b) it can be grown in brackish water; (c) the cellulose synthesized by this organism can be hydrolyzed by cellulases without the pretreatment procedures required when utilizing lignocellulosic feedstocks, such as switchgrass, for ethanol production; and, (d) after digestion with cellulases, cells can be returned unharmed to photobioreactors for continued cellulose production. Additionally, this organism is amenable to genetic manipulation by both natural transformation and conjugation. Thus, the potential for increased production by genetic manipulation exists. Indeed, our laboratory has preliminary data suggesting not only the possibility of increased cellulose production, but also the production of soluble oligomers and direct production of glucose from this transgenic strain of *S. leopoliensis*.

The possible synergy between the innate systems of *S. leopoliensis* and the cellulose synthesizing machinery of *G. xylinus* are suggestive of the possibility for the production of designer saccharides tailored for specific applications such as biofuels or novel materials in a very efficient photosynthetic system which can be grown on non-arable lands. Indeed, the future of cellulose biosynthesis and saccharide secretion from microbes can lead to great advances in making our planet a better place to live.

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