Supporting Information

Omadjela et al. 10.1073/pnas.1314063110

SI Materials and Methods

Constructs. The bacterial cellulose synthase (bcs)A and bcsB genes were cloned into the pETDuet (Novagen) expression vector as described (1). BcsA was expressed with a C-terminal dodecahistidine tag to facilitate purification, and the mature region of BcsB was fused to an N-terminal pectate lyase B (PelB) signal sequence for correct targeting. All N-terminal truncation mutants of BcsB were cloned as C-terminally FLAG-tagged species into the pETDuet expression vector containing the WT *bcsA* gene using NcoI and HindIII restriction sites. The expression of the truncated complexes was as described for the WT complex (1).

Protein Expression and Purification. All BcsA-B complexes were expressed in *Escherichia coli* C43 (2) in auto-induction medium and were purified by metal affinity and size exclusion chromatography as described previously (1). The protein was solubilized from the membrane fraction in Triton X-100 detergent, followed by detergent exchange into 1 mM LysoFosCholine Ether 14 (LFCE14) or 5 mM *N,N*-dimethylamine oxide (LDAO) during metal affinity chromatography. The purified complexes were concentrated to a 50 μM final concentration using an extinction coefficient of (161,925 $M^{-1}{\cdot}cm^{-1}$) and reconstituted into proteoliposomes (PLs) or nanodiscs (NDs).

Preparation of Inverted Membrane Vesicles. The cell pellet obtained from a 2-L culture of *E. coli* C43 overexpressing the BcsA-B complex was resuspended in RB buffer containing 20 mM sodium phosphate, pH 7.2, 100 mM NaCl, and 10% glycerol and lysed in a bench-top microfluidizer. The whole cell extract was cleared from cell debris by centrifugation for 20 min at 12,500 rpm in a Beckman JA-20 rotor at 4 °C, and the supernatant was floated on a 1.8 M sucrose cushion by centrifugation at 100,000 × *g* for 120 min at 4 °C in a Beckman 45Ti rotor. The membrane vesicles were recovered from the top of the sucrose cushion, diluted fivefold in RB buffer, and sedimented overnight at 100,000 × *g* in a 45Ti rotor. The purified inverted membrane vesicles (IMVs) were resuspended in 1 mL RB buffer, homogenized in a tissue grinder, and stored in aliquots at −80 °C.

Reconstitution into Proteoliposomes and Nanodiscs. The purified and concentrated BcsA-B complex was incubated at a 5 μM final concentration with 5 mg/mL *E. coli* total lipid extract solubilized in 8 mM LFCE14 in AB buffer containing 25 mM sodium phosphate, pH 7.5, 0.3 M NaCl, 5 mM cellobiose and 10% (vol/vol) glycerol. The detergent was removed by addition of SM-2 BioBeads (BioRad) until the turbidity of the solution indicated the formation of lipid vesicles. The obtained PLs were stored in aliquots at −80 °C.

For reconstitution into NDs, the apoA1-derived membrane scaffold protein (MSP) was expressed and purified as described previously (3) and incubated at 120 μM with 30 μM of purified BcsA-B and 1 mg/mL *E. coli* total lipid extract solubilized in 8 mM LFCE14. The detergent was removed by addition of BioBeads, and the reconstituted NDs were purified over a S200 analytical gel filtration column in 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM cellobiose, and 10% glycerol. The purified NDs were concentrated to 5 μM assuming an additive extinction coefficient of 185,875 $M^{-1}{\cdot}cm^{-1}$ for BcsA-B and MSP (4).

