Impairment of O-antigen production confers resistance to grazing in a model amoeba–cyanobacterium predator–prey system

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The grazing activity of predators on photosynthetic organisms is a major mechanism of mortality and population restructuring in natural environments. Grazing is also one of the primary difficulties in growing cyanobacteria and other microalgae in large, open ponds for the production of biofuels, as contaminants destroy valuable biomass and prevent stable, continuous production of biofuel crops. To address this problem, we have isolated a heterolobosean amoeba, HGG1, that grazes upon unicellular and filamentous freshwater cyanobacterial species. We have established a model predator–prey system using this amoeba and *Synechococcus elongatus* PCC 7942. Application of amoebae to a library of mutants of *S. elongatus* led to the identification of a grazer-resistant knockout mutant of the *wzm* ABC O-antigen transporter gene, SynPCC7942_1126. Mutations in three other genes involved in O-antigen synthesis and transport also prevented the expression of O-antigen and conferred resistance to HGG1. Complementation of these rough mutants returned O-antigen expression and susceptibility to amoebae. Rough mutants are easily identifiable by appearance, are capable of autoflocculation, and do not display growth defects under standard laboratory growth conditions, all of which are desired traits for a biofuel production strain. Thus, preventing the production of O-antigen is a pathway for producing resistance to grazing by certain amoebae.

Biofuels derived from algal biomass represent a carbon-neutral and potentially sustainable alternative to fossil fuels that can be developed without massive infrastructure changes for distribution and utilization. The next generation of biofuels is being developed through the mass growth of photosynthetic microorganisms, such as eukaryotic algae and cyanobacteria, in open pond production systems (1, 2). Whereas these systems avoid the high economic costs associated with bioreactor production and maintenance, they are susceptible to contamination with organisms that predate upon or infect the desired production strain, resulting in a "pond crash" that can destroy almost the entire yield of high-density biomass, as occurs with agricultural crops (1, 3, 4). Strategies for crop protection in algal biotechnology must be developed to overcome the impact of grazers and infectious agents that otherwise prevent the growth of biomass for the production of biofuels, nutraceuticals, agricultural feedstocks, waste-water treatment, and other applications (5, 6).

In their natural environments, cyanobacteria are subjected to grazing pressure by a variety of organisms, including protistan predators such as ciliates, flagellates, and amoebae (7). Grazing affects both mortality and population structure (8), as has recently been observed in the rapid decline of biomass of *Microcystis* in a natural pond and in the restructuring of the bloom with a shift from a susceptible *Microcystis* species to a resistant one upon amoebal grazing (9). Whereas it is known that amoebae graze on cyanobacteria (10–12), these predators have not received much attention. Van Wichelen et al. (2010) attribute this omission to the fact that population densities of amoebae peak for short periods of time and, hence, may be overlooked during monthly or even biweekly samplings of freshwater environments (9).

Bacteria have evolved a variety of mechanisms to evade detection, capture, ingestion, and digestion (13), but very little is known about the molecular mechanisms that govern the interactions between cyanobacteria and their protistan predators (14). The cell surface appears to be important as S layers (15), a giant cell-surface protein (16), and lipopolysaccharide (LPS) heterogeneity (17) have been implicated as defenses against nanoflagellates and a dinoflagellate. Xinyao et al. (2006) studied the grazing of an amoeba of the *Naegleria* genus on a variety of freshwater unicellular and filamentous cyanobacteria and showed that prey morphology such as filament form and aggregation affects ingestion (18). Other undetermined cell properties also affected food selection as some unicellular cyanobacteria were excreted after ingestion.

Defenses against amoebae have been characterized in a number of opportunistically pathogenic bacteria, which range from modifications of the cell surface to the production and delivery of cytotoxic compounds. Epitope differences in the O-antigen of otherwise genetically identical serovars of *Salmonella enterica* are sufficient to account for feeding preferences among intestinal amoebae (19). *Legionella pneumophila* evades lysosomal digestion in the soil amoeba *Acanthamoeba castellani* by shedding LPS outer membrane vesicles (20). A type III secretion system confers cytotoxicity of *Pseudomonas aeruginosa* to *A. castellani* (21) and of *Vibrio parahaemolyticus* to both *A. castellani* and *Dictyostelium discoideum* (22). A type VI secretion system confers cytotoxicity of *Vibrio cholera* to *D. discoideum* (23) through the translocation of an actin cross-linking protein (24). The soil bacterium *Pseudomonas fluorescens* induces the expression of genes involved in the production of certain extracellular toxins in response to chemical cues from *A. castellani* (25).

