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Mutation of the *murC* and *murB* Genes Impairs Heterocyst Differentiation in *Anabaena* sp. Strain PCC 7120

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ABSTRACT

To stabilize cellular integrity in the face of environmental perturbations, most bacteria, including cyanobacteria, synthesize and maintain a strong, flexible, three-dimensional peptidoglycan lattice. *Anabaena* sp. strain PCC 7120 is a filamentous cyanobacterium capable of differentiating morphologically distinct nitrogen-fixing heterocyst cells in a periodic pattern. While heterocyst development has been shown to require proper peptidoglycan remodeling, the role of peptidoglycan synthesis has remained unclear. Here we report the identification of two peptidoglycan synthesis genes, *murC* (*alr5065*) and *murB* (*alr5066*), as required for heterocyst development. The *murC* and *murB* genes are predicted to encode a UDP-*N*-acetylmuramate:L-alanine ligase and a UDP-*N*-acetylenolpyruvoylglucosamine reductase, respectively, and we confirm enzymatic function through complementation of *Escherichia coli* strains deficient for these enzymes. Cells depleted of either *murC* or *murB* expression failed to differentiate heterocysts under normally inducing conditions and displayed decreased filament integrity. To identify the stage(s) of development affected by *murC* or *murB* depletion, the spatial distribution of expression of the patterning marker gene, *patS*, was examined. Whereas *murB* depletion did not affect the pattern of *patS* expression, *murC* depletion led to aberrant expression of *patS* in all cells of the filament. Finally, expression of *gfp* controlled by the region of DNA immediately upstream of *murC* was enriched in differentiating cells and was repressed by the transcription factor NtcA. Collectively, the data in this work provide evidence for a direct link between peptidoglycan synthesis and the maintenance of a biological pattern in a multicellular organism.

IMPORTANCE

Multicellular organisms that differentiate specialized cells must regulate morphological changes such that both cellular integrity and the dissemination of developmental signals are preserved. Here we show that the multicellular bacterium *Anabaena*, which differentiates a periodic pattern of specialized heterocyst cells, requires peptidoglycan synthesis by the murine ligase genes *murC* (*alr5065*) and *murB* (*alr5066*) for maintenance of patterned gene expression, filament integrity, and overall development. This work highlights the significant influence that intracellular structure and intercellular connections can have on the execution of a developmental program.

Bacteria have evolved diverse strategies to combat environmental changes, including osmotic pressure, oxidative damage, and nutrient deprivation (reviewed in references 1 and 2). To prevent lysis from such environmental stressors, two different cell wall architectures have been adopted that also serve as a basis for classification. Gram-positive bacteria have a characteristically thick peptidoglycan layer (20 to 80 nm) outside an inner membrane, while Gram-negative strains have an inner membrane and outer membrane separated by a thin peptidoglycan layer (1 to 7 nm). Cyanobacteria have a Gram-negative cell wall organization, except that the peptidoglycan layer is thicker—normally between 15 and 35 nm for filamentous strains (3–6). The peptidoglycan layer must be rigid enough to contribute to cell stabilization but flexible enough to respond to changing conditions.

Peptidoglycan is a mesh-like structure built from two repeating amino sugars, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), with a pentapeptide chain that cross-links glycan strands (reviewed in references 7 and 8). Based primarily on work from *Escherichia coli* and a few other model organisms, peptidoglycan is built in three stages: (i) the formation of the GlcNAc and MurNAc disaccharide with the pentapeptide chain, (ii) the translocation of subunits across the membrane by a lipid transporter, and finally (iii) the transglycosylation and transpeptidation of the new glycan strands into the preexisting matrix. After the initial synthesis of GlcNAc, the MurB enzyme (a UDP-*N*-acetylenolpyruvoylglucosamine reductase) converts UDP-*N*-acetylglucosamine enolpyruvate into UDP-*N*-acetylmuramic acid (MurNAc). Following the synthesis of MurNAc, the amino acids that comprise the pentapeptide chain are sequentially added to MurNAc by a series of Mur ligases. The first is MurC, a UDP-*N*-acetylmuramate:L-alanine ligase that adds the first D-alanine subunit to the chain, followed by MurD, MurE, and MurF. GlcNAc

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and MurNAc are then attached to a lipid transport precursor, and the GlcNAc-MurNAc-pentapeptide complex is flipped from the cytoplasm to the outside the cell membrane for incorporation into the existing peptidoglycan matrix and cross-linking. Gram-negative bacteria have 20 to 30% cross-linking, while cyanobacteria have a higher cross-linking percentage closer to that of Gram-positive bacteria—between 56 and 63% (3, 9, 10).

The filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 (herein, *Anabaena*) differentiates a periodic pattern of morphologically distinct, nitrogen-fixing heterocyst cells in response to the depletion of environmental combined nitrogen (reviewed in references 11 and 12). Heterocysts create a microoxic environment for the oxygen-labile nitrogenase complex to fix atmospheric dinitrogen. Nitrogen fixed by heterocysts is exported to neighboring vegetative cells in exchange for a source of reductant, thus delineating two mutually dependent cell types within a multicellular organism. The master regulator of differentiation, HetR, and its inhibitors, PatS and HetN, control the formation and maintenance of a periodic pattern that places heterocysts at regular intervals along the filament (approximately every 10th cell) (13). HetR is an autocatalytic transcriptional regulator that is upregulated 0.5 h after the induction of differentiation (14), which in turn promotes the expression of its own inhibitor, *patS* (15). It is the interaction of HetR and PatS, and later HetN, along the length of an *Anabaena* filament that defines and maintains the pattern of cells that will develop into heterocysts (16–19). This pattern can be visualized within 8 to 10 h after upregulation by marking *patS* expression with a reporter such as green fluorescent protein (GFP) or yellow fluorescent protein (YFP) (20). Following pattern formation, cells commit to differentiation and undergo the morphological changes necessary to create functional heterocyst cells by 24 h after sensing nitrogen starvation.