Sedimentation Assays. Standard cellulose synthase sedimentation assays were performed by incubating 1 μM of cellulose synthase complexes, either in PLs, NDs, or detergent micelles, in the presence of 30 μM cyclic-di-GMP (c-di-GMP), 20 mM $MgCl_2$, 5 mM UDP-glucose (UDP-Glc), and 0.25 μCi UDP-[^{3}H]-Glc in AB buffer lacking glycerol and containing only 0.1 M NaCl. Following incubation at 37 °C for 45 min, the polymerization reaction was terminated by addition of 2% SDS, and the water-insoluble polymer was pelleted by centrifugation at 15,000 × *g* at room temperature. The obtained pellet was resuspended in 20 μL 50 mM Tris, pH 7.5, and 0.1 M NaCl and spotted at the origin of a descending Whatman-2MM chromatography paper, which was developed in an aqueous solution of 60% ethanol. For enzymatic degradation, the pellet was resuspended in 20 μL 50 mM sodium acetate, pH 4.5, and 100 mM NaCl and was digested with 0.1 mg/mL of endo-β-1,4- or endo-β-1,3 glucanase from *Aspergillus niger* (TCI) or *Trichoderma* sp. (Megazyme), respectively. Following paper chromatography, the high-molecular-weight polymer retained at the origin was quantified by scintillation counting.

To ensure a constant ratio of UDP-Glc to ^{3}H-labeled UDP-Glc for the titration of UDP-Glc in the presence of 0.7 mM UDP (Fig. 4*C*), a fourfold concentrated stock solution containing 20 mM UDP-Glc and 1.0 μCi UDP-[^{3}H]-Glc was diluted to the final substrate concentration required for the individual experiments. Following synthesis, the reactions were treated as described above.

Enzyme-Coupled Activity Assays. Pyruvate kinase (PK)- and lactate dehydrogenase (LDH)-coupled activity assays were performed by incubating 0.5 μM cellulose synthase with 1 U PK and 1 U LDH, 0.5 mM NADH, 1 mM phosphoenolpyruvate (PEP), and 30 mM $MgCl_2$ in 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM cellobiose, and 10% glycerol in a total volume of 20 μL. The cellulose synthase complex was added last to the reaction mix after a preincubation for 10 min at room temperature. The decrease in absorbance at 340 nm was measured in a SpectraMax plate reader in Corning 384 well clear flat bottom assay plates. Control reactions in the absence of cellulose synthase were performed to determine the background NADH oxidation. Data were plotted and analyzed in Origin (5) and fitted to monophasic Michaelis-Menten kinetics as described (3).

Western Blot Analysis. Proteins were separated by SDS/PAGE on a 12.5% polyacrylamide gel and transferred to nitrocellulose membranes at 100 V and constant current (350 mA) for 60 min at 4 °C in a BioRad Mini-Transfer Cell according to the manufacturer's specifications. The nitrocellulose membrane was blocked in 5% milk/TBS-Tween solution for 30 min and incubated overnight with an anti-penta-His (Qiagen) or anti-FLAG (Sigma) primary mouse antibody. The membranes were washed three times in 5% milk/TBS-Tween before incubating with an IRDye800-conjugated anti-mouse secondary antibody (Rockland) for 45 min at room temperature. After washing, the membranes were scanned on an Odyssey Infrared Imager (Licor).

Linkage Analysis. The freeze-dried in vitro product obtained from 20 μL of 1 μM PL-reconstituted BcsA-B was dispersed in 200 μL dry DMSO. The mixture was incubated for 6 h at room temperature combining sonication (10-min intervals every hour) and agitation with a magnetic stirrer. Samples were maintained under argon atmosphere during the dispersion and methylation steps. Methylation reactions were performed using the NaOH/CH_3I method (6) by repeating five times the methylation step on each sample, thereby avoiding any risk of undermethylation. Partially methylated polysaccharides were hydrolyzed in the presence of 2 M trifluoroacetic acid at 121 °C for 2 h and further

derivatized to permethylated alditolacetates (7). The latter was separated and analyzed by gas chromatography/electron-impact MS (GC/EI-MS) on a SP-2380 capillary column (30 m × 0.25 mm inner diameter; Supelco) using an HP-6890 GC system and an HP-5973 electron-impact mass spectrometer as a detector (Agilent Technologies). The temperature program increased from 160 °C to 210 °C at a rate of 1 °C/min. The mass spectra of the fragments obtained from the permethylated alditolacetates were compared with those of reference derivatives.

Data Analysis. All measurements were performed at least in triplicate, and error bars represent deviations from the means.