As amoebae are known to be a problem in open ponds and little is known about natural mechanisms of amoebal grazing resistance in cyanobacteria, we sought to establish a model system with which we could study the interaction between amoebae and cyanobacteria at the molecular level. Here, we describe the isolation of a heterolobosean amoeba, HGG1, which grazes on the unicellular freshwater cyanobacterium *Synechococcus elongatus* PCC 7942 and certain filamentous freshwater cyanobacteria. Through a genetic screen using this amoeba–cyanobacterium model system, we have identified O-antigen synthesis and transport as a major pathway that, when impaired, produces resistance to certain amoebal grazers.

Results

Isolation and Characterization of Amoeba HGG1. Amoeba HGG1 was isolated from a freshwater pond using the filamentous cyanobacterial model production strain, *Leptolyngbya* sp. BL0902 (26) as a food source. HGG1 also forms plaques on lawns of the

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filamentous, nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120 and of the unicellular *S. elongatus*. HGG1 can reduce the turbidity of liquid cultures of the filamentous cyanobacteria BL0902 and *Anabaena* as well, but no macroscopic indications of grazing on liquid *S. elongatus* cultures, such as decreased turbidity or increased amoebal numbers, were observed. Microscopic examination revealed amoeboid cells with a length of 14.4–22.9 μm (mean 17.7 μm), and a width of 8.0–16.2 μm (mean 12.3 μm), which move with eruptive pseudopodia. Uroidal filaments are visible at the trailing end (Fig. 1*A*). HGG1 forms spherical cysts with an average diameter of 10.1 μm (Fig. 1*B*). Incubation of trophozoites of HGG1 in BG-11 growth medium in the absence of a cyanobacterial food source, in deionized water, or in 2 mM Tris, pH 7.2, as described in (27, 28), failed to elicit the formation of a flagellate form. Chlorophyll fluorescence is detectable in food vacuoles of amoebae sampled from the edge of an expanding plaque on a lawn of *S. elongatus*, indicating the cyanobacterial cells have been ingested (Fig. 1 *C* and *D*).

The 18S rRNA gene of HGG1, as amplified with the MoonA and MoonB primers (29), is 1813 bp long. Direct sequencing of both strands of the PCR product revealed no heterogeneity. However, when the product was cloned and both strands of the inserts of four independent clones were sequenced, they were not identical and showed a few polymorphisms (Fig. S1). This result is not uncommon in eukaryotic organisms, which can have several hundred nonidentical copies of the 18S rRNA gene within a single genome (30). A comparison of all of the sequences to 18S sequences in the NCBI nr database using Blastn (31) and Megablast (32) revealed that the closest match is to the heterolobosean soil amoeba *Vrihiamoeba italica* (93% nucleotide identity with 98% coverage). This result was consistent with a phylogenetic analysis, which showed that HGG1 is most closely related to *V. italica* (100% bootstrap) (Fig. S2).

Screening a Library of Mutants of *S. elongatus* for Grazer Resistance. To investigate the potential for natural mechanisms of resistance, we screened a library of transposon insertional mutants, the unigene set (UGS) of mutants of *S. elongatus* (33, 34), for knockout mutations that confer resistance to grazing. The UGS library contains ~2,600 individual gene insertions, representing mutations in over 88% of genomic loci. We spotted liquid cultures of all individual mutants on agar plates, allowed the spots to grow for 13 d under high light conditions, and then uniformly distributed amoebae suspensions to every spot culture. We monitored the plates for amoebal grazing activity in the form of yellowing of spots for 25 d.

Two different phenotypes of resistance were observed on the plates. The first was a phenotype of true resistance where the colony was not grazed upon for the entirety of the 25 d. Only one spot from the entire screen displayed this phenotype, 7-D-3 (Fig. 2 *A*–*C* and Movies S1 and S2). The second resistant phenotype, that of revival, was one in which the culture turned yellow in response to the amoeba, but eventually regrew. An example of this phenotype is spot 7-B-12, which was grazed upon, then revived, and subsequently showed a second round of amoebal activity (Fig. 2 *A*–*C* and Movies S1 and S3). Of the 2,592 spots tested in this screen, 1,065 spots revived after initial HGG1 grazing.

Mutant 7-D-3 contains a transposon insertion in the gene SynPCC7942_1126. Similarity searches using Blastp (35) and functional predictions using EMBL STRING (36) indicated that this gene encodes a homolog of the ABC O-antigen transporter, Wzm. The Wzm protein has been shown to transport fully polymerized O-antigen from the cytoplasm to the periplasm in *Escherichia coli* serotypes O8 and O9a (37–40) (Fig. 3*A*). We hypothesized that this mutation prevents the expression of O-antigen on the outer membrane of *S. elongatus*, thereby truncating the LPS, a possible recognition factor for amoebae (Fig. 3*B*). We identified *S. elongatus* homologs of other proteins involved in O-antigen synthesis and transport pathway, as highlighted in Fig. 3*A*, and examined the library clones related to those loci. Each was represented by a duplication that results from integration of the transforming plasmid, rather than a segregated insertion mutation, explaining why they were missed in the original screen. We remade these mutants and verified true interruption alleles to test the hypothesis that truncated LPS confers grazer resistance.

