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Cell Envelope Components Influencing Filament Length in the Heterocyst-Forming Cyanobacterium *Anabaena* sp. Strain PCC 7120

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Heterocyst-forming cyanobacteria grow as chains of cells (known as trichomes or filaments) that can be hundreds of cells long. The filament consists of individual cells surrounded by a cytoplasmic membrane and peptidoglycan layers. The cells, however, share a continuous outer membrane, and septal proteins, such as SepJ, are important for cell-cell contact and filament formation. Here, we addressed a possible role of cell envelope components in filamentation, the process of producing and maintaining filaments, in the model cyanobacterium *Anabaena* sp. strain PCC 7120. We studied filament length and the response of the filaments to mechanical fragmentation in a number of strains with mutations in genes encoding cell envelope components. Previously published peptidoglycan- and outer membrane-related gene mutants and strains with mutations in two genes (*all5045* and *alr0718*) encoding class B penicillin-binding proteins isolated in this work were used. Our results show that filament length is affected in most cell envelope mutants, but the filaments of *alr5045* and *alr2270* gene mutants were particularly fragmented. All5045 is a DD-transpeptidase involved in peptidoglycan elongation during cell growth, and Alr2270 is an enzyme involved in the biosynthesis of lipid A, a key component of lipopolysaccharide. These results indicate that both components of the cell envelope, the murein sacculus and the outer membrane, influence filamentation. As deduced from the filament fragmentation phenotypes of their mutants, however, none of these elements is as important for filamentation as the septal protein SepJ.

Multicellularity appears to have arisen several times during the course of evolution and has evolved in different phylogenetic groups, including bacteria, fungi, algae, plants, and metazoans (1, 2). Multicellularity generally involves cell-cell adhesion, intercellular communication, and the differentiation of specialized cells. Bacterial manifestations of multicellularity range from undifferentiated chains of cells to morphologically differentiated structures, and the behavior of cells within multicellular structures is coordinated by both shared and unique molecular mechanisms (3). Because they present differentiated cells that carry out distinct functions, some prokaryotes, including myxobacteria, streptomycetes, and cyanobacteria, are classified as patterned multicellular organisms (3). Cyanobacteria are characterized by performing oxygenic photosynthesis, but they show very diverse morphologies that include both unicellular and multicellular forms (4). Among multicellular forms, some cyanobacteria, such as those of the genera *Anabaena* and *Nostoc*, make filaments (also known as trichomes) that can be hundreds of cells long. When deprived of nitrogen, the cells of these genera are characterized by specialized cells that have differing nutritional tasks, oxygenic photosynthesis and nitrogen fixation, which contribute to the growth of the filament as an organismic unit (5, 6).

Because an exchange of carbon and nitrogen compounds occurs between CO_2-fixing vegetative cells and N_2-fixing heterocysts (5, 6), filament integrity is essential for the diazotrophic growth of heterocyst-forming cyanobacteria. In the model heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 (here referred to as *Anabaena*), several genes whose mutation results in filament fragmentation are known (7–13), and some of these genes encode cell-cell joining proteins. The *fraCDE* operon and the *sepJ* gene encode integral membrane proteins that are required for *Anabaena* to make long filaments, mainly when deprived of fixed nitrogen, although inactivation of *sepJ* produces a stronger filament fragmentation phenotype than inactivation of any of the *fra* genes (9–12). Available evidence suggests that SepJ on one hand and FraC and FraD on the other are part of septal junction complexes that are likely involved not only in filament integrity but also in intercellular molecular exchange (14, 15).

Cyanobacteria are diderm bacteria (16) that bear a cytoplasmic membrane (CM) and an outer membrane (OM), the latter lying outside the peptidoglycan (PG) layer or murein sacculus (17, 18). Whereas the CM is a phospholipid bilayer, the OM is an asymmetrical bilayer consisting of phospholipids and lipopolysaccharide (LPS) in the inner and outer leaflet, respectively (19). The permeability of the OM is due to the presence of porins, proteins with a β-barrel fold that permit the passage of small ions and molecules (20). In a proteomic analysis of *Anabaena*, some OM porins could be identified (21). Omp85 proteins are required for the incorporation of porins and other β-barrel proteins into the OM (22). The *Anabaena* genome encodes three Omp85-like proteins, Alr0075, Alr2269, and Alr4893, among which Alr2269 likely represents the principal Omp85-like protein in this cyanobacterium (23–26). LPS is composed of a hydrophobic membrane anchor (lipid A), an oligosaccharide core, and an O antigen consisting of a repeating oligosaccharide (19, 27). LPS is a barrier for hydrophobic molecules (28), which, coupled with a porin diffusion limit for hydrophilic molecules of about 700 Da, makes the OM an effective selective permeability barrier (20). Although showing some char-

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TABLE 1 Cell envelope mutant strains used in this work

Strain	Genotype	Protein product/predicted function	Reference
AFS-I-*alr2269*[a]	*alr2269*::pCSV3	Chloroplast outer envelope membrane protein homolog (Omp-85-like protein); involved in outer membrane protein biogenesis	26
AFS-I-*alr0075*[a]	*alr0075*::pCSV3	Omp-85-like protein; not expressed in heterocysts	26
AFS-I-*alr4893*[a]	*alr4893*::pCSV3	Omp-85-like protein	26
AFS-I-*alr2270*[a]	*alr2270*::pCSV3	LpxC; UDP-3-*O*-acyl *N*-acetylglucosamine deacetylase; involved in lipid A biosynthesis	26
DR1822	Tn*5*::Ω within *all4829*	RfbP; UDP-galactophosphotransferase; involved in O antigen biosynthesis	29
DR1963	Tn*5*::Ω within *all4830*	RfbZ; mannosyl transferase; involved in O antigen biosynthesis	29
DR1967[a]	*all4828*::Ω	RfbD; GDP-D-mannose dehydratase; involved in O antigen biosynthesis	29
AFS-I-*all1861*[a]	*all1861*::pCSV3	Peptidoglycan-binding protein	Rudolf et al., unpublished
CSR27[a]	*alr2458*::pCSV3	Alanine racemase; likely involved in peptidoglycan biosynthesis	
DR1992	*alr0093*::Tn*5*-1058	HcwA (AmiC2); *N*-acetylmuramoyl–L-alanine amidase; involved in peptidoglycan metabolism	38
CSMI23	Δ*alr5045*::C.K3	PBP	This study
CSMI24[a]	Δ*alr0718*	PBP; FtsI	This study
CSVM34	Δ*alr2338*	Septal protein SepJ	55
CSR10	*alr4167*::pCSV3	BgtA; ATPase subunit of a basic amino acid ABC transporter	56