To exclude molecular oxygen for continued nitrogenase function, heterocysts deposit two external layers (an outer polysaccharide layer and an inner glycolipid layer) during morphogenesis (21). Previous studies suggest that the peptidoglycan must also be remodeled during this process. A transposon insertion in the *N*-acetylmuramoyl:L-alanine amidase *hcwA* gene resulted in the formation of heterocysts that were incapable of fixing nitrogen under oxic conditions (22). *hcwA* encodes a protein that is homologous to amidases that are involved in the construction, recycling, and remodeling of peptidoglycan, and the gene product was shown to have cell wall lytic activity in *E. coli*. Similarly, transposon inactivation of the gene encoding the penicillin-binding protein Pbp6, a member of a class of proteins known to be involved in peptidoglycan formation and maintenance, resulted in a normal pattern of heterocysts that could fix nitrogen only under anoxic conditions (23). The amidase-encoding genes *amiC1* and *amiC2* were both found to be required for proper heterocyst differentiation in *Anabaena* and in the closely related strain *Nostoc punctiforme* ATCC 29133, respectively (24, 25). Mutation of *amiC1* in *Anabaena* resulted in the greatly reduced formation of heterocysts, and the mutant was impaired in the movement of the fluorescent dye calcein-AM between cells (25). Although also impaired in intercellular dye movement, an *N. punctiforme amiC2* mutant was entirely incapable of forming heterocysts and, instead, created cellular aggregates of fragmented filaments due to incomplete septal cleavage between dividing cells (24). Additionally, peptidoglycan structure was altered in the *amiC2* mutant compared to the wild type, as shown by transmission electron microscopy (TEM) analysis. Recent work has shown that the SjcF1 protein binds to peptidoglycan and is required for the movement of fluorescent dyes between cells due, at least in part, to its role in the proper formation of septal nanopores used for transport between adjacent cells (26).

Each of the phenotypes described above is due to mutations in genes involved in peptidoglycan maintenance, which present a growing body of evidence that the peptidoglycan layer surrounding heterocysts may require extensive remodeling during cellular differentiation. Here we show that the *murC* and *murB* genes are required for heterocyst differentiation in *Anabaena* and are also required for maintenance of patterned expression of the *patS* gene during development. This work is the first to provide evidence of a direct link between the formation of peptidoglycan subunits, rather than remodeling of the layer, and the maintenance of a biological pattern leading to cellular differentiation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The growth of *Escherichia coli* and *Anabaena* sp. strain PCC 7120 (wild type) and its derivatives, concentrations of antibiotics, the induction of heterocysts in media lacking a source of combined nitrogen, and conditions for photomicroscopy were as previously described (27, 28). Growth medium containing ammonia as a nitrogen source was prepared as previously described (29). Plasmids were introduced into *Anabaena* strains by conjugation from *E. coli* as previously described (30). Transposon mutagenesis, screening for mutants unable to grow diazotrophically, and determination of the transposon insertion site were performed as previously described (31, 32). The ability of strains to grow diazotrophically was assessed visually following 2 weeks of growth on BG-11 medium lacking a combined nitrogen source as previously described (33). Expression from the copper-inducible *petE* promoter was achieved with the addition of copper to a final concentration of 2 μM (34). Medium deficient in copper was prepared as previously described (27). To determine heterocyst percentages, 500 cells were counted. All results are expressed as the average from three replicates, and error bars represent 1 standard deviation. Mean filament length was determined by counting the cells in 125 filaments and taking the average as previously described (31). *E. coli* temperature-sensitive strains ST5 and ST222 were cultured at 28°C, and growth medium was supplemented with 0.3% thymidine (35). To inactivate the *murB* and *murC* genes in strains ST5 and ST222, respectively, cultures were grown at 42°C. Transcription from the arabinose-inducible *BAD* promoter was induced with the addition of 0.2% L-arabinose (36).

Plasmid construction. The strains and plasmids used in this study are listed in Table 1. The oligonucleotide primers used in this study are listed in Table S1. The integrity of all PCR-derived products was verified by sequencing. Plasmid pPJAV265 is a mobilizable shuttle vector based on pAM504 (37) carrying the *patS* promoter (P_{patS}) transcriptionally fused to the yellow fluorescent protein (YFP). The coding region of YFP was amplified by PCR from pUC57-PS12-yfp (38) with the primers Turbo-OEX-F and YFP-MunI-R, and P_{patS} was amplified by PCR from pAM1951 (39) with the primers PpatS-XKS-MunI-F and PpatS-OEX-R. These products were fused by overlap extension PCR (40) and cloned as a MunI fragment into the EcoRI site of pAM504, and directionality was determined by PCR to create pPJAV265.

Plasmid pPJAV308 is a suicide plasmid based on pRL277 (14) used to inactivate the *murB* gene. A 409-bp fragment of the *murB* coding region was amplified by PCR from *Anabaena* chromosomal DNA with the primers murB-insert-F and murB-insert-R. The product was cloned as a BamHI-SacI fragment into the BglII-SacI sites of pRL277 to create pPJAV308.

Plasmid pPJAV309 is a suicide plasmid based on pRL277 used to inactivate the *murC* gene. A 505-bp fragment of the *murC* coding region was amplified by PCR from *Anabaena* chromosomal DNA with the primers

TABLE 1 Strains and plasmids utilized in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
Strains		
Anabaena		
PCC 7120	Wild type	Pasteur Culture Collection
UHM101	Δ*patA*	52
UHM103	Δ*hetR*	27
UHM350	Δ*murB* insertion carrying pPJAV329	This study
UHM351	Δ*murC* insertion carrying pPJAV328	This study
BJP002	Δ*alr5067* insertion	This study
E. coli		
ST5	*murB*(Ts) mutant; Sm^r	35
ST222	*murC*(Ts) mutant; Sm^r	35
Plasmids		
pAM504	Shuttle vector for replication in *E. coli* and *Anabaena*; Km^r Nm^r	37
pAM1956	pAM504 with promoterless *gfp*	39
pAM1951	pAM504 with P_{patS}-*gfp*	39
pRL277	Suicide vector; Sp^r Sm^r	14
pRR106	Suicide plasmid with transposon; Sp^r Sm^r	31
pUC57-PS12-yfp	Plasmid used as template for YFP	8
pPJAV360	pAM504 with the arabinose-inducible *BAD* promoter	41
pPJAV265	pAM504 with P_{patS}-YFP	This study
pPJAV308	pRL277 used to inactivate *murB*	This study
pPJAV309	pRL277 used to inactivate *murC*	This study
pPJAV328	pAM504 with P_{petE}-*murCB* transcribed divergently from P_{patS}-YFP	This study
pPJAV329	pAM504 with P_{petE}-*murB* transcribed divergently from P_{patS}-YFP	This study
pPJAV336	pAM504 with P_{petE}-*murC* transcribed divergently from P_{patS}-YFP	This study
pPJAV371	pAM504 with P_{BAD}-*murB*	This study
pPJAV372	pAM504 with P_{BAD}-*murC*	This study
pPJAV425	pAM504 with P_{murB}-*gfp*	This study
pPJAV426	pAM504 with P_{murC}-*gfp*	This study
pPJAV427	pAM504 with P_{murC}-*gfp* with a mutated NtcA binding site	This study
pPJAV611	pRL277 used to inactivate *alr5067*	This study

[a] Ts, temperature sensitive; Sm, streptomycin; Km, kanamycin; Nm, neomycin; Sp, spectinomycin.

murC-insert-F and murC-insert-R. The product was cloned as a BamHI-SacI fragment into the BglII-SacI sites of pRL277 to create pPJAV309.