1. Morgan JL, Strumillo J, Zimmer J (2013) Crystallographic snapshot of cellulose synthesis and membrane translocation. *Nature* 493(7431):181–186.
2. Wagner S, et al. (2008) Tuning Escherichia coli for membrane protein overexpression. *Proc Natl Acad Sci USA* 105(38):14371–14376.
3. Hubbard C, McNamara JT, Azumaya C, Patel MS, Zimmer J (2012) The hyaluronan synthase catalyzes the synthesis and membrane translocation of hyaluronan. *J Mol Biol* 418(1-2):21–31.
4. Denisov IG, Grinkova YV, Lazarides AA, Sligar SG (2004) Directed self-assembly of monodisperse phospholipid bilayer Nanodiscs with controlled size. *J Am Chem Soc* 126(11):3477–3487.
5. OriginLab (2012) Data Analysis and Graphing Software, Northampton, MA.
6. Ciucanu I, Kerek F (1984) A simple and rapid method for the permethylation of carbohydrates. *Carbohydr Res* 131(2):209–217.
7. Albersheim P, Nevins PD, English PD, Karr A (1967) A method for the analysis of sugars in plant cell wall polysaccharides by gas liquid chromatography. *Carbohydr Res* 5(3): 340–345.

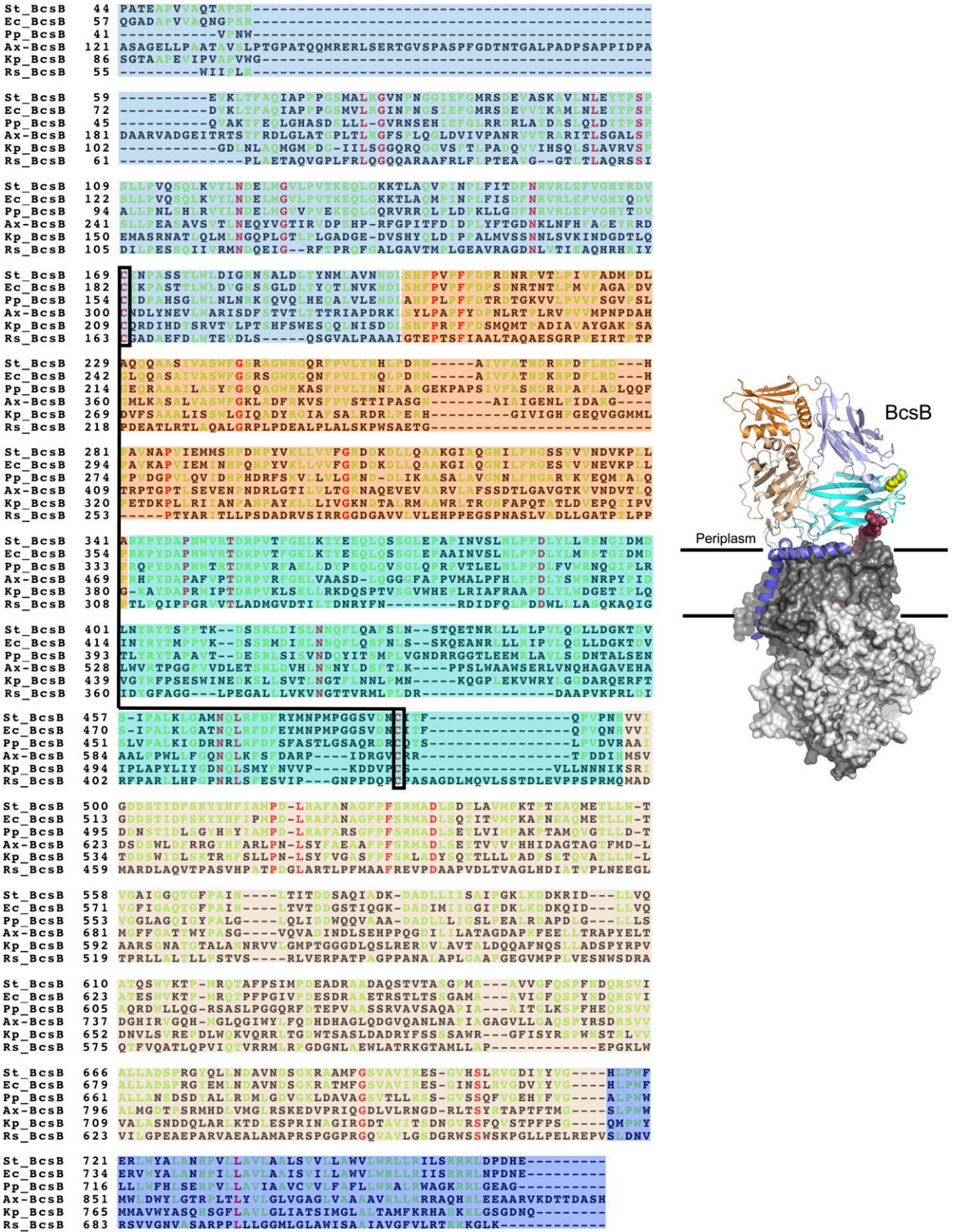

Fig. S1. Sequence alignment of BcsB. (*Left*) BcsB sequences from *Salmonella typhimurium* (St), *Escherichia coli* (Ec), *Pseudomonas putida* (Pp), *Gluconacetobacter xylinus* (Ax), *Klebsiella pneumoniae* (Kp), and *Rhodobacter sphaeroides* (Rs) are compared. The alignment is colored according to the individual BcsB domains: carbohydrate-binding domain (CBD)-1, blue; flavodoxin-like domain (FD)-1, orange; CBD-2, cyan; FD-2, sand, transmembrane (TM) anchor, and preceding interface helix, dark blue. Conserved cysteines forming a disulfide bridge are framed black and are shown as yellow spheres in the right panel. (*Right*) Structure of the *R. sphaeroides* BcsA-B complex. BcsA is shown as a surface in shades of gray; BcsB is shown as cartoon colored according to the sequence alignment.