[a] PCR analysis with genomic DNA and primers listed in Table S1 in the supplemental material showed that the strain is heterozygous, containing both wild-type and mutant chromosomes (see Fig. S2 to S4 in the supplemental material).

acteristic differences, the cyanobacterial OM conserves the main features of the OM of Gram-negative bacteria (18). In *Anabaena*, genes encoding lipid A biosynthetic proteins (*alr2270* to *alr2274*) are clustered in an operon with Omp85-encoding *alr2269* (26), and a cluster of genes encoding O antigen-related enzymes has also been characterized (29). The cyanobacterial PG differs from the best-investigated peptidoglycans in that it is thicker and possesses a higher degree of cross-linking between PG chains than those in other Gram-negative bacteria (17, 18). Nonetheless, the essential enzymatic machinery of PG biogenesis and metabolism, including glycosyltransferases, DD-transpeptidases, and hydrolases (30), is conserved in cyanobacteria (18, 31). Bifunctional enzymes with transpeptidase and glycosyltransferase activities are known as class A penicillin-binding proteins (PBPs), and monofunctional transpeptidases are known as class B PBPs (30).

In filamentous cyanobacteria, the OM is continuous along the filament, determining the presence of a continuous periplasmic space that contains the murein sacculus (32–35). *Anabaena* bears a murein sacculus composed of two or three PG layers, which surround each cell in the filament, so that PG is also present in the intercellular septa (35). As observed by transmission electron microscopy (TEM) and electron tomography, the PG layers of adjacent cells are fused in a number of intercellular septa, but in some other septa, they are seen well separated (33, 35). The murein sacculus has been isolated from some heterocyst-forming cyanobacteria, and sacculi corresponding to several cell units have been observed, implying that the PG layers from adjacent cells are indeed fused (chemically interlinked) in a substantial number of the intercellular septa of the filament (36, 37).

The aim of this study was to elucidate a possible role of cell envelope components, PG and OM, in filamentation, the process of producing and maintaining filaments, in heterocyst-forming cyanobacteria. For this study, available *Anabaena* strains with mutations in genes encoding proteins involved in the biosynthesis of the cell envelope were investigated (Table 1). With respect to the OM, strains with mutations in genes encoding Omp85-like proteins and a lipid A biosynthesis protein (26), as well as proteins involved in the biosynthesis of the O antigen of LPS (29), have been studied. For the murein sacculus, strains with mutations in genes encoding an amidase known as HcwA (38) or AmiC2 (39), the All1861 protein that contains two PG-binding domains, and an alanine racemase (M. Rudolf, N. Tetik, N. Flinner, G. Ngo, M. Stevanovic, M. Burnat, R. Pernil, E. Flores and E. Schleiff, unpublished data) have been studied. In addition, two genes, *alr0718* and *alr5045*, that encode class B PBPs have been inactivated and characterized. Below, we describe the last of these and then present our analysis of cell envelope properties and filament integrity. We have found that both components of the cell envelope, PG and OM, contribute to make *Anabaena* filaments long.

MATERIALS AND METHODS

Strains and growth conditions. *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120 was grown axenically in BG11 medium (containing $NaNO_3$), $BG11_0$ medium (free of combined nitrogen), or $BG11_0$ NH_4^+ medium {$BG11_0$ containing 4 mM NH_4Cl and 8 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]-NaOH buffer, pH 7.5}. In every case, ferric citrate replaced the ferric ammonium citrate used in the original recipe (4). For plates, the medium was solidified with 1% separately autoclaved Difco agar. Cultures were grown at 30°C in the light (25 $\mu E\ m^{-2}\ s^{-1}$ [1 E is the energy in 1 mol of photons]), with shaking (80 to 90 rpm) for liquid cultures. For the mutants described below, antibiotics were used at the following concentrations: streptomycin sulfate (Sm) and spectinomycin dihydrochloride pentahydrate (Sp), 5 $\mu g\ ml^{-1}$ each for both liquid and solid media, and neomycin sulfate (Nm), 40 $\mu g\ ml^{-1}$ for solid media and 25 $\mu g\ ml^{-1}$ for liquid media. DNA was isolated from *Anabaena* by the method of Cai and Wolk (40). All *Anabaena* strains used in this work are listed in Table 1. *Escherichia coli* DH5α was used for plasmid constructions. It and strains HB101 and ED8654, used for conjugation with *Anabaena*, were grown in Luria-Bertani medium supplemented when appropriate with antibiotics at standard concentrations.

Plasmid construction and genetic procedures. To inactivate *alr5045*, two DNA fragments, one encompassing 566 bp from sequences upstream of the central region of the gene and the other one including 601 bp from sequences downstream of the central region of the gene, were amplified by standard PCR using DNA from *Anabaena* as the template and primer pairs alr5045–1/alr5045-2 and alr5045-3/alr5045-4, respectively. (All oli-

godeoxynucleotide primers and plasmids used in this work are listed in Table S1 in the supplemental material.) The two DNA fragments were cloned in pSpark (Canvax, Biotech SL), producing plasmid pCSMI50, and, after corroboration by sequencing and digestion, ligated in direct orientation separated by gene cassette C.K3 encoding neomycin phosphotransferase, producing plasmid pCSMI51. The insert of the resulting plasmid, excised with SacI, was transferred to SacI-digested pCSRO (41), producing pCSMI52.

To inactivate *alr0718*, two DNA fragments, one encompassing 710 bp from sequences upstream of the central region of the gene and the other one including 554 bp from sequences downstream of the central region of the gene, were amplified by standard PCR using *Anabaena* DNA as the template and primer pairs alr0718-7/alr0718-8 and alr0718-9/alr0718-10, respectively. The two DNA fragments were cloned in pSpark (Canvax, Biotech SL), producing plasmid pCSMI48, and after corroboration by sequencing, the insert of the resulting plasmid, excised with SacI, was transferred to SacI-digested pCSRO (41), producing pCSMI49.

Conjugation of *Anabaena* with *E. coli* HB101 carrying the cargo plasmid (pCSMI52 or pCSMI49) with the helper and methylation plasmid pRL623 was effected by the conjugative plasmid pRL443, carried in *E. coli* ED8654, and performed as described previously (42), with selection for resistance to Sm and Sp for pCSMI49 and to Nm for pCSMI52. Exconjugants were isolated, and double recombinants were identified as clones resistant to sucrose (and Nm for pCSMI52) (40) and sensitive to the antibiotic for which the resistance determinant was present in the vector portion of the transferred plasmid (Sm/Sp). The genetic structures of selected clones were studied by PCR with DNA isolated from those clones and primers alr5045-5/alr5045-6 and alr5045-5/C.K3-5′ for *alr5045* and alr0718-11/alr0718-12 and alr0718-11/alr0718-1 for *alr0718*. A clone homozygous for the chromosomes with mutations in *alr5045* was chosen for further analysis and named strain CSMI23 (see Fig. S1 in the supplemental material). Although no clone containing only chromosomes with *alr0718* mutated was obtained, one with a low number of wild-type chromosomes was selected for further analysis and named CSMI24 (see Fig. S2 in the supplemental material).