Plasmids pPJAV328, pPJAV329, and pPJAV336 are mobilizable shuttle vectors based on pAM504 carrying P_{patS}-YFP divergently transcribed from P_{petE}-*murC/B*, P_{petE}-*murB*, or P_{petE}-*murC*, respectively. The *petE* promoter was amplified by PCR from chromosomal DNA with the primers PpetE-XhoI-F and PpetE-OEX-R, and *murC/B*, *murB*, and *murC* were amplified by PCR from *Anabaena* chromosomal DNA with the primer sets murC-OEX-F and murB-OEX-BglII-R, murB-OEX-F and murB-OEX-BglII-R, and murC-OEX-F and murC-OEX-BglII-R, respectively. These products were overlap extended together and cloned into the SmaI site of pPJAV265, and directionality was determined by PCR to create pPJAV328, pPJAV329, and pPJAV336.

Plasmids pPJAV371 and pPJAV372 are mobilizable shuttle vectors based on pAM504 carrying P_{BAD}-*murB* and P_{BAD}-*murC*, respectively. The coding regions of *murB* and *murC* were amplified by PCR from *Anabaena* chromosomal DNA with the primer pairs murB-BamHI-F and murB-SacI-R and murC-BamHI-F and murC-SacI-R, respectively. The products were cloned as BamHI-SacI fragments into the same sites of pPJAV360 (41) to create pPJAV371 (P_{BAD}-*murB*) and pPJAV372 (P_{BAD}-*murC*).

Plasmids pPJAV425 and pPJAV426 are mobilizable shuttle vectors based on pAM504 carrying P_{murC}-*gfp* or P_{murB}-*gfp*, respectively. The promoter regions of *murC* and *murB* were amplified by PCR from *Anabaena* chromosomal DNA with the primer sets PmurC-F and PmurC-R and alr5066-up-FM475L and alr5066-up-RM475L, respectively. These products were cloned into the SmaI site of pAM1956, and directionality was determined by PCR to create pPJAV425 (P_{murC}-*gfp*) and pPJAV426 (P_{murB}-*gfp*).

Plasmid pPJAV427 is a mobilizable shuttle vector based on pAM504 carrying P_{murC}-*gfp* with transversions at each of the 6 nucleotides comprising the proposed NtcA binding site (from 5′-GTA-N_8-TAC-3′ to 5′-TGC-N_8-GCA-3′). Fragments upstream and downstream of the NtcA binding site were amplified by PCR from *Anabaena* chromosomal DNA with the primer sets PmurC-F and PmurC-NtcAmut-R and PmurC-NtcAmut-F and PmurC-R. The resulting products were fused by overlap extension PCR, with the region of overlap introducing the mutations to the NtcA binding site, and cloned into the SmaI site of pAM1956, and directionality was determined by PCR to create pPJAV427.

Plasmid pPJAV611 is a suicide plasmid based on pRL277 used to inactivate *alr5067*. A 252-bp fragment of the *alr5067* coding region was amplified by PCR from *Anabaena* chromosomal DNA with the primers alr5067-int-BamHI-F and alr5067-int-SacI-R. The product was cloned as a BamHI-SacI fragment into the BglII-SacI sites of pRL277 to create pPJAV611.

Strain construction. Suicide plasmids pPJAV309 and pPJAV308, carrying fragments of the coding regions of *alr5065* (*murC*) and *alr5066* (*murB*), respectively, were introduced into the wild type as previously described (31). Introduction of these plasmids creates single-crossover mutations that interrupted the desired gene via insertion of an entire plasmid at the recombination site. This insertion results in duplication of the homologous region used for recombination and produces truncated

alleles of *alr5065* or *alr5066*. To determine whether plasmid insertion into *alr5065* or *alr5066* was achieved, colonies resistant to spectinomycin and streptomycin were verified by PCR with the primers murC-OEX-F and murB-OEX-F, respectively, which anneal outside the regions of genomic DNA incorporated onto the plasmids, and pRL-out-SacI-R, which anneals to the plasmid. To facilitate complete segregation of the desired mutations to all copies of the chromosome, colonies containing insertions into the *alr5065* or *alr5066* gene had plasmids harboring *alr5065*, *alr5066*, or both introduced. These plasmids were utilized because complete segregation of the insertions would not be achieved if the *alr5065* and *alr5066* genes are essential. Colonies containing insertions that were resistant to spectinomycin, streptomycin, and neomycin were restreaked at least three times and then assessed for complete segregation of the chromosomal insertion. Complete segregation of the *alr5065* or *alr5066* insertions was determined by the inability to amplify a specific PCR product with the primers murC-OEX-F and murC-OEX-BglII-R or murB-OEX-F and murB-OEX-BglII-R, respectively, which anneal outside the regions of genomic DNA incorporated onto the plasmids to create strains UHM350 and UHM351. In all cases in which UHM350 and UHM351 were used, spectinomycin and streptomycin selection was maintained to ensure that disruption of the *alr5065* or *alr5066* gene was maintained. To cure the plasmids inserted into *alr5065* or *alr5066*, UHM350 and UHM351 were grown without spectinomycin and streptomycin selection and plated on BG-11 containing 5% sucrose to select for cells in which the *sacB* gene present on the plasmid integrant had been resolved (42). Cured strains were verified by amplification of a specific PCR product using the primers murC-OEX-F and murC-OEX-BglII-R or murB-OEX-F and murB-OEX-BglII-R, respectively, and sensitivity to spectinomycin and streptomycin.