Absence of O-Antigen Confers Resistance to Grazing. Segregated, insertional knockout mutants were regenerated in genes homologous to the O-antigen transporter *wzm* (SynPCC7942_1126), the transporter-associated ATPase *wzt* (SynPCC7942_1244), the O-antigen chain length determining factor *wbdD* (SynPCC7942_1903), and one of the O-antigen synthetases *wbdC* (SynPCC7942_1887). To test complementation of these mutants, the wild-type (WT) ORFs were expressed from neutral site I (NS1) in the segregated knockout mutants and WT-like antibiotic-resistant controls (Table S1). All genotypes were confirmed by PCR amplification (Fig. S3).

These strains were tested for resistance to HGG1 using a plaque assay (Fig. 2 *D* and *E*), in which lawns of each mutant were grown under high light conditions for 6–7 d before the addition of amoebae to the center of the plate as a single 4-μL spot containing $\sim 2 \times 10^4$ amoebae. Amoebae plaques formed and grew on all WT controls, but did not grow on mutants defective for the transporter-related (Fig. 2*D*) or synthesis-related genes (Fig. 2*E*). Mutant strains that were complemented *in trans* with a WT copy of the mutated gene, such as the *wzm* mutant expressing Wzm from NS1, allowed plaque formation and growth, demonstrating that genetic disruption of any one of these genes is sufficient to confer resistance to the grazing amoeba HGG1. In contrast, mutant strains expressing another of the O-antigen–related genes, such as the *wzm* mutant expressing Wzt from NS1, remained resistant to HGG1.

To confirm that these mutations disrupt the synthesis or transport of O-antigen, outer membranes were purified from each of the strains, resolved by Tris-glycine SDS gel electrophoresis and visualized with a carbohydrate-specific stain (Fig. 4 and Fig. S4). Lipopolysaccharide from smooth *E. coli* expressing O-antigen displays a characteristic ladder pattern of bands, which is absent from rough *E. coli* lacking O-antigen (41) (Fig. 4*A*). Outer membrane preparations from WT *S. elongatus* and antibiotic-

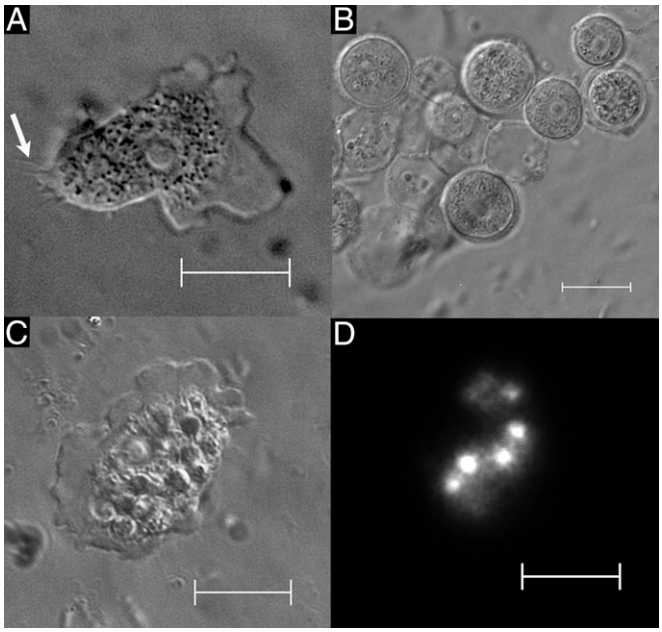

Fig. 1. Amoebae morphology. (*A*) Phase contrast micrograph of amoeba HGG1; arrow points to uroidal filaments. (*B*) Differential interference contrast (DIC) image of HGG1 cysts. (*C*) DIC image of HGG1 feeding on *S. elongatus*. (*D*) Epifluorescence image of *C* with autofluorescence from cyanobacterial chlorophyll visible. (Scale bars, 10 μm.)