Mutant chromosome segregation. Genomic DNA from strains AFS-I-*alr2269*, AFS-I-*alr0075*, AFS-I-*alr4893*, and AFS-I-*alr2270* (26) and DR1822, DR1963, and DR1967 (29), and from DR1992 (38), was subjected to PCR analysis to test the segregation of mutant chromosomes. The primers used are shown in Table S1 and the PCR results in Fig. S3 and S4 in the supplemental material.

Growth tests. The protein concentrations and chlorophyll *a* (Chl) contents of the cultures were determined by a modified Lowry procedure (43) and by the method of Mackinney (44), respectively. The growth rate constant ($\mu = \ln 2/t_d$, where t_d is the doubling time) was calculated from the increase of the protein content, determined in 0.2-ml samples, of shaken liquid cultures (45). The cultures were inoculated with an amount of cells containing about 5 μg of protein ml^{-1} and grew logarithmically until they reached about 40 μg of protein ml^{-1}. For tests of growth on solid media, cultures grown in BG11 medium (supplemented with antibiotics when appropriate) were harvested and washed three times with 50 ml of $BG11_0$ medium, and dilutions were prepared in $BG11_0$ medium. Ten-microliter samples of the resulting suspensions were spotted on agar plates with different nitrogen sources and incubated at 30°C in the light (25 μE m^{-2} s^{-1}).

Sensitivity to harmful compounds was tested by spotting 2 μl of a cell suspension containing 2.5 μg Chl ml^{-1} on agar plates with BG11 medium supplemented with SDS, lysozyme, or proteinase K at 5 to 30 μg ml^{-1} or with erythromycin at 0.005 to 0.1 μg ml^{-1}. Because some of the mutants still contained wild-type copies of the mutated genes, for these mutants, the plates were supplemented with 5 μg ml^{-1} each of Sp and Sm to maintain the selection pressure for the mutated chromosomes. In these plates, strain CSR10 (Sm^r Sp^r) was used as a control instead of PCC 7120. The plates were incubated at 30°C in the light (25 μE m^{-2} s^{-1}) and observed over a period of 2 weeks.

Nitrogenase activity. Filaments grown in BG11 medium were harvested, washed, and resuspended in $BG11_0$ medium. After 24 h of incubation under growth conditions, the filaments were used in acetylene reduction assays performed under oxic or anoxic conditions, as described previously (45). For the anoxic assays, the cell suspensions, placed in sealed flasks, were supplemented with 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), bubbled with argon for 3 min, and incubated for 90 min under assay conditions before starting the reaction by addition of acetylene.

Substrate uptake assays. Cells grown in BG11 medium were harvested by centrifugation at 4,000 rpm at room temperature, washed twice with 25 mM Tricine [*N*-tris(hydroxymethyl)-methylglycine]-NaOH buffer (pH 8.1), and resuspended in the same buffer. Transport assays were carried out at 30°C in the light (white light from fluorescent lamps; about 175 μE m^{-2} s^{-1}). Amino acid uptake was determined as described previously (45) in 10-min transport assays that were started by mixing a suspension of cells (1.1 ml) containing 10 μg of Chl with a solution (0.1 ml) of L-[U-^{14}C]glutamic acid (253 mCi $mmol^{-1}$) or L-[U-^{14}C]aspartic acid (207 mCi $mmol^{-1}$) (both radiolabeled amino acids were purchased from Amersham, GE Healthcare, United Kingdom). The final concentration of amino acids in the assays was 10 μM. The amount of amino acid taken up was determined in a 1-ml sample of the cell suspension. The sample was filtered (0.45-μm-pore-size Millipore HA filters were used), and the cells on the filters were washed with 5 to 10 ml of 5 mM Tricine-NaOH buffer (pH 8.1). The filters carrying the cells were then immersed in 5 ml of scintillation cocktail, and their radioactivity was measured. The retention of radioactivity by boiled cells was used as a blank.

Microscopy. Cells grown in shaken liquid BG11 medium or incubated in shaken liquid $BG11_0$ medium were observed and photographed with a Zeiss Axioscop microscope equipped with a Zeiss ICc1 digital camera. To determine the percentage of heterocysts, about 3,000 cells of each strain from each tested growth condition were counted. For TEM, 50-ml cultures from the wild type and strain CSMI23 grown in BG11 medium were harvested and fixed in 2.5% glutaraldehyde-$BG11_0$ medium for 1 h at room temperature and washed two times with 10 ml of wash buffer (0.05 M Na-cacodylate, 0.4 M sucrose, pH 7.2) for 15 min at room temperature. Samples were postfixed in 1% OsO_4 at 4°C for 1 h and washed three times with wash buffer. They were then dehydrated in a graded series (30, 50, 70, 80, 90, and 100%) of ethanol and embedded in araldite resin. Ultrathin sections (40 to 50 nm) were mounted on carbon-coated copper grids and stained with uranyl acetate. Samples were visualized in a transmission electron microscope (Philips CM12). Septa and longitudinal axes of the cells were measured in TEM micrographs using ImageJ software (http://imagej.nih.gov/ij).

In vivo labeling with fluorescent vancomycin (Van-FL) (Bodipy FL Conjugate; Invitrogen) was done as described by Lehner et al. (46). Samples (50 μl) from cultures grown in BG11 medium were used. Filaments were incubated for 1 h in the dark with Van-FL (1 mg ml^{-1}) and washed twice with BG11 medium. The samples were visualized with a Leica DM6000B fluorescence microscope and an Orca-ER camera (Hamamatsu). Fluorescence was monitored using a fluorescein isothiocyanate (FITC) L5 filter (excitation, band-pass [BP] 480/40 filter; emission, BP 527/30 filter) and analyzed with ImageJ. The fluorescence per surface area was determined for the indicated numbers of septal regions, and the background fluorescence per surface area in the micrograph was subtracted.