Suicide plasmid pPJAV611, carrying a fragment of the coding region of *alr5067*, was introduced into the wild type as described above to create strain BJP002. Spectinomycin- and streptomycin-resistant colonies were verified for proper insertion by PCR with the primers alr5067-up-out and pRL-out-SacI-R and for complete segregation with the primers alr5067-up-out and alr5067-dn-out.

Alcian blue staining, acetylene reduction assays, and bioinformatic analyses. Alcian blue staining of the exopolysaccharide layer to identify mature heterocysts was conducted as previously described (28). Aerobic and anaerobic acetylene reduction assays to assess heterocyst functionality were conducted as previously described (27, 43, 44). The amino acid sequences of MurB and MurC from *Anabaena* were aligned against 15 and 7 other protein sequences, respectively, utilized in previous work (45, 46). The sequences were aligned using the program PRALINE with server time provided by VU University, Amsterdam, The Netherlands (47–50). Analysis was conducted using the residue substitution matrix BLOSUM62 with the default program settings.

RESULTS

Identification of a transposon mutant impaired in diazotrophic growth. During a screen for mutants impaired in diazotrophic growth, a strain was found to contain a transposon insertion at nucleotide position +476 relative to the annotated translational start site of *alr5065*. This mutant, designated C6, was unable to grow diazotrophically and displayed a deficit in heterocyst differentiation, producing 4.5% heterocysts compared to the 9% produced by the wild type (see Fig. S1 in the supplemental material). Proper heterocyst production was restored in C6 when the mutation was complemented with both *alr5065* and *alr5066* but not when either gene was introduced individually, which suggests that *alr5065* and *alr5066* may be in an operon and the transposon insertion affected the expression of both genes. After repeated rounds of subculturing, C6 lost viability and ceased to grow in culture. Multiple attempts were made to create clean or insertional mutants of *alr5065* and *alr5066* individually, but no double recombinants were ever recovered.

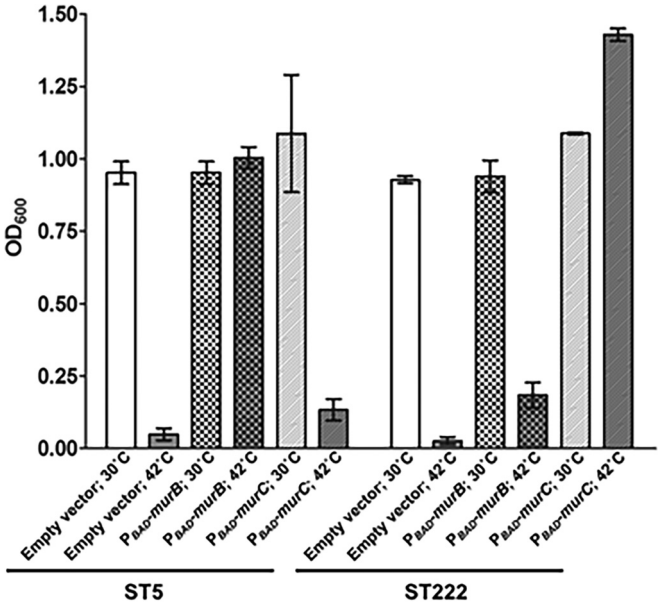

FIG 1 The *murB* and *murC* genes from *Anabaena* can complement the temperature-sensitive *E. coli* strains ST5 (a Δ*murB* strain) and ST222 (a Δ*murC* strain), respectively. Strains ST5 and ST222, harboring a plasmid with either *murB* (pPJAV371) or *murC* (pPJAV372) controlled by the arabinose-inducible *BAD* promoter or an empty vector, were grown at 30°C or 42°C. Cultures were inoculated at an optical density at 600 nm (OD_{600}) of 0.05, expression was induced with the addition of a final concentration of 0.2% L-arabinose, and the final OD_{600} was recorded after overnight growth at either 30°C (the permissive growth temperature) or 42°C (the nonpermissive growth temperature). All treatments were conducted in triplicate, and error bars represent standard deviations.

***alr5065* and *alr5066* encode MurC and MurB, respectively.** On the basis of amino acid homology, *alr5065* and *alr5066* are predicted to encode a UDP-*N*-acetylmuramate:L-alanine ligase (MurC) and a UDP-*N*-acetylenolpyruvoylglucosamine reductase (MurB), respectively. Earlier work on the amino acid sequences of the MurC and MurB proteins from other bacteria identified conserved residues required for function (45, 46). An *in silico* analysis of MurC and MurB from *Anabaena* demonstrated that all of the conserved residues are present in the previously defined positions in both proteins (see Fig. S3 and S4 in the supplemental material). To determine whether the protein products of *alr5065* and *alr5066* have MurC and MurB activities, these genes were expressed in the temperature-sensitive Δ*murC* and Δ*murB E. coli* mutant strains ST222 and ST5 (35), respectively, and complementation was assessed. Strain ST222 harboring the empty vector control or a construct expressing *alr5066* was unable to grow at 42°C (the nonpermissive temperature) (Fig. 1). Expression of *alr5065* in strain ST222 complemented the strain and facilitated growth at 42°C. Similarly, strain ST5 only grew at 42°C when complemented by *alr5066* expression. We conclude that *alr5065* and *alr5066* encode a UDP-*N*-acetylmuramate:L-alanine ligase (MurC) and a UDP-*N*-acetylenolpyruvoylglucosamine reductase (MurB), respectively, capable of catalyzing the same reactions in *E. coli*.