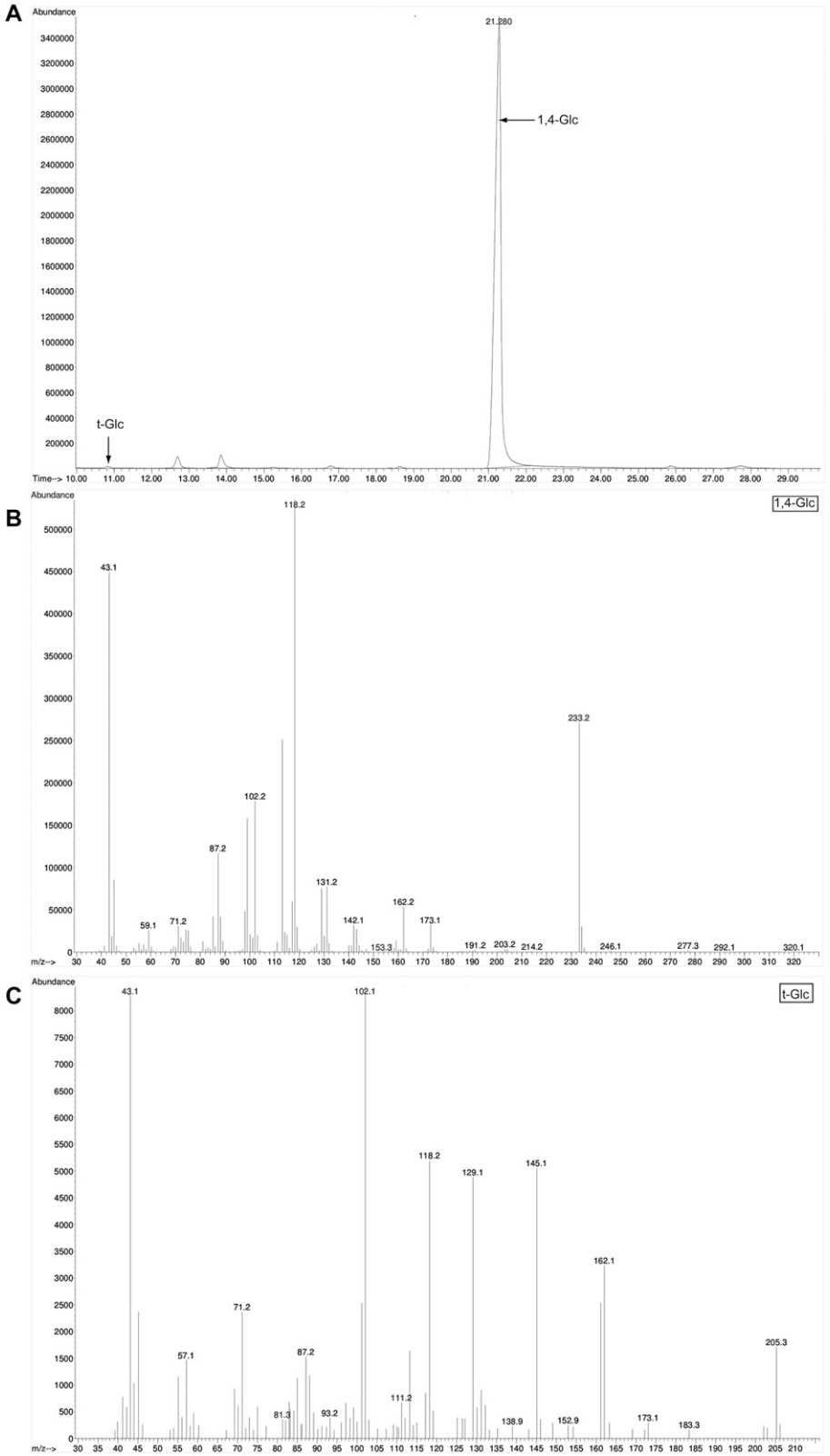

Fig. S2. Characterization of the BcsA-B in vitro product by linkage analysis (see experimental details in *SI Materials and Methods*). (*A*) Shown is a typical gas chromatogram corresponding to the separated permethylated alditolacetates. The derivative corresponding to terminal glucose residues (nonreducing ends; t-Glc) was quantified from multiple chromatograms obtained from different dilutions of the alditolacetates and represents no more than 0.3–0.5% of the total alditolacetates. The only other peak in the chromatogram arising from a sugar residue corresponds to 1,4-linked glucosyl residues (1,4-Glc) and represents 99.5–99.7% of the total derivatives. (*B* and *C*) EI-MS fragmentation spectra of the permethylated alditolacetates obtained from the product synthesized in vitro by the BcsA-B complex. The identity of the 1,5-di-*O*-acetyl, 2,3,4,6-tetra-*O*-methyl-D-glucitol and 1,4,5-tri-*O*-acetyl, 2,3,6-tri-*O*-methyl-D-glucitol corresponding to

Legend continued on following page

the t-Glc and 1,4-Glc residues, respectively, was verified after fragmentation by EI-MS. EI-MS analysis performed on the other minor peaks visible on the chromatogram (e.g., before retention times of 13, 14, 17, and 19 min) revealed that they did not correspond to any sugar derivative. The fragmentation spectra obtained are characteristic of 1,4,5-tri-*O*-acetyl, 2,3,6-tri-*O*-methyl-D-glucitol (*B*) (GC retention time of 21.3 min) and 1,5-di-*O*-acetyl, 2,3,4,6-tetra-*O*-methyl-D-glucitol (*C*) (GC retention time of 10.8 min) and correspond to 1,4-Glc and t-Glc residues, respectively.