Mechanical fragmentation of filaments. To determine the filament size distribution, samples from BG11-grown cultures were taken with great care to prevent disruption of the filaments and visualized by standard light microscopy. For mechanical fragmentation of filaments, 10-ml samples from the same cultures were passed three times through a 25-gauge (G) (length, 5/8 in.) syringe, and the filaments in the resulting suspension were visualized by standard light microscopy. To determine the filament size (the number of cells per filament), 170 to 1,700 filaments were counted from each strain. Comparison of the filament lengths from

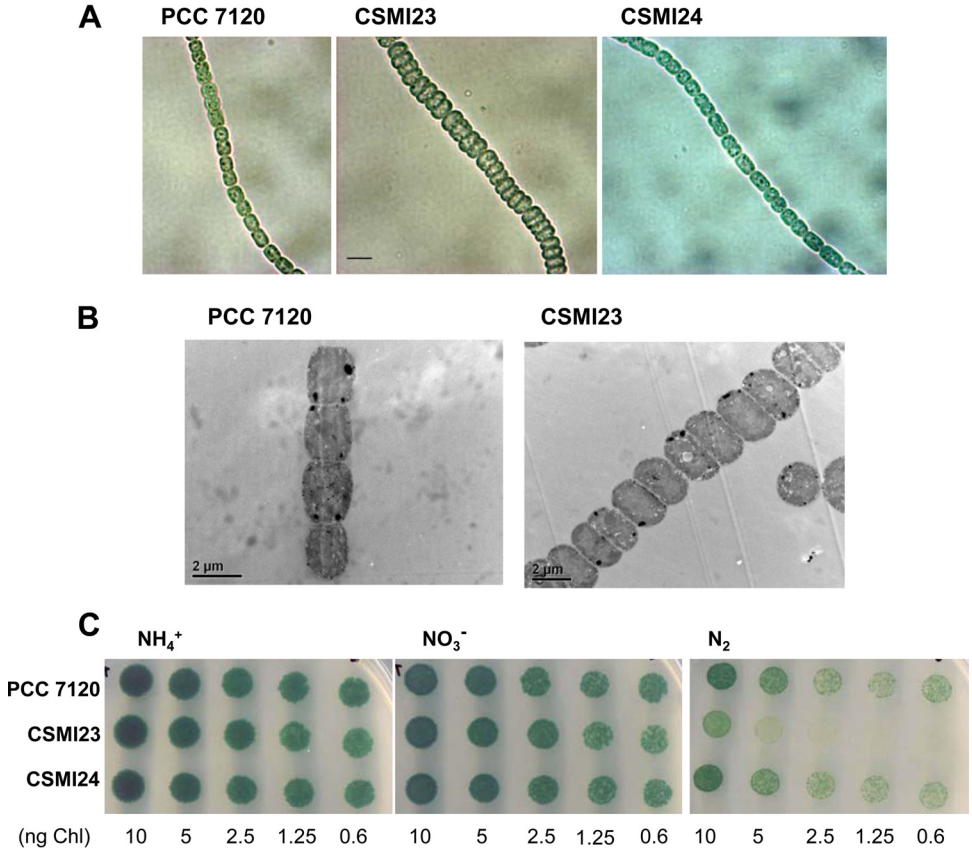

FIG 1 Characterization of *Anabaena alr5045* and *alr0718* mutants. (A) Filaments of *Anabaena* sp. strains PCC 7120, CSMI23, and CSMI24 from cultures incubated in BG11 medium visualized by light microscopy (scale bar, 5 μm; the same magnification was used for the three micrographs). (B) Filaments of *Anabaena* sp. strains PCC 7120 and CSMI23 from cultures incubated in BG11 medium visualized by transmission electron microscopy. (C) Growth tests of strains PCC 7120, CSMI23, and CSMI24 in solid medium using ammonium ($BG11_0NH_4^+$), nitrate (BG11), or N_2 ($BG11_0$) as the nitrogen source. Each spot was inoculated with an amount of cells containing the indicated amount of chlorophyll *a* (Chl), and the plates were incubated under culture conditions for 9 days and photographed.

mechanically fragmented filaments and the distributions of filament sizes in different strains were assessed by Student's *t* test and the χ^2 test, respectively.

RESULTS

Isolation and characterization of *alr5045* and *alr0718* mutants. In order to extend the set of cell envelope mutants used in this study, two genes, *alr5045* and *alr0718*, both predicted to encode class B PBPs (31, 47), were inactivated. Based on their protein sequences, Alr0718 and Alr5045 are 609- and 610-amino-acid-residue proteins, respectively, both bearing two functional domains: a dimerization domain close to their N termini and a β-lactamase-type transpeptidase fold in their C-terminal halves (47). Open reading frame (ORF) *alr5045* was inactivated by deletion of a 1,329-bp internal fragment of the gene with insertion of a neomycin resistance-conferring gene cassette (C.K3). After transferring the gene construct by conjugation into *Anabaena*, a selected clone that was homozygous for chromosomes bearing the Δ*alr5045*::C.K3 mutation was named strain CSMI23 (see Fig. S1 in the supplemental material). To create an *Anabaena alr0718* mutant, a 1,392-bp fragment internal to the gene was deleted without leaving any gene marker behind to avoid polar effects on neighboring genes (see Fig. S2 in the supplemental material). Several clones bearing this deletion were obtained, but genotypic characterization performed by PCR using genomic DNA from those clones indicated that none was homozygous for the mutant chromosome. Thus, *alr0718* is likely an essential gene in *Anabaena*. Although not fully segregated, a clone with a low number of wild-type chromosomes, strain CSMI24 (see Fig. S2 in the supplemental material), was used in this study.

As observed by light microscopy, strain CSMI23 showed an evident alteration in cell morphology both in medium containing (BG11) and medium lacking ($BG11_0$) combined nitrogen (Fig. 1A shows filaments from BG11 medium). In contrast, no evident alteration in cell morphology was observed in mutant CSMI24. In order to determine the sizes of the cells from these mutants, the cell axis parallel to the filament axis (which is called the longitudinal axis) and, as an indication of cell width, intercellular septa were measured for a number of cells in filaments visualized by light microscopy. Strain CSMI23 cells showed a shorter longitudinal axis and wider septum than the cells from the wild type, whereas strain CSMI24 cells presented dimensions that differed little from those of the wild-type cells (Table 2). Nonetheless, Student's *t* tests indicated that the dimensions of the cells were significantly different ($P \leq 10^{-6}$) in each of the mutants and the wild type. In order to corroborate the altered cell dimensions of strain CSMI23, filaments of the mutant were also visualized by TEM,

TABLE 2 Cell dimensions, growth rates, and nitrogenase activities of CSMI23 and CSMI24 mutants

Strain	Cell dimensions[a] (μm)		Growth rate constant (μ) (day^{-1})[b]		Nitrogenase activity (μmol mg Chl^{-1} min^{-1})[c]	
	Longitudinal axis	Septum	NO_3^-	N_2	Oxic	Anoxic
PCC 7120	3.74 ± 0.88 (415)	2.36 ± 0.36 (428)	0.83 ± 0.13 (4)	0.76 ± 0.20 (6)	8.35 ± 1.58 (3)	15.26 ± 3.05 (5)
CSMI23	2.68 ± 0.68 (302)	3.58 ± 0.69 (288)	0.82 ± 0.12 (4)	0.71 ± 0.31 (6)	2.34 ± 0.60 (3)	3.66 ± 0.83 (3)
CSMI24	3.39 ± 0.74 (231)	2.13 ± 0.32 (235)	0.88 ± 0.16 (4)	0.77 ± 0.24 (6)	6.71 ± 1.89 (3)	ND