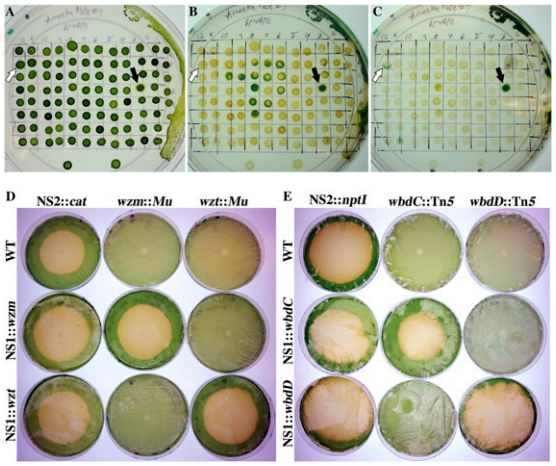

Fig. 2. UGS library screen for grazer resistance and amoeba lawn plaque assays with strains affecting O-antigen production. (*A*–*C*) Images showing the UGS screen plate 7 (*A*) just before addition of amoeba HGG1 to every spot and 11 d (*B*) and 25 d (*C*) after addition of HGG1. Of all UGS library mutants tested, only spot 7-D-3 (closed arrows) survived all 25 d without turning yellow upon amoebal grazing. Position 7-B-12 (open arrows) was completely yellow by day 11, but subsequently regrew and was grazed upon again. Time-lapse videos of the complete time course for plate 7, position 7-D-3, and position 7-B-12 are available as Movies S1, S2, and S3, respectively. (*D* and *E*) Nine days following amoebae addition to lawn plates, large plaques had grown on all antibiotic-resistant WT controls (NS2::*cat* and NS2::*nptI*) and on the four complemented strains (*wzm*::*Mu*;NS1::*wzm*, *wzt*::*Mu*;NS1::*wzt*, *wbdC*::Tn*5;NS1*::*wbdC*, and *wbdD*::Tn*5;NS1*::*wbdD*). In contrast, only a slight thinning at the HGG1 inoculation point was observed on the plates of mutants defective for (*D*) transporter genes (*wzm*::*Mu* and *wzt*::*Mu*) and (*E*) synthesis genes (*wbdC*::Tn*5* and *wbdD*::Tn*5*). Mutant strains expressing genes other than the one mutated (*wzm*::*Mu*;NS1::*wzt*, *wzt*::*Mu*;NS1::*wzm*, *wbdC*::Tn*5*;NS1::*wbdD*, and *wbdD*::Tn*5*;NS1::*wbdC*) were also resistant to plaque formation. Genotypes of plates are identified by a combination of column and row labels.

resistant WT-like controls produce a ladder of bands analogous to those observed in *E. coli*. Mutants defective for either the O-antigen transport genes, *wzm* or *wzt* (Fig. 4*B*), or the O-antigen synthesis genes, *wbdC* or *wbdD* (Fig. 4*C*), lack this banding pattern. Complementation of these mutants causes the return of the O-antigen signature, whereas strains that carry other, noncomplementing genes from the pathway continue to lack the O-antigen ladder. Thus, the four genes investigated here function in the synthesis and transport of the O-antigen to the outer membrane of *S. elongatus* and the lack of extracellular O-antigen in these rough mutants confers resistance to HGG1.

Phenotypes Associated with Resistance and a Lack of O-Antigen. A number of physical phenotypes are associated with the grazer-resistance phenotype and defects in O-antigen production. These phenotypes include autoflocculation in liquid cultures (Fig. 5 *A* and *B*), altered appearances on solid media (Fig. 5*C*), and altered pelleting characteristics upon centrifugation (Fig. S5). When dense liquid cultures are held stationary, mutants lacking O-antigen clump together, settle to the bottom of the culture, and often cling to the glass vessel (Fig. 5 *A* and *B*). In contrast, WT *S. elongatus* grows planktonically and does not settle (Fig. 5*A*). When grown on plates, WT is reflective, moist in appearance (Fig. 5*C*), and viscous when scraped. Rough mutants, in contrast, are nonreflective, dry in appearance, and are dry and flakey when scraped. When collected by centrifugation, WT forms a compact pellet, whereas rough mutants form a smear along the side of the centrifuge bottle (Fig. S5). Taken together, these phenotypes clearly distinguish rough and smooth strains of *S. elongatus* and allow for the rapid visual identification of rough mutants.

Rough mutants lack O-antigen and, therefore, lack a major component of the outer membranes of Gram-negative bacteria. Whereas decades of previous investigations have shown that this difference can have a major impact on recognition by the cascade system of the innate immune system or by bacteriophage (42), it is still unclear whether deletion of the O-antigen has any deleterious effects on the isolated growth of the bacterium. For example, deleting O-antigen does not appear to affect solvent tolerance (43). Because growth rate and culture density are important criteria for a biofuel production strain, we compared growth of WT and the O-antigen transporter mutants as determined by increases in dry biomass (Fig. S6), a measure that is directly relevant to biofuel production. Dry biomass analysis was preferred over turbidity tests because reductions in absorbance measurements are known to occur as a result of autoflocculation in microalgae (44). Triplicate cultures of WT-like and autoflocculating, rough strains were grown at 30 °C while shaking under high light for 10 d. The amount of biomass collected from the *wzm* mutant was not significantly different ($P > 0.05$) from WT throughout the entire time course, whereas the *wzt* mutant biomass was not significantly different ($P > 0.05$) from WT at the end of the experiment. We concluded that the lack of O-antigen does not affect the growth of the cyanobacterium.