Mutation of *murC* or *murB* inhibits heterocyst differentiation. In an effort to determine if *murC* and *murB* were individually involved in heterocyst differentiation, conditional mutants were created. To do so, *murC* and *murB* were individually disrupted by plasmid insertion and immediately complemented by the addi-

TABLE 2 Filament integrity and heterocyst differentiation in various strains of *Anabaena*

Strain	Copper present in medium[a]	Avg filament length (no. of cells)[b]	% of heterocysts at 24 h N− [c]	Diazotrophic growth[d]
Wild type	Yes	100.9 ± 52.8	7.9 ± 0.50	Yes
	No	101.9 ± 61.6	8.4 ± 0.87	Yes
Wild type/pPJAV329	Yes	109.8 ± 57.3	8.9 ± 0.42	Yes
	No	105.0 ± 57.9	8.1 ± 0.61	Yes
Wild type/pPJAV328	Yes	100.7 ± 36.0	8.2 ± 0.92	Yes
	No	103.7 ± 29.6	7.5 ± 0.64	Yes
UHM350	Yes	40.3 ± 13.2	0	No
	No	12.8 ± 7.2	0	No
UHM351	Yes	46.1 ± 15.6	0	No
	No	13.5 ± 8.3	0	No
UHM350 cured of pPJAV308	Yes	108.9 ± 35.0	7.5 ± 1.01	Yes
UHM351 cured of pPJAV309	Yes	109.0 ± 39.2	8.3 ± 0.76	Yes
BJP002	Yes	102.4 ± 17.4	8.1 ± 0.61	Yes

[a] Copper was either absent from growth medium or maintained at 2 μM.
[b] From 20-ml BG-11 cultures, 125 filaments were counted. The average filament length ± standard deviation is presented.
[c] Heterocyst percentages were determined by counting 500 cells in triplicate. Data are expressed as the average ± standard deviation.
[d] Diazotrophic growth was determined by streaking BG-11 plates lacking a combined nitrogen source, and growth was assessed visually after 2 weeks of incubation.

tion of a replicative plasmid carrying the corresponding genes under transcriptional control of the copper-inducible *petE* promoter (34). Introduction of P_{petE}-*murB* (pPJAV329) into the *murB* mutant in the presence of copper was sufficient for complete segregation of the *murB* insertion and created a viable strain (UHM350). Complete segregation of the *murC* insertion mutant was only achieved with the introduction of both *murC* and *murB* genes, as P_{petE}-*murC/B* (pPJAV328) in the presence of copper. The latter combination was designated strain UHM351. Strains UHM350 and UHM351 displayed a growth defect and spontaneously lysed if not passaged every 2 weeks once a sufficient density was reached during plate-based culture containing copper. These results further suggest that *murC* and *murB* may be cotranscribed in an operon and indicate that the *murC* and *murB* genes were effectively expressed from the copper-inducible *petE* promoter. The impaired growth of these strains on medium that supports normal growth of the wild type indicates a defect in vegetative growth.

The filament integrity and ability to differentiate functional heterocysts were first determined for all strains in the presence of copper to ensure expression of the plasmid-borne *murC* and *murB* genes. The pattern of heterocysts produced by the wild-type strain, as well as average filament length, was unaffected by additional copies of *murC* or *murB* 24 h after the removal of combined nitrogen (Table 2; see Fig. S2A and B in the supplemental material). In contrast, no heterocysts were produced by either of the conditional mutant strains in the presence of copper and the average filament length was significantly reduced compared to that of the wild type, from 100.9 ± 52.8 cells in the wild type to 46.1 ± 15.6 and 40.3 ± 13.2 cells in strains UHM350 ($P = 1.3 \times 10^{-23}$) and UHM351 ($P = 5.9 \times 10^{-28}$), respectively (Table 2; see Table S2 in the supplemental material). Contrary to expectations, the presence of the *murC* and *murB* genes on multicopy plasmids only partially complemented the chromosomal inactivation of these genes.

To mimic *murB* and *murC* null mutants, strains UHM350 and UHM351 were grown on BG-11 containing ammonia but lacking copper for 48 h and then transferred to medium lacking both a combined nitrogen source and copper; incubation for longer than 48 h resulted in culture lysis, likely due to defects in peptidoglycan synthesis. During growth in medium lacking copper, the wild type harboring pPJAV328 or pPJAV329 yielded heterocyst percentages and average filament lengths similar to those observed under copper-replete conditions (Fig. 2A and B). In contrast to the wild type, the conditional mutants UHM350 and UHM351 did not differentiate heterocysts following growth in medium lacking copper and the removal of combined nitrogen (Fig. 2C and D, Table 2). The average filament length of strains UHM350 (12.8 ± 7.2 cells) and UHM351 (13.5 ± 8.3 cells) was significantly lower in medium lacking copper than in medium containing copper ($P = 4.4 \times 10^{-59}$ for UHM350; $P = 5.4 \times 10^{-51}$ for UHM351) (Table 2; see Table S2 in the supplemental material). These results indicate that *murC* and *murB* are required for heterocyst differentiation and contribute to maintaining filament integrity. It is interesting to note that though the presence of an extrachromosomal copy of *murC/B* is sufficient for strain viability, it appears that a functional copy of these genes at the native chromosomal locus is necessary for heterocyst differentiation. These data also suggest that the transposon insertion in the C6 mutant described above was not fully segregated because complete segregation of mutations at this locus abrogates heterocyst formation.

During times of increased stress, bacteria can accumulate suppressor mutations to overcome detrimental characteristics and allow the strain to continue growing. To verify that the phenotypes of UHM350 and UHM351 were due to insertions in *murB* and *murC*, respectively, rather than suppressor mutations, the