[a] The cell dimensions were determined in filaments grown in BG11 (nitrate-containing) medium and viewed by light microscopy. Septum dimensions were used as a measure of cell width. Shown are means and standard deviations of the data from the number of cells indicated in parentheses.
[b] Growth rate constants were determined from the increase in protein content of the cultures with the indicated nitrogen source, measured as described in Materials and methods. Shown are means and standard deviations of the data from the number of independent cultures indicated in parentheses.
[c] Nitrogenase activity was determined as the ethylene produced in acetylene reduction assays performed under oxic and anoxic conditions in filaments incubated for 24 h in the absence of combined nitrogen. Shown are means and standard deviations of the data from the number of independent cultures indicated in parentheses. Student's *t* test analysis indicated that nitrogenase activity was significantly different in strain CSMI23 and the wild type under both oxic ($P = 0.007$) and anoxic ($P = 0.001$) conditions. ND, not done.

which confirmed the morphological alteration of the cells (Fig. 1B). Strain CSMI23 cells showed a shorter longitudinal axis (1.51 ± 0.33 μm [mean ± standard deviation]; $n = 136$) and wider septum (1.94 ± 0.51 μm; $n = 114$) than the cells from the wild type (2.57 ± 0.48 μm, $n = 33$, and 1.16 ± 0.26 μm, $n = 28$, respectively). Student's *t* test showed that both the lengths of longitudinal axes ($P = 10^{-31}$) and widths of the septa ($P = 10^{-11}$) differed significantly in the mutant relative to the wild type. The cell dimensions determined by light or electron microscopy were somewhat different, but the cells could have been affected by the fixation procedure used for electron microscopy.

Strains CSMI23 and CSMI24 grew well on solid media supplemented with a source of combined N, either nitrate or ammonium, but on solid medium free of combined nitrogen, strain CSMI23 exhibited weak growth compared to the wild type (Fig. 1C). Determination of the growth rate constants (μ) in liquid cultures showed, however, that the growth rate of CSMI23 under diazotrophic conditions was only 7% lower than that of the wild type, whereas no difference was found between the growth rate constant of strain CSMI24 and that of the wild type (Table 2). The growth rates of strains CSMI23 and CSMI24 in nitrate-containing medium were similar to that of the wild type (Table 2). The nitrogenase activities of strains PCC 7120, CSMI23, and CSMI24 were determined in filaments incubated for 24 h without combined nitrogen (Table 2). The nitrogenase activities assayed under oxic conditions were about 28% and 80% of the wild-type activity for strains CSMI23 and CSMI24, respectively. Under anoxic conditions, the activity of CSMI23 was still about 24% that of the wild type. At 24 and 48 h after removal of combined nitrogen, heterocysts were 2.7 and 7.4% of total cells, respectively, in strain CSMI23 and 7.6 and 9.2%, respectively, in the wild type. Thus, heterocyst differentiation appears delayed in strain CSMI23. Whereas these results confirm a deficit in heterocyst differentiation and physiology in strain CSMI23, we cannot rule out the possibility that lack of alteration in the growth phenotype of strain CSMI24 is due to lack of segregation of the mutant chromosomes.

Vancomycin recognizes the free D-Ala-D-Ala dipeptide of lipid II or uncrosslinked murein, and a fluorescently labeled derivative of vancomycin, Van-FL, binds to regions of the cell that are actively synthesizing PG (46, 48, 49). Here, we used Van-FL to look into the morphology of CSMI23 and CSMI24. In the wild type, Van-FL labeling was mainly observed at intercellular septa, and different filaments showed intercellular septa labeled to different levels, perhaps indicative of differences in the growth stage, although most septa showed fluorescence of 90 to 190 units (frequencies above 0.02) (Fig. 2). In strain CSMI23, strong label was also observed in the intercellular septa, which showed a tendency to accumulate more label than wild-type septa (Fig. 2). In contrast, relatively weak labeling was observed in the intercellular septa of strain CSMI24 (Fig. 2).

OM permeability. Because the OM is generally a permeability barrier for some harmful substances (20) and, in the case of *Anabaena*, also for some metabolites, including acidic amino acids (26), we tested OM permeability in the different cell envelope mutants by studying sensitivity to erythromycin, SDS, lysozyme, and proteinase K and uptake of aspartate and glutamate. *Anabaena* is polyploid (50), and although some of the investigated mutants were not homozygous for the mutant chromosomes, they could be studied as heterozygous strains. Strains AFS-I-*alr2269*, AFS-I-*alr0075*, AFS-I-*alr4893*, and AFS-I-*alr2270* have been previously investigated and showed the presence of an OM with increased permeability in the strains bearing a low number of wild-type *alr2269* or *alr2270* genes (26). The sensitivity of strains AFS-I-*all1861* and CSR27 to some harmful compounds will be described in detail elsewhere (Rudolf et al., unpublished). Examples of tests of the sensitivities of the other mutants investigated in this work to the harmful compounds are shown in Fig. S5 in the supplemental material. Relevant information is summarized in Table 3. Strains DR1822, DR1963, and DR1967 (strains with mutations in genes related to the biosynthesis of the O antigen of LPS) were sensitive to SDS and, in the cases of DR1822 and DR1963, also to lysozyme, proteinase K, and erythromycin. Strains AFS-I-*all1861*, CSR27, DR1992, and CSMI23 (with genes related to PG biogenesis mutated) were sensitive to SDS and, in the cases of CSR27 and CSMI23, also to lysozyme and proteinase K. These results show that the OM has increased permeability in LPS mutants (AFS-I-*alr2270*, DR1822, DR1963, and DR1967) and also that some PG mutants (AFS-I-*all1861*, CSR27, DR1992, and CSMI23) are sensitive mainly to SDS.