Rough Phenotype Allows for Identification of Resistant Mutants. To test the observed association between the rough phenotype and resistance, we performed a combined selection and screen for

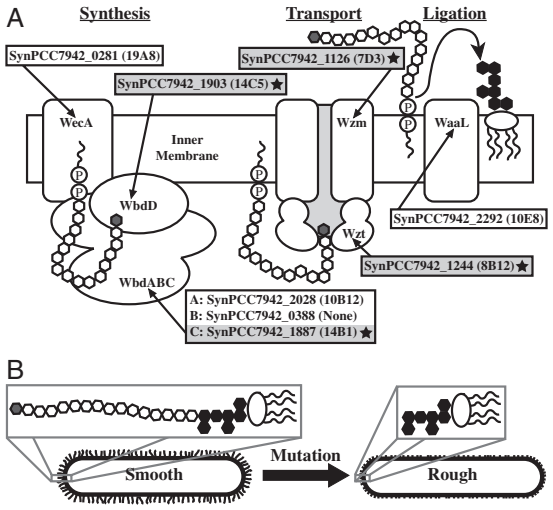

Fig. 3. Model of O-antigen synthesis and transport in *S. elongatus* PCC 7942 and the effects of mutations in these genes. (*A*) Diagram of the O-antigen transport and synthesis components previously discovered in serotype O8 and O9 of *E. coli* and predicted *S. elongatus* homologs, adapted from Greenfield and Whitfield (40). WecA initiates synthesis on the cytoplasmic side of the inner membrane via the linkage of the initial sugar moeity to an undecaprenyl phosphate anchor. The mannosyltransferases WbdA, WbdB, and WbdC synthesize the O-antigen polymer, whereas WbdD terminates polymerization and determines the length of the O-antigen. The complete O-antigen molecule is recognized by the nucleotide binding protein Wzt and transported to the periplasmic space. Once in the periplasm, the O-antigen is transferred by WaaL from the undecaprenyl anchor to the outer core of the lipopolysaccharide molecule. We have identified the homologs of most of these genes in the genome of *S. elongatus* PCC 7942, as listed in arrowed boxes around the figure. UGS identifiers representing insertional mutants of those genes are given in parentheses. The four mutants investigated in this study are starred and highlighted. (*B*) Insertional mutations in the genes involved in O-antigen synthesis and transport, such as those investigated here, are hypothesized to prevent the appearance of O-antigen in the periplasmic space, thereby preventing its attachment to the rest of the lipopolysaccharide molecule. This leads to the rough strain whose outer membrane is primarily composed of lipopolysaccharide molecules lacking the O-antigen moiety.

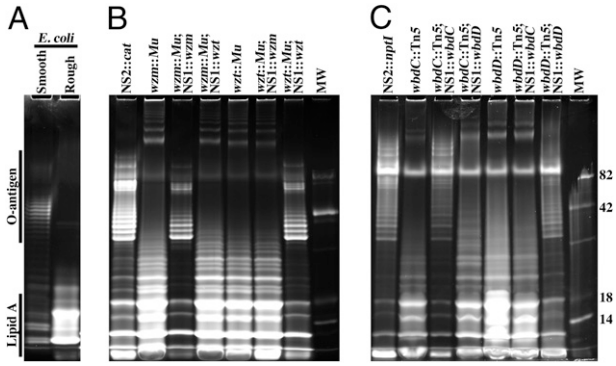

Fig. 4. Analysis of outer membrane preparations from WT and mutant strains. Outer membrane preparations were resolved by Tris-glycine SDS gel electrophoresis and carbohydrates were visualized by Pro-Q Emerald staining. (*A*) Lipopolysaccharides (10 μg/lane) from smooth (strain O55:B5) and rough (strain EH100 Ra mutant) *E. coli* (Sigma) were run as controls for the presence and absence of the O-antigen ladder pattern. Outer membrane preparations from strains related to (*B*) O-antigen transport and (*C*) O-antigen synthesis. MW, CandyCane Glycoprotein Molecular Weight Standards (Life Technologies). Numbers at the right indicate positions and molecular weights in kilodaltons of marker bands.