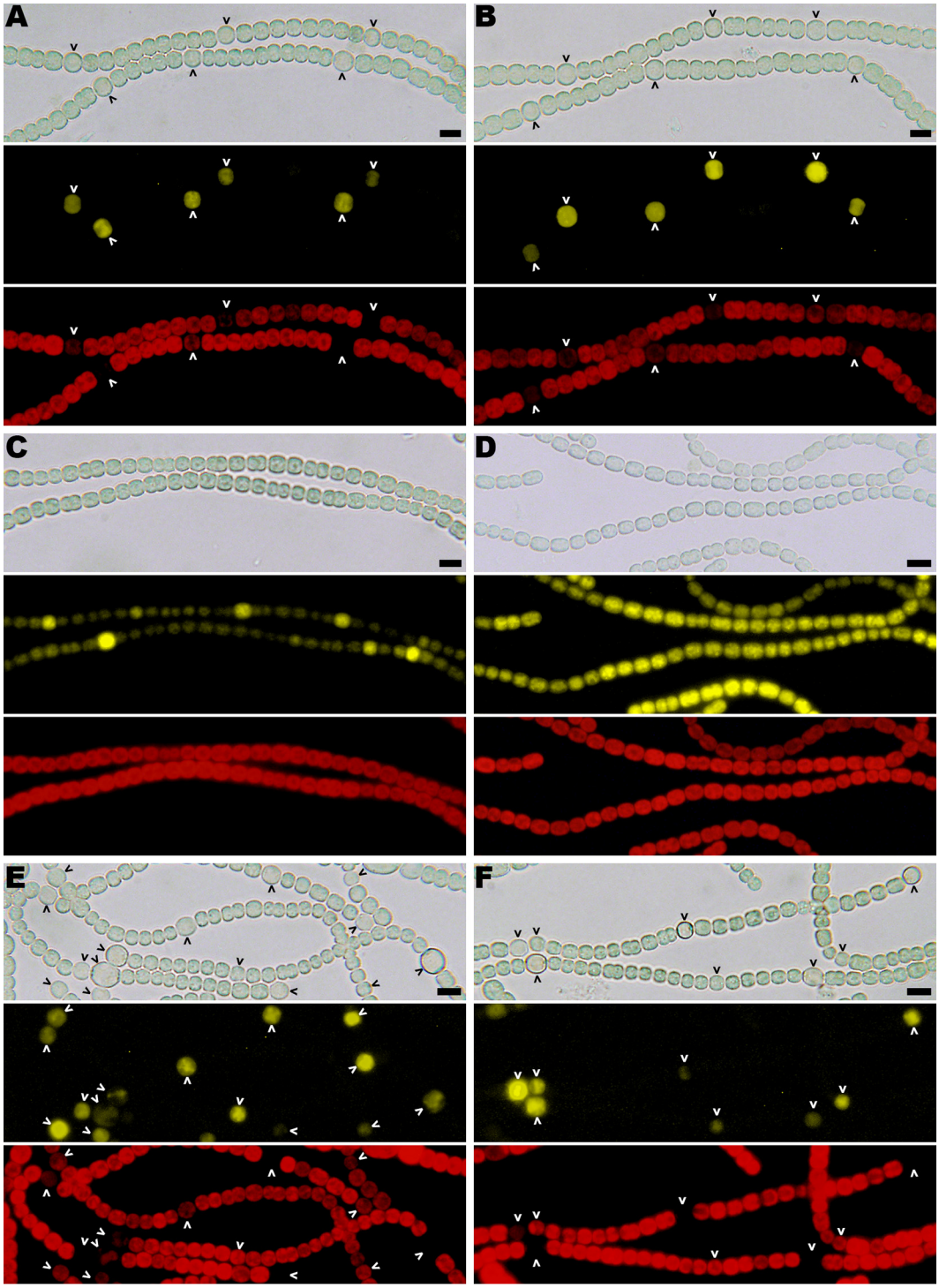

FIG 2 The *murC/B* genes are required for heterocyst differentiation. Cells of the wild-type strain harboring pPJAV329 (P_{petE}-*murB*, P_{patS}-YFP) (A) or pPJAV328 (P_{petE}-*murC/B*, P_{patS}-YFP) (B), UHM350 (in which *murB* is inactivated by pPJAV308) (C) or UHM351 (in which *murC* is inactivated by pPJAV309) (D), UHM350 after curing of pPJAV308 (E), or UHM351 after curing of pPJAV309 (F) were imaged 24 h after the induction of heterocyst differentiation in the absence of copper. From top to bottom are shown the bright-field image with yellow fluorescence from P_{patS}-YFP and red autofluorescence. Carets indicate heterocysts. Bars, 10 μm.

strains were cured of the plasmids inserted into these genes through *sacB*-mediated counterselection on sucrose. Both cured strains displayed a pattern of functional heterocysts and average filament lengths equivalent to those of the wild type (Fig. 2E and F and Table 2; see Table S2 in the supplemental material). Because the cured strains returned to a wild-type phenotype, we conclude that the inability of UHM350 and UHM351 to differentiate heterocysts is due to inactivation of *murB* and *murC*, re-

spectively, by plasmid insertion and not the product of secondary mutations.

It was possible, however, that the phenotypes observed above, including reversion to wild type after curing, were due to the insertional mutations of *murB* and *murC* creating a polar effect on a downstream gene. To determine if a polar mutation was responsible for the inability of UHM350 and UHM351 to differentiate heterocysts, the downstream gene, *alr5067*, was inactivated by plasmid insertion and the phenotype of the resulting strain, BJP002, was assessed. Strain BJP002 developed a wild-type pattern of functional heterocysts 24 h after the induction of differentiation and displayed filament lengths similar to those of the wild type (Table 2; see Table S2 in the supplemental material). These data show that mutation of *alr5067* does not impair heterocyst formation or function or filament integrity. From this, we conclude that the phenotypes of UHM350 and UHM351 were not due to a polar mutation and infer that *murB* and *murC* are therefore required for heterocyst development and filament integrity.

Heterocyst pattern maintenance is impaired in strain UHM351. In response to fixed-nitrogen starvation, *Anabaena* must establish and maintain a regularly spaced distribution of heterocysts along the multicellular filament to continue growing in the absence of a combined nitrogen source. Soon after nitrogen starvation is sensed, cells in the filament upregulate expression of *hetR* (14), the master regulator of differentiation, which in turn upregulates *patS* (15), an inhibitor of HetR. The interaction of PatS and HetR is responsible for *de novo* pattern formation in *Anabaena*, which establishes the pattern of cells that will differentiate into heterocysts (16–18). As shown by a *patS* promoter fusion to *gfp*, transcription of *patS* initially increases in all cells of the filament but resolves to a pattern of about every tenth cell by 8 to 10 h after combined nitrogen removal (8 to 10 h N−) (20). This expression pattern is maintained until about 27 h N−, when transcription of *patS* is halted in mature heterocysts (39, 51).

The inability of strains UHM350 and UHM351 to differentiate heterocysts indicates that some part of the genetic cascade involved in differentiation is impaired. To define the stage at which differentiation has been interrupted, pattern formation and maintenance were assessed by observation of patterned yellow fluorescence from an introduced *patS* promoter fusion with YFP. When strains UHM350 and UHM351, which contain a P_{patS}-YFP transcriptional fusion on the plasmids they harbor, were grown in the presence of copper, a normal pattern of *patS* transcription was evident at 9 h N− (see Fig. S5C and E in the supplemental material). Interestingly, despite the failure to differentiate phenotypically distinct heterocysts, the pattern of *patS* expression was maintained at 24 h N− (see Fig. S5D and F). Because a pattern of *patS* expression was established and maintained similarly to that of the wild type, these results indicate that pattern formation and maintenance were not the cause of the inability to differentiate heterocysts in strains UHM350 and UHM351 in the presence of copper.