The abilities of aspartate and glutamate to be taken up were tested in the cell envelope mutants and compared with those of the wild type and of strains AFS-I-*alr2269*, AFS-I-*alr2270*, AFS-I-*alr4893*, and AFS-I-*alr0075*, which have been previously reported (26). Substantial increases in glutamate and aspartate uptake in strains AFS-I-*alr2269* and AFS-I-*alr2270* has been interpreted to result from increased permeability of the OM in these mutants (26). OM mutants DR1963 and DR1967 showed increased transport of glutamate (1.9-fold and 1.7-fold, respectively) and aspar-

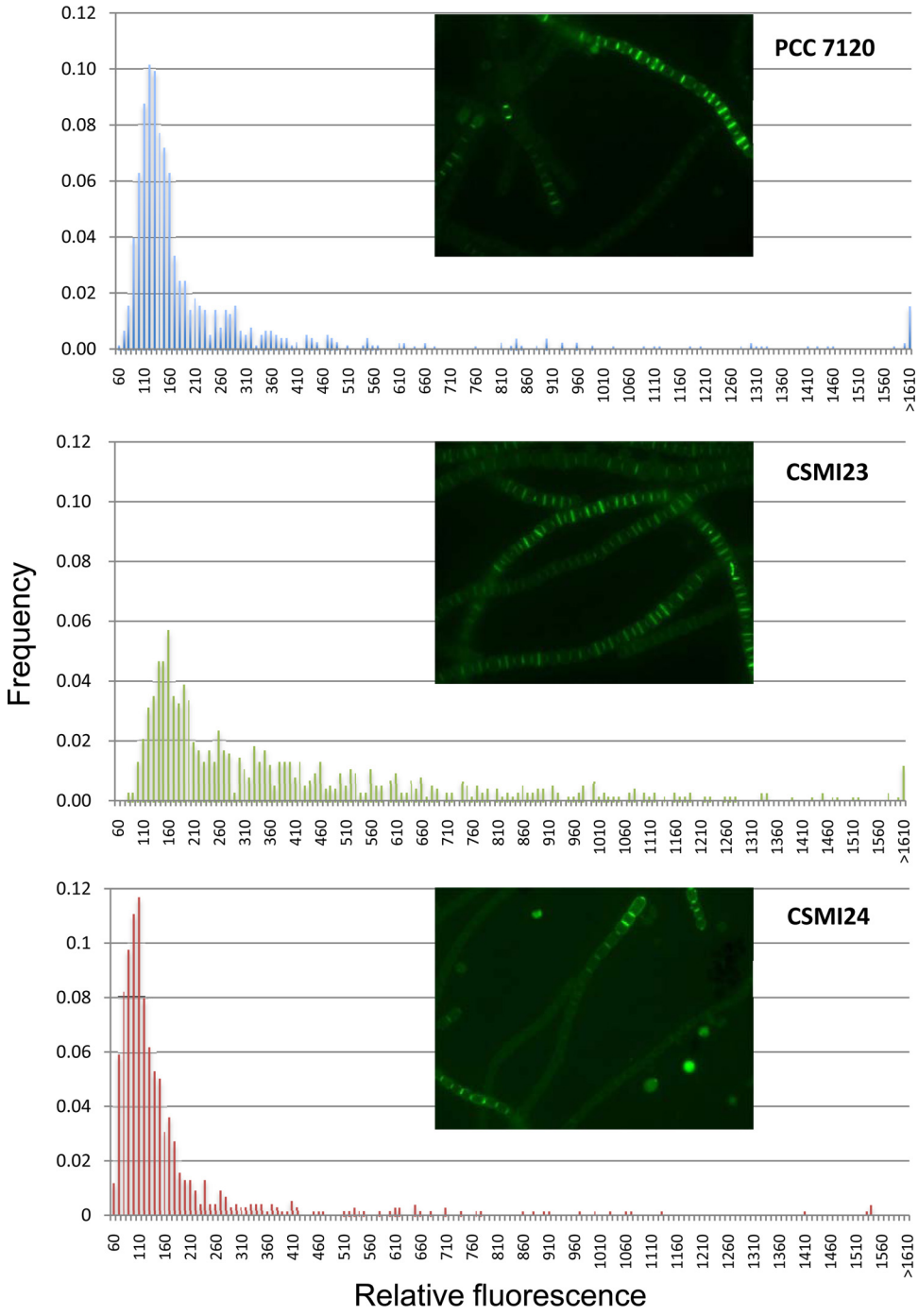

FIG 2 Staining with Van-FL of *Anabaena* wild type and *alr5045* and *alr0718* mutants and quantification of fluorescence from the intercellular septa. The insets show representative examples of filaments of strains PCC 7120, CSMI23, and CSMI24 from cultures incubated in BG11 medium, stained with Van-FL, and visualized by fluorescence microscopy. (The brightness and contrast were increased to improve visibility.) The histograms show the distribution of labeling quantified in 777 (strains PCC 7120 and CSMI24) or 769 (strain CSMI23) intercellular septa.

tate (2.2-fold and 1.6-fold, respectively) compared to the wild type (Table 3). The PG biosynthesis mutant CSR27 showed increased glutamate (2.1-fold) and aspartate (2.1-fold) uptake compared to the wild type. All other cell envelope mutants, however, showed transport activities for the two amino acids that could not be considered significantly different from those of the wild type (with the possible exception of strain CSMI24 and, only for aspartate uptake, strain DR1822). Thus, the increased permeability resulting from inactivation of some components of the cell envelope was also observed as increased uptake of aspartate and glutamate in strains DR1963, DR1967, and CSR27.

Filament lengths. Once the permeability of the OM in the cell envelope mutants was assessed through studies of sensitivity to harmful compounds and transport of acidic amino acids, we de-

TABLE 3 Sensitivity to harmful compounds and uptake of acidic amino acids in *Anabaena* cell envelope mutants

Strain	Segregation of mutant chromosomes[a]	Sensitivity to harmful compounds[b]	Uptake (nmol mg Chl^{-1})[c]			
			Glu	*P*	Asp	*P*
PCC 7120	Wild type		18.23 ± 7.75 (11)		43.48 ± 11.32 (11)	
AFS-I-*alr2269*[d]	No	Em, SDS, Lsz, PK	152.7 ± 13.1 (5)		161.9 ± 15.1 (5)	
AFS-I-*alr0075*[d]	No	NS	13.8 ± 0.7 (12)		40.7 ± 1.7 (10)	
AFS-I-*alr4893*[d]	No	NS	13.6 ± 0.5 (11)		39.9 ± 2.0 (12)	
AFS-I-*alr2270*[d]	No	Em, SDS, Lsz, PK	238.5 ± 11.3 (4)		250.7 ± 8.0 (4)	
DR1822	Yes	Em, SDS, Lsz, PK	30.32 ± 22.63 (6)	0.170	63.10 ± 20.85 (6)	0.039
DR1963	Yes	Em, SDS, Lsz, PK	34.18 ± 9.20 (6)	0.004	93.73 ± 31.64 (6)	0.001
DR1967	No	SDS	31.06 ± 9.45 (6)	0.015	70.95 ± 11.22 (5)	0.001
AFS-I-*all1861*	No[e]	SDS[e]	23.30 ± 6.23 (2)	0.445	57.58 ± 3.40 (2)	0.142
CSR27	No[e]	SDS, Lsz, PK[e]	38.24 ± 0.74 (2)	0.008	90.09 ± 9.65 (2)	0.001
DR1992	Yes	SDS	16.75 ± 8.15 (4)	0.774	39.48 ± 15.91 (4)	0.634
CSMI23	Yes	SDS, Lsz, PK	25.76 ± 4.72 (4)	0.119	38.75 ± 13.79 (4)	0.551
CSMI24	No	NS	28.72 ± 7.72 (4)	0.055	55.46 ± 7.63 (4)	0.097
CSVM34	Yes[f]	ND	ND		ND	