randomly generated rough mutants. A freshly plated lawn of WT *S. elongatus* cells was mutagenized with 302 nm UV light for 5 min, allowed to recover for 7 d, and subsequently grazed upon in an HGG1 plaque assay. One hundred survivors of the grazing were regrown and screened for a rough appearance. Five percent of the revived colonies were rough, compared with 100 survivors picked from a nonmutagenized plate of WT that were all smooth in appearance. This disproportionately high frequency of rough mutants, compared with only four characterized rough mutants in the entire 2,600 strain collection (0.15%), suggests that amoebal grazing selects for the rough phenotype. When revivers were tested again for resistance to HGG1 using the lawn plaque assay, only the five rough strains were resistant; all 195 smooth strains proved susceptible to the amoeba. Rough strains generated by UV mutagenesis were stably maintained in liquid media without antibiotic or grazer selection for over a month. These results provide further evidence that the rough phenotype is a major determinant of amoeba resistance and that spontaneous grazer-resistant mutants can be obtained and maintained through screens for this phenotype.

Discussion

Establishment of a Model Predator–Prey System. Heterolobosean amoebae are a diverse group present in a variety of freshwater and marine habitats, and terrestrial soils (45). Their diversity is likely to be high. In recent years, many new species have been discovered (28), including halophiles (46), thermophiles (47), and a single human pathogen, *Naegleria fowleri*, which causes primary amoebic meningoencephalitis, a nearly uniformly fatal disease of the central nervous system (48). Like other free-living amoebae, the heterolobosean are phagotrophic protists and consume bacteria, including cyanobacteria (18). HGG1 ingests *S. elongatus* and forms expanding plaques on lawns. This property has allowed us to establish a model predator–prey system and successfully screen mutant collections for resistant clones, which led to the discovery of O-antigen transport and synthesis genes.

Discovery of O-Antigen Synthesis and Transport Genes. Although the O-antigen of *Anacystis nidulans* KM, an isolate likely to be essentially identical to *S. elongatus* PCC 7942, was shown to structurally and chemically resemble that of *E. coli* O8 (49), the genes involved in O-antigen synthesis and transport in *S. elongatus* had not been previously identified and characterized. Our discovery that *S. elongatus* uses a Wzm/Wzt O-antigen transport system homologous to that of *E. coli* O8 and O9 is consistent with the O-antigen structure of *A. nidulans* KM, whose lipopolysaccharide is involved in adsorption of the cyanophage AS-1 (50), which also infects *S. elongatus* PCC 7942 (51). Whereas the dominant mechanism of O-antigen production in enterics is the Wzx/Wzy flippase pathway (52), the Wzm/Wzt system appears to predominate among the cyanobacteria. Blastp searches using the Wzm and Wzt proteins of *E. coli* IAI1 revealed representative homologs (*e* values < 1*e*-15) throughout the diverse cyanobacterial clades, whereas searches using the Wzx and Wzy sequences from *E. coli* O157:H7 produced only three remote Wzx homologs (*e* values between 2*e*-4 and 3*e*-7) and no potential Wzy homologs (*e* values > 1*e*-4) among all cyanobacterial sequences.

In the case of *Anabaena* sp. strain PCC 7120, the filamentous cyanobacterium has two homologs each of the *wzm* transporter (*alr4485* and *all0917*) and the associated *wzt* ATP-binding protein genes (*alr4486* and *all0916*). Transcriptomics data (53) indicate that one copy of each gene is constitutively expressed (*alr4485* and *alr4486*), whereas the second copy is expressed only upon nitrogen starvation and heterocyst development (*all0917* and *all0916*). It is known that heterocyst development requires the formation of additional glycolipid and envelope polysaccharide layers to generate an anoxic environment for nitrogen fixation (54); however, it is unclear whether a change in O-antigen serotype is associated with heterocyst formation and how such a change would remain localized to the heterocyst in a filament where the outer membrane is continuous between cells. The identification of *wzm* and *wzt* paralogs whose expression is dependent upon nitrogen depletion suggests that O-antigen is produced during heterocyst development that is different enough from the vegetative O-antigen as to warrant the expression of an alternative transport complex. Future investigations of these genes in *Anabaena* sp. PCC 7120 will be important not only for testing the generality of the mechanism of amoeba resistance both on solid media and in liquid cultures that are relevant to biofuel production, but will also be important for understanding localization of extracellular layers appearing during heterocyst development.