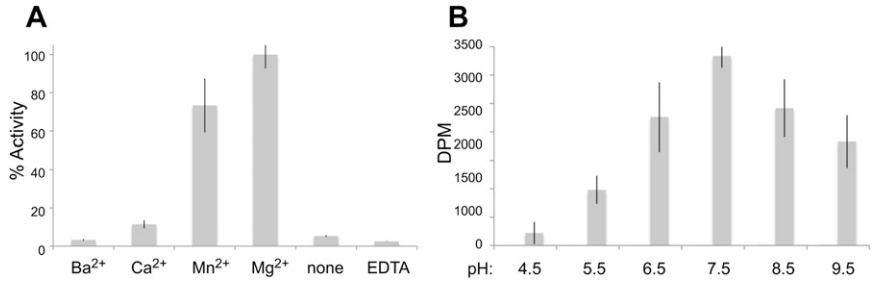

Fig. S3. Cation selectivity and pH optimum of cellulose synthase. (*A*) Cellulose synthesis reactions were performed in the presence of 20 mM of the indicated cations or in the absence of any additional divalent cations with and without addition of 20 mM EDTA. The activities are shown relative to the activity in the presence of magnesium. (*B*) The BcsA-B complex exhibits a maximum catalytic activity at neutral pH. Activity assays were performed in phosphate buffer adjusted at the indicated pH, revealing a pH optimum at pH 7.5. All activity assays were performed for 45 min at 37 °C with 1 μM PLs-reconstituted *R. sphaeroides* BcsA-B and by quantifying the accumulation of ^{3}H-labeled water insoluble cellulose as described.

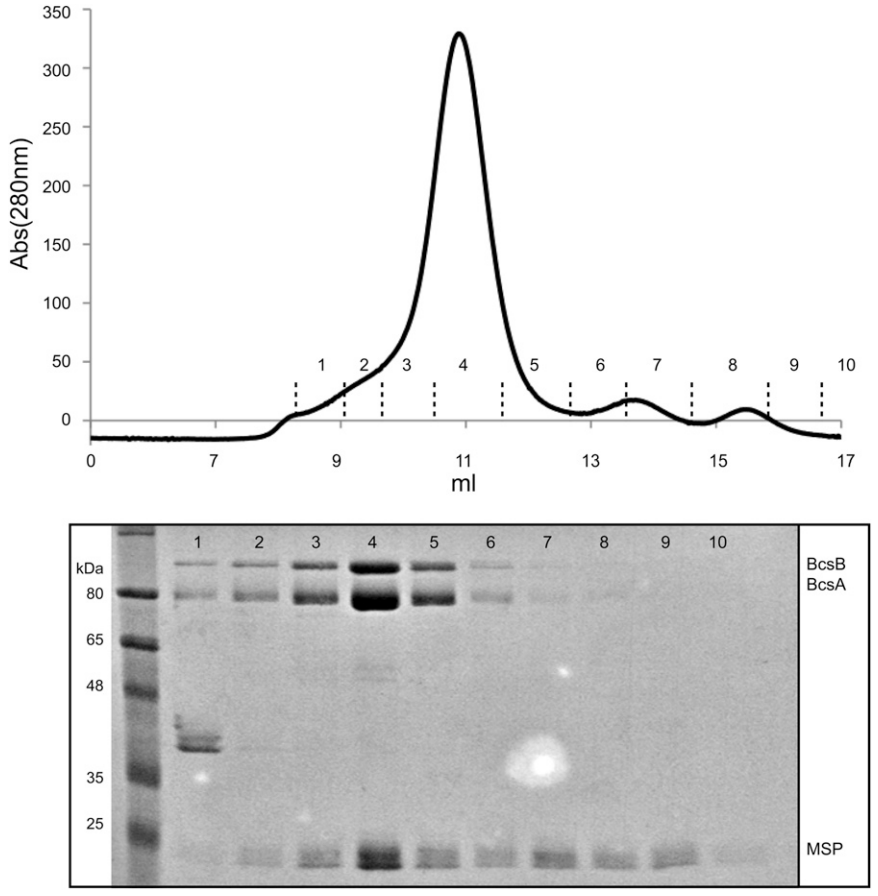

Fig. S4. Reconstitution of BcsA-B into lipid nanodiscs. 30 μM of the purified BcsA-B complex was incubated with 120 μM purified apo-A1 protein MSP in the presence of 1 mg/mL detergent-solubilized *E. coli* total lipid extract. After detergent removal by addition of SM-2 BioBeads, the NDs were purified by gel filtration chromatography over an analytical S200 Superdex size-exclusion chromatography column (*Upper*) in 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM cellobiose, and 10% (vol/vol) glycerol. The eluted fractions were analyzed by SDS/PAGE and Coomassie staining (*Lower*), pooled, and concentrated to 5 μM.