[a] The presence of wild-type and mutant chromosomes was tested by PCR, as shown in Fig. S1 to S4 in the supplemental material.
[b] Sensitivity to erythromycin (Em), SDS, lysozyme (Lsz), or proteinase K (PK) was tested on solid BG11 medium as described in Materials and Methods and shown in Fig. S5 in the supplemental material (see also the work of Nicolaisen et al. [26]). The substance(s) to which the indicated strain was more sensitive than the control strain (PCC 7120 or CSR10 [see Fig. S5 in the supplemental material and reference 26]) are indicated. NS, the indicated strain was not more sensitive than the control strain to any of the tested substances; ND, not determined.
[c] Uptake assays were performed as described in Materials and Methods, with 10 μM glutamate (Glu) or aspartate (Asp) as the substrate in 10-min assays. Shown are the means and standard deviations from the number of experiments indicated in parentheses, which were performed with samples from independent cultures. The significance (*P*) of the differences between the mutants and the wild-type figures was assessed by Student's *t* test.
[d] Data from Nicolaisen et al. (26).
[e] Data from Rudolf et al., unpublished.
[f] Mariscal et al. (55).

termined the lengths of the filaments of the mutants. Samples from cultures of the wild type and cell envelope mutants grown for 4 days in liquid BG11 medium were visualized by light microscopy, and filament sizes were determined as the number of cells per filament. The distributions of filament sizes in the mutants and the wild type were compared (Fig. 3). Strain CSVM34, a *sepJ* gene mutant whose filaments fragment extensively, was used for comparison. As shown in Fig. 3, each of the cell envelope mutants presented a different distribution of filament sizes. Strain AFS-I-*alr0075* was the only mutant in which the distribution of filament sizes did not differ significantly from that in the wild type. Other than strain CSVM34, strains AFS-I-*alr2270*, CSMI23, DR1992, and DR1967 had the highest proportions of short filaments. In contrast, strains AFS-I-*all1861* and DR1822 had a smaller proportion of short filaments than the wild type but did not show an increased percentage of long filaments. Thus, except for strain AFS-I-*all1861*, the PG mutants produced shorter filaments than the wild type, although mutant AFS-I-*alr2270*, altered in lipid A biosynthesis, was the strain showing the shortest filaments, with more than 60% of filaments with a length not greater than 20 cells. Therefore, alteration by mutation of almost any component of the cell envelope results in an altered distribution of filament sizes. Because mutants AFS-I-*alr2269*, AFS-I-*alr0075*, AFS-I-*alr4893*, AFS-I-*alr2270*, DR1967, AFS-I-*all1861*, CSR27, and CSMI24 were heterozygous, i.e., contained both wild-type and mutant chromosomes, a decrease in the numbers of the corresponding wild-type genes appears sufficient to alter filament length.

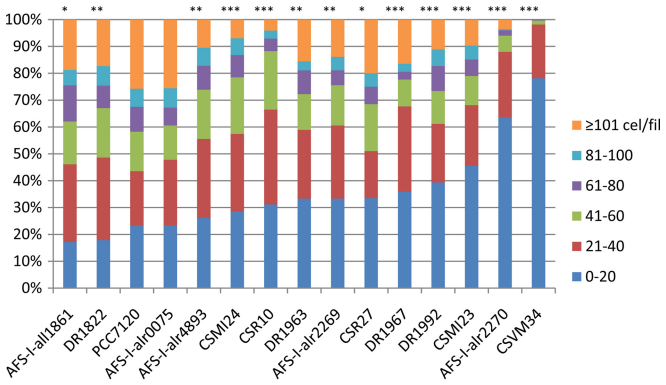

FIG 3 Distribution of filament lengths in *Anabaena* cell envelope mutants. Samples of shaken cultures of the wild type and mutants grown in BG11 medium (with the corresponding antibiotics for the mutants) were taken with great care to avoid disruption of the filaments and counted. A total of 170 filaments (from three or four independent cultures) from each strain were ascribed to the indicated size intervals (size is expressed as cells per filament [cel/fil], with the color code on the right; percentages of filaments are indicated on the left). The distribution of the filament sizes in each mutant was compared to that of the wild type using the χ^2 test; *P* values are indicated with asterisks to denote the level of significance (*, $P \leq 0.05$; **, $P \leq 0.001$; ***, $P \leq 0.0001$). Strain CSVM34 (*sepJ*) was used for comparison. The wild-type and mutant strains are arranged based on increasing percentages of the 0- to 20-cell/filament size interval.

We then asked whether the cell envelope mutants were more sensitive to mechanical fragmentation than the wild type. Samples from the same cultures used for the analysis of the distribution of the filament sizes were subjected to mechanical fragmentation by passing them through a syringe (see Materials and Methods) and visualized by light microscopy. As a result of this treatment, the filaments from most mutants had a mean size significantly different from that of the wild-type filaments ($P < 0.05$) (Table 4), including two mutants (strains DR1967 and CSR27) showing somewhat larger filaments. The filaments from strains AFS-I-*alr2270* and CSMI23 were particularly fragmented by the treat-

TABLE 4 Lengths of mechanically fragmented filaments from *Anabaena* wild type and cell envelope mutants

Strain	Segregation of mutant chromosomes[a]	Size of mechanically fragmented filaments[b]	
		No. of cells per filament	*P*
PCC 7120	Wild type	22.03 ± 17.18 (1,700)	
AFS-I-*alr2269*	No	20.35 ± 19.91 (210)	0.175
AFS-I-*alr0075*	No	21.70 ± 16.11 (209)	0.794
AFS-I-*alr4893*	No	22.32 ± 16.30 (210)	0.815
AFS-I-*alr2270*	No	12.71 ± 11.31 (343)	10^{-16}
DR1822	Yes	25.27 ± 19.80 (209)	0.012
DR1963	Yes	21.05 ± 17.56 (210)	0.438
DR1967	No	25.26 ± 21.17 (207)	0.013
AFS-I-*all1861*	No[c]	19.41 ± 15.53 (290)	0.015
CSR27	No[c]	26.64 ± 21.45 (217)	0.0003
DR1992	Yes	20.00 ± 16.14 (1,091)	0.002
CSMI23	Yes	14.14 ± 10.12 (1,703)	10^{-56}
CSMI24	No	20.47 ± 16.57 (1,642)	0.008
CSVM34	Yes[d]	6.27 ± 3.36 (310)	10^{-54}