Inhibition of O-Antigen Production Confers Resistance to Grazing by HGG1. Inactivation of any of the four genes investigated here, each of which prevents the maturation of O-antigen on the outside of the cell, confers resistance to grazing by the amoeba HGG1. This finding is consistent with previous observations that serotype differences in *Salmonella* (19) or the marine *Synechococcus* sp. WH7803 (17) affect predation by protists. The discovery that

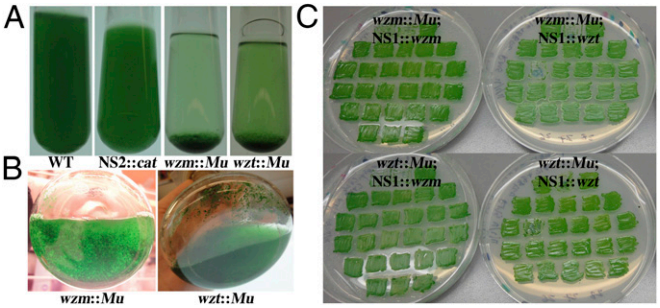

Fig. 5. Autoflocculation and rough phenotypes of O-antigen production mutants. (*A*) Test tube cultures were inoculated at an optical density of 0.03 at 750 nm. Cultures were grown on a roller drum at 30 °C under constant light for 10 d. WT *S. elongatus* grows planktonically, whereas pelleting was observed with the *wzm*::*Mu* and *wzt*::*Mu* mutant strains. (*B*) Clumping of the *wzm*::*Mu* and *wzt*::*Mu* strains in dense cultures grown in flasks. (*C*) Patches of complemented strains *wzm*::*Mu*;NS1::*wzm* and *wzt*::*Mu*;NS1::*wzt* displayed the WT-like smooth phenotype, which is defined by a moist, reflective appearance. In contrast, noncomplemented mutants *wzm*::*Mu*;NS1::*wzt* and *wzt*::*Mu*;NS1::*wzm* displayed the rough phenotype, which is defined by a dry, matte appearance.

alterations in O-antigen in *Anabaena* sp. PCC 7120 via mutations of the *rfbZ* (*all4830*) or *rfbP* (*all4829*) loci prevents cyanophage infectivity (55) further supports the hypothesis that removal of the conserved O-antigen transport or synthesis genes may be a general mechanism for generating cyanobacterial resistance to protistan grazers and viral pathogens. The resistance of rough, reviver strains generated by UV mutagenesis further indicates that O-antigen deletion is a primary pathway for generating amoeba-resistant strains.

Grazer-Resistant and Autoflocculating Cyanobacteria for Biofuel Production. Resistant strains not only display a visibly identifiable rough phenotype but are also capable of autoflocculating. Bioflocculation is a desirable trait for biofuel production strains as it allows for a cost- and energy-efficient method of harvesting and dewatering biomass, as opposed to costly and labor-intensive methodologies such as the use of chemical flocculants, filtration, centrifugation, and ultrasonic aggregation (2, 3, 5, 6). Thus, mutations that impair O-antigen production in *S. elongatus* confer two highly desirable biofuel production traits: resistance to grazing and the ability to autoflocculate.

Using what we know about the resistance pathway, we envision that cyanobacterial strains can be generated with the ability to turn on and off the expression of O-antigen, allowing resistance and autoflocculation to be finely controlled by an external signal such as cell density through quorum sensing or the presence of predators. Alternatively, because some null mutations confer resistance, markerless deletion strains or spontaneous rough mutants can be generated that do not incorporate exogenous DNA. Our experiments demonstrated that resistant strains can be selected through application of the HGG1 amoeba in combination with visual identification of the rough phenotype. The ability to generate resistant strains that do not carry genes from other genera will facilitate deployment of these organisms in field production, because such strains are not considered new under the Toxic Substances Control Act of the Environmental Protection Agency.

Materials and Methods

Strains, Plasmids, and Culture Conditions. See Table S1 for a list of vectors and strains used in this study. All *S. elongatus* strains were derived from WT strain AMC06. WT-like antibiotic-resistant strains were constructed using either pAM1573, a neutral site II (NS2) recombination vector encoding chloramphenicol resistance (*cat*), or pAM1579, a NS2 recombination vector encoding kanamycin resistance (*nptI*) (56). Insertional knockout mutants were generated using transposon-mutagenized cosmids from the UGS library (33, 34). Cultures were grown in BG-11 media (57) or BG-11M media (58) both in liquid or on solid medium under constant light levels ranging from 200 to 350 μmol photons $m^{-2}s^{-1}$ at 30 °C.