To mimic *murC* and *murB* null mutations, strains UHM350 and UHM351 were first grown on BG-11 with fixed nitrogen but lacking copper and then stepped down to medium lacking both components. At 9 h N−, both the mutants and wild type produced a normal pattern of *patS* transcription (see Fig. S5 in the supplemental material). By 24 h N−, the wild type and UHM350 displayed patterned *patS* expression; however, *patS* expression in UHM351 was no longer patterned, and fluorescence from *patS* expression was instead observed in all cells of the filament (Fig. 2C and D). We conclude that the pattern of *patS* expression was not maintained after its formation in UHM351, although this loss of patterning does not explain the inability to differentiate heterocysts in either conditional mutant.

***murC* transcription is patterned, and its expression is influenced by NtcA.** Experimental evidence has shown that a few of the genes involved in heterocyst differentiation in *Anabaena* are expressed in a cell-type-specific manner (12). Because the data presented here suggest a role for *murC* and *murB* in differentiation, transcriptional fusions of the promoter regions of these genes to the green fluorescent protein (GFP) were introduced into the wild type, Δ*hetR* (UHM103) (27), and Δ*patA* (UHM101) (52) strains and fluorescence was assessed qualitatively at the individual cell level and quantitatively at the population level. A P_{murB}-*gfp* fusion failed to display fluorescence in any strain under any condition tested (Fig. 3C), consistent with the idea that *murC* and *murB* may be transcribed as an operon. In contrast, a P_{murC}-*gfp* fusion displayed even fluorescence in all cells of all strains tested when grown with a source of combined nitrogen (Fig. 3A). Following the induction of heterocyst differentiation, elevated levels of green fluorescence were visible in differentiating cells (Fig. 3B), which indicates cell-type-specific activation of the *murC* promoter region. Fluorescence quantification showed that the average expression of *gfp* from the *murC* promoter region was higher from cultures grown in the presence of a combined nitrogen source than 22 h after its removal in all strains tested (Fig. 3C). Expression was also elevated in the Δ*hetR* (UHM103) and Δ*patA* (UHM101) strains, which are both greatly impaired in heterocyst formation. It is possible that in strains lacking the elements to enact a complete developmental program, a repressor is absent, thus facilitating increased *murC* expression.

Analysis of the direct NtcA regulon identified a GTA-N_8-TAC binding site upstream of the *murC* gene, between nucleotides 6036446 and 6036459 (53). To determine the role of this potential binding site in *murC* expression, the GTA-N_8-TAC sequence within the *murC* promoter region was mutated to TGC-N_8-GCA and expression was assessed as described above. While mutation of the NtcA binding site did not alter heterocyst-specific upregulation of *murC* (data not shown), average expression from P_{murC}(Δ*ntcA*)-*gfp* was higher than that from the wild-type promoter in the absence of a combined nitrogen source in all strains tested (Fig. 3C). These data are consistent with increased fluorescence from the P_{murC}-*gfp* fusion described above in Δ*hetR* (UHM103) and Δ*patA* (UHM101) strains in which *hetR* is either absent or patterned gene expression is impaired and, thus, *ntcA* is not properly expressed. Together, these findings suggest that NtcA represses *murC* expression during differentiation, and we infer that repression is likely direct.

DISCUSSION

In this work, we showed that the *murC* and *murB* genes of *Anabaena* are required for heterocyst differentiation and contribute to maintaining the pattern of gene expression necessary for proper heterocyst placement. MurC and MurB were shown to complement *E. coli* strains harboring mutations in homologous muramyl ligases. Because muramyl ligases are critical for peptidoglycan synthesis in *E. coli*, this suggests the mutation of these genes likely results in defects in the peptidoglycan layer in *Anabaena* as well. Additionally, a *murC* promoter fusion demonstrated that this gene is upregulated in a cell-type-specific manner