[a] The presence of wild-type and mutant chromosomes was tested by PCR, as described in Fig. S1 to S4 in the supplemental material.
[b] To determine filament lengths, samples (10 ml) were subjected to mechanical fragmentation by passing them three times through a 25-G (length, 5/8 in.) syringe and visualized by light microscopy, as described in Materials and Methods. Shown are the means and standard deviations of the number of filaments indicated in parentheses, which were taken from 3 or 4 independent cultures. The significance (*P*) of the differences between the wild type and each mutant was assessed by Student's *t* test.
[c] Rudolf et al., unpublished.
[d] Mariscal et al. (55).

ment (Table 4). However, none of these mutants fragmented as much as the *sepJ* mutant. Thus, the strains that showed more short filaments in the distribution of filament sizes (AFS-I-*alr2270* and CSMI23) were also more sensitive to mechanical fragmentation, indicating that the cell envelope contributes to the maintenance of the integrity of the filament. Microscopic inspection of fragmented filaments predominantly showed filaments ending in round cells and only a few filament ends evidently corresponding to a broken cell, but we have not addressed the mechanism through which filaments break down.

DISCUSSION

Class B PBPs have only transpeptidase activity, with a role in the processes of elongation and division of rod-shaped bacterial cells (51, 52). *Anabaena* Alr5045 is a predicted class B PBP that is homologous to *E. coli* PBP2 (31), which is involved in cell elongation (30). The inactivation of *alr5045* results in a strain (CSMI23) whose vegetative cells show an altered morphology. Specifically, the cells of strain CSMI23 are shorter and wider than the wild-type cells. This morphology could result from a deficit in cell elongation, which would be consistent with a role of Alr5045 similar to that of *E. coli* PBP2 in cell elongation. Strain CSMI23 shows a delay in heterocyst differentiation and is impaired in aerobic diazotrophic growth specifically on solid media. In *Anabaena*, *alr5101* encodes a class A PBP (18) whose inactivation results in a deficit in aerobic N_2 fixation and in altered morphology of both vegetative cells and heterocysts (53). Two other class A PBPs, All2981 and Alr4579, are also specifically required for aerobic N_2 fixation, and their inactivation results in an altered polysaccharide layer of the heterocyst envelope (31). In addition, an *N*-acetylmuroamoyl-L-alanine amidase is required specifically for heterocyst maturation (38, 39). Therefore, PG metabolism-related enzymes appear to be important during the differentiation of heterocysts: they may be needed for the proper assembly of the glycolipid and polysaccharide layers of the heterocyst envelope or for remodeling of the vegetative-cell–heterocyst intercellular septa.

In contrast to strain CSMI23, the *alr0718* mutant CSMI24 could not be segregated. Alr0718 likely represents the *Anabaena* ortholog of *E. coli* FtsI/PBP3 (47), a protein specifically involved in septal-PG synthesis during cell division (30). It seems that whereas other PBPs can fulfill, at least partly, the role of Alr5045 in cell elongation, no protein can replace Alr0718. The *ftsI* ortholog of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 is also an essential gene (54). Labeling of strains CSMI23 and CSMI24 with Van-FL increased in the septa of the former and decreased in the septa of the latter compared to the wild type, consistent with a role of Alr5045 and Alr0718 in PG synthesis during cell elongation and septation, respectively. Perhaps cells of strain CSMI23 respond to diminished cell elongation by increasing synthesis of septal PG.

To investigate a possible role of the cell envelope in filamentation, we examined PG and OM mutants of *Anabaena*. Regarding PG-related genes, in addition to *alr5045* (encoding a PBP2 protein), *alr0718* (*ftsI*), and *alr0093* (*hcwA* or *amiC2*) mutants, strains CSMI23, CSMI24, and DR1992, respectively, we investigated *alr2458* (encoding an alanine racemase) and *all1861* (encoding a PG-binding protein) mutants, strains CSR27 and AFS-I-*all1861*, respectively. Strains CSMI23 and DR1992 formed filaments significantly shorter than those of the wild type when grown in standard BG11 medium, whereas filaments of strain CSR27 were not very different in length compared to those of the wild type and those of AFS-I-*all1861* were somewhat longer than those of the wild type. Although all strains were somewhat affected, only the filaments of strain CSMI23 were severely fragmented by mechanical shearing. Short filaments in BG11 medium and, in the case of strain CSMI23, the response to mechanical fragmentation indicate a role of PG in maintaining long filaments in *Anabaena*. The role of peptidoglycan in filamentation may be related to its chemical nature as a covalently linked macromolecule that can span several cell units in the filament. On the other hand, the sensitivity of most PG-related mutants to SDS may imply a malformation of their OMs resulting from impaired anchoring to an altered PG.

Regarding OM-related genes, mutants of the Omp85-like protein Alr2269 and of the lipid A biosynthesis protein Alr2270 have previously been shown to produce an OM with increased permeability (26), resulting in very high activities of transport of acidic amino acids (Table 3). We have shown here that mutations that affect the biosynthesis of the O antigen of the LPS may also increase the permeability of the OM, because at least strains DR1963 and DR1967 show significantly increased levels of uptake of acidic amino acids. In addition, these strains and DR1822 are sensitive to SDS and, in the cases of DR1822 and DR1963, to the other harmful substances tested. Strains DR1963 and DR1967 form significantly more abundant short filaments than does the wild type, whereas among the OM mutants investigated, strain AFS-I-*alr2270* is notable for producing a large fraction of short filaments under standard growth conditions and being very sensitive to mechanical fragmentation. Available data suggest that this mutant bears a much altered OM, correlating with its production of short, weak filaments. Although the components of the OM are kept together

mainly, if not exclusively, by noncovalent chemical interactions, our results imply that the OM also contributes to filamentation. The molecular basis for the role of the OM in filamentation, however, remains to be determined. We note, for instance, that strain DR1822, which completely lacks the O antigen of LPS, unexpectedly makes longer filaments than strain DR1967, which produces substantial amounts of LPS (29).

In summary, we have found in *Anabaena* that a PG metabolism mutant, strain CSMI23, and a lipid A biosynthesis mutant, AFS-I-*alr2270*, make very short, weak filaments, although other cell envelope mutants are also affected in the length of the filaments that they produce. None of the investigated cell envelope components, however, is as important as the septal protein SepJ for filamentation. Our results are consistent with the idea that specific elements, such as SepJ, are key for filamentation but also that filaments become and remain lengthy, in part, due to characteristics of their cell envelopes.

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