Isolation and Growth of Amoeba HGG1. A water sample from the Huntington Gardens' Chinese Garden lake (Pasadena, CA) was serially diluted in the wells of a flat bottom multiwell cell culture dish (Costar, Corning) using a *Leptolyngbia* sp. BL0902 log-phase culture as diluent. The dish was incubated in the light (30 μmol photons $m^{-2}s^{-1}$) at 22 °C for 5 d. One of the wells developed an orange clearing and when this area was sampled, amoebae were evident by phase contrast microscopy. This material was inoculated in 10-μL spots on a freshly plated lawn of BL0902 on a BG-11 plate. Following growth of the lawn (5 d) at 22 °C, clear enlarging plaques with orange rims were evident. Microscopic examination of the rims of the plaques revealed actively feeding amoebae. A small inoculum from the rim of a plaque was mixed with an actively growing liquid culture of BL0902, serial 10-fold dilutions were made using the BL0902 culture as diluent, and samples were spread on BG-11 plates. Following a 5-d incubation at 22 °C in the light (30 μmol photons $m^{-2}s^{-1}$), several isolated plaques were evident. Amoebae from the center of two of these plaques were used to infect BL0902, and the 18S rRNA DNA was amplified and sequenced as described below. The sequences were identical, and only one of these isolates, designated HGG1, was maintained and used for all subsequent work. With exception of the initial isolation of HGG1, all subsequent experiments were carried out at 30 °C at an illumination of 30–50 μmol photons $m^{-2}s^{-1}$ unless otherwise noted. HGG1 was routinely maintained on lawns of *S. elongatus* PCC 7942 by transferring material from the outer rim of a plaque to the center of a 4-d-old lawn.

Microscopy was carried out with a Zeiss Axioskop microscope equipped with phase contrast and differential interference optics and a filter set (excitation 510 nm, emission 560 nm) to detect fluorescence from chlorophyll.

Amoebal DNA Isolation and 18S rRNA Gene Amplification, Sequencing, and Phylogeny. Trophozoites were collected by scraping the edge of a plaque and resuspended in BG-11 followed by pelleting in a microfuge. Total DNA was extracted using the DNeasy kit (Qiagen) protocol for cultured cells. The 18S rRNA gene was amplified using the MoonA and MoonB primers (29) complementary to conserved sequences near the 5′ and 3′ termini of the 18S rRNA gene (Table S2). For detailed PCR parameters, see *SI Materials and Methods*. Following cleanup, both strands of the PCR product were directly sequenced using the following primers: MoonA, 527F and 1102F; MoonB, 543R and 1265R (Table S2). In addition, the PCR product was cloned into the TOPO TA 2.1 vector (Invitrogen) and both strands of the inserts from four independent clones were sequenced using the above primers.

One of the cloned HGG1 18S rRNA gene sequences (that of plasmid A, Fig. S1) was placed into an existing alignment of selected heterolobosean sequences (59) and manually aligned to this set. A neighbor-joining phylogenetic tree was constructed using the Seaview platform (60) using 1,000 replicates to generate bootstrap values. Genetic distances were calculated using the Kimura 2 parameter method.

Transformation of *S. elongatus*, Genotyping, and Cloning. Transformation and homologous recombination of vectors into *S. elongatus* were performed according to standard methods that take advantage of its natural competence (61–63). The genotypes of strains were confirmed by standard colony or whole cell PCR protocols using Taq DNA polymerase (NEB) or *Phusion* High-Fidelity DNA polymerase (NEB). ORFs were cloned by standard whole cell PCR protocols using *Phusion* High-Fidelity DNA polymerase and primers that added the TOPO-cloning tag to the 5′ end of the ORF. PCR products were cloned directly into the pSyn_1/D-TOPO expression vector (Life Technologies) according to the manufacturer's instructions. Expression clones were confirmed by sequencing before transformation of *S. elongatus*. Primers for genotyping, segregation testing, cloning, and sequencing are listed in Table S2.

Plaque Assays. Library screen plates were generated by spotting 4 μL of dense cultures from the UGS library onto BG-11M plates [1.5% (wt/vol) agar] containing the appropriate antibiotics. Plate cultures were grown at 30 °C and 350 μmol photons $m^{-2}s^{-1}$ for 13 d before amoebae addition. Lawn plates were generated by concentrating 1 mL of late-log or stationary phase cultures fourfold and spreading the concentrate onto BG-11 plates [1.5% (wt/vol) agar] containing the appropriate antibiotics. Plates were grown at 30 °C and 350 μmol photons $m^{-2}s^{-1}$ for 6–7 d before amoebae addition. Amoebae addition was performed by scraping the growing edges of plaques aseptically and resuspending the scrapings in BG-11 medium to generate a visibly dense mixture. A sample (4 μL) of this mixture was then applied to lawn plates or each spot of the library screen plates. After addition of amoebae, plates were incubated at 30 °C and 10 μmol photons $m^{-2}s^{-1}$.

Purification and Analysis of Outer Membranes. Outer membrane purifications were performed according to a modification of the EDTA-sucrose method of Brahamsha (64). For specific details, see *SI Materials and Methods*. Purified outer membrane samples were denatured according to the Novex Tris-glycine SDS gel manual (Life Technologies) and then digested with 1.2 mg/mL of proteinase K (Sigma) at 50 °C for 20 min. Samples were analyzed by gel electrophoresis through a 14% (wt/vol) Tris-glycine SDS gel. Gels were stained and visualized for carbohydrates according to the Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit manual (Life Technologies).

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