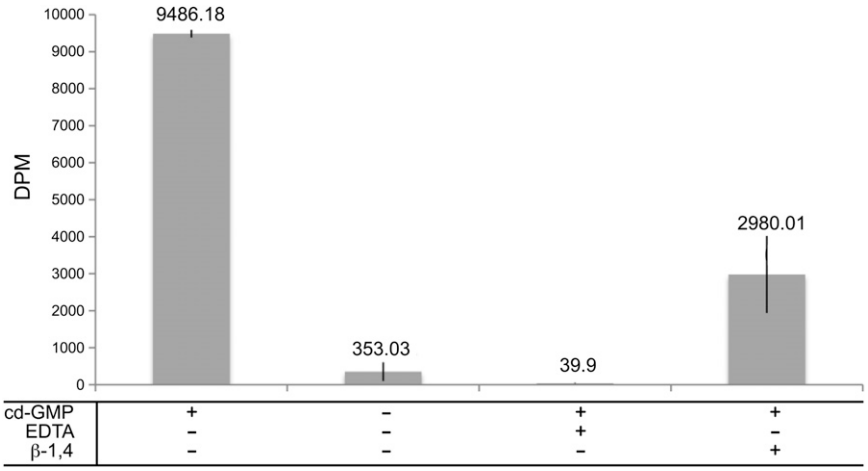

Fig. S5. Nanodiscs provide a native-like environment for BcsA-B. NDs containing the purified BcsA-B complex were incubated at 0.5 μM for 60 min at 37 °C with 5 mM UDP-Glc and 0.03 mM c-di-GMP as described for the PLs-reconstituted complex. No product is formed in the presence of 20 mM EDTA. After synthesis, the polymer can be digested with 0.1 mg/mL β-1,4 cellulase from *Aspergillus niger* (β-1,4). The synthesized, ^{3}H-labeled polymer was quantified as described in *SI Materials and Methods*.

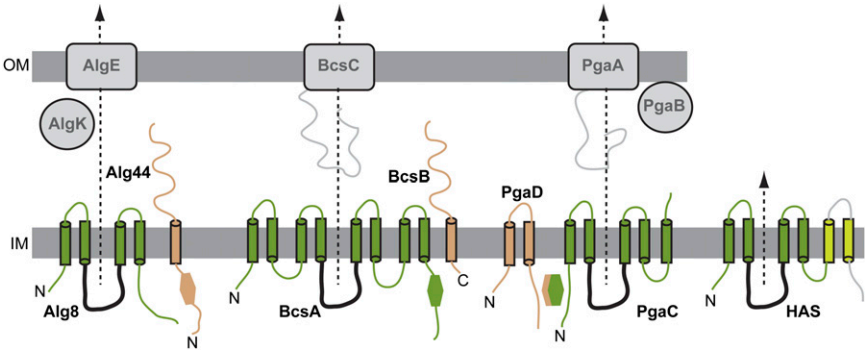

Fig. S6. Comparison of bacterial exopolysaccharide synthases. The biosynthesis of bacterial alginate, cellulose, and poly-β-1,6 *N*-acetylglucosamine is activated by c-di-GMP, whereas hyaluronan (HA) formation is independent of c-di-GMP. c-di-GMP-binding domains (green and khaki hexagons) can either be a part of the catalytic subunits (shown in green) or are localized in associating subunits that interact with the synthases, as shown for BcsA and BcsB and Alg44 and Alg8, respectively. PgaC and PgaD of the poly-β-1,6 *N*-acetylglucosamine synthase bind c-di-GMP at their interface and do not contain PilZ domains. The GT domains are shown in black and the periplasmic and outer-membrane components required for activity in vivo are shown in gray. Predicted TM segments are shown as cylinders. HA synthase (HAS) is predicted to contain four to six TM helices (shown in dark and light green) and is sufficient for HA synthesis and membrane translocation. IM and OM, inner and outer membrane, respectively.