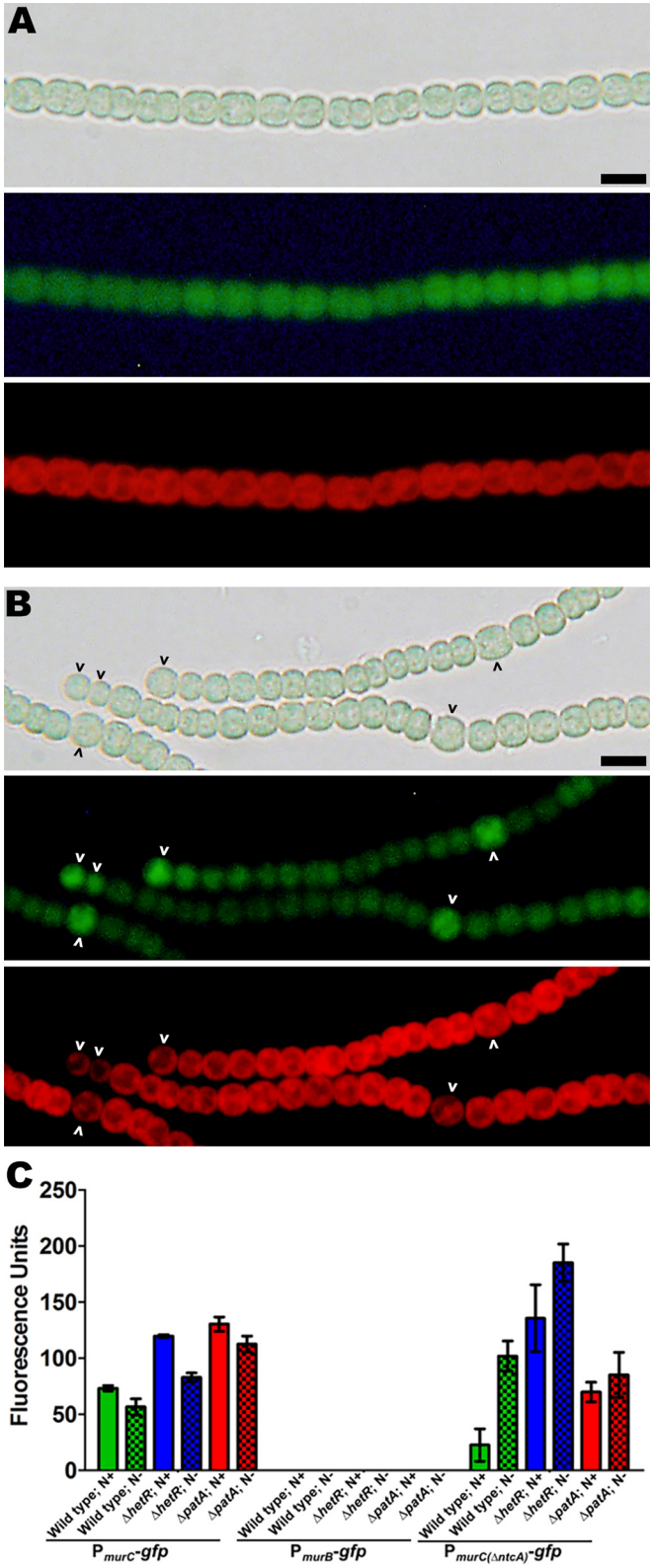

FIG 3 Qualitative and quantitative analyses of expression from the *murC* and *murB* promoter regions. The wild-type strain harboring a P_{murC}-*gfp* transcriptional fusion on pPJAV425 was imaged in the presence of (A) or 22 h after the removal of (B) combined nitrogen. From top to bottom are shown the bright-field image with green fluorescence from P_{murC}-*gfp* and red autofluorescence. Carets indicate heterocysts. Bars, 10 μm. Fluorescence was quantified from the wild-type (green bars), Δ*hetR* (UHM103 [blue bars]), and Δ*patA* (UHM101 [red bars]) strains in the presence of combined nitrogen (N+) or 22 h after the removal of combined nitrogen (N−) harboring P_{murC}-*gfp* (pPJAV425), P_{murB}-*gfp* (pPJAV426), and P_{murC}(Δ*ntcA*)-*gfp* (pPJAV427) transcriptional fusions. All fluorescence measurements were taken at an OD_{750} of 0.1. Error bars show standard deviations.

and that a previously annotated NtcA binding site is required to be intact for normal expression (53). This expression profile is opposite that of the *sjcF1* gene, encoding a peptidoglycan-binding protein, which is downregulated in heterocysts (26). Interestingly, a transposon insertion in *murF* (*all0036*), another muramyl ligase gene, was isolated in a similar, but independent, screen for mutants impaired in diazotrophic growth (P. Videau and L. Cozy, unpublished data). Based on the results presented here and throughout the literature, three main phenotypes are observed when genes involved in peptidoglycan synthesis or remodeling are mutated in heterocystous cyanobacteria: heterocysts are often not formed or are nonfunctional, filament integrity is compromised, and intercellular signaling is disrupted. Based on the genes responsible for these phenotypes and the detrimental nature of these mutations to the organisms, it is apparent that peptidoglycan synthesis and remodeling are essential to heterocyst differentiation.

The conditional *murC* and *murB* mutants presented here were incapable of producing heterocysts under any condition tested. Mutation of the *amiC2* locus in *Nostoc punctiforme* also resulted in a strain that could not differentiate heterocysts, however, in contrast to the normal morphology of the *murC* and *murB* mutants following the removal of combined nitrogen to induce differentiation, the *amiC2* mutant produced an aberrant phenotype consisting of groups of fused cells with incomplete septal cleavage (24). Mutation of the *amiC1* and *alr5045* genes from *Anabaena* resulted in a reduced percentage of heterocysts that formed after a time delay (25, 54). Finally, mutation of the *pbp6* and *hcwA* genes in *Anabaena* yielded strains that produced a normal pattern of heterocysts that could not fix nitrogen under aerobic conditions (22, 23). These three distinct levels of heterocyst production roughly correspond to the stage at which differentiation halted. Mutants that fail to differentiate heterocysts likely disrupt the genetic cascade sometime during the induction or patterning phases. The delayed production of a reduced number of heterocysts indicates that differentiation was perturbed during pattern formation or in the ability to commit to a heterocyst cell fate. Production of a normal pattern of nonfunctional heterocysts is usually the result of a failure during morphogenesis. It remains unknown whether the above defects in differentiation result from improperly formed peptidoglycan itself disrupting the developmental program or if any of these genes' products have additional regulatory roles during differentiation.

In the conditional *murB* and *murC* insertion mutants (UHM350 and UHM351), heterocysts were not formed following complete chromosomal segregation of the insertion, even when complemented with plasmid-borne copies of *murB* and *murC*. It is possible that patterned expression of one or both of these genes is required for differentiation, but because transcription was controlled by the copper-inducible *petE* promoter during complementation rather than the native promoter, normal expression dynamics were not achieved. This is unlikely, however, because overexpression of *murC* and *murB* from the *petE* promoter did not alter heterocyst patterning or forma-

tion in the wild type. It can also be hypothesized that the *murC/B* locus must be intact for heterocyst formation. Perhaps interruption of this locus changes the production of a small RNA or some other regulatory element required for differentiation. Seven transcriptional start sites have been annotated within the coding regions of *murC* and *murB*, so it is conceivable that a small RNA originates from this region (29). A similar result has been described in *Chlamydia trachomatis*, in which disruption of *murC* could not be complemented via reintroduction of the *murC* coding region into a neutral site on the chromosome (55). In this case, the specific genomic locus was required to be intact for proper peptidoglycan formation. It is also possible that the plasmid insertion used to create UHM350 and UHM351 created dominant-negative mutations that resulted in the production of truncated MurC and MurB protein products. In this scenario, the chromosomally encoded truncated MurC and MurB proteins interact with the full-length plasmid-borne *murC* and *murB* protein products, perhaps via dimerization or competition for substrate, leading to inactivation of the complementing proteins and the inability to restore the phenotype.

Mutation of many of the genes associated with peptidoglycan formation or remodeling compromises filament integrity. Filament integrity is maintained, at least in part, by the shared junctional components of adjacent cells. Incorrect peptidoglycan remodeling can therefore disrupt these stabilizing junctions, leading to fragmentation, either through improper formation of the junctions themselves or due to the inability of required proteins to localize to the cell-cell interface. The latter was the case for strains harboring mutations of the septal junction protein genes *sepJ* (*fraG*), *fraCDE*, and *fraH*, where each displayed significant fragmentation following the induction of heterocyst differentiation (31, 56–58). Though mutation of *murC* or *murB* (UHM350 and UHM351) led to fragmentation similar to that in strains with mutations in septal junction and peptidoglycan-associated proteins, UHM350 and UHM351 produced fragmented filaments irrespective of combined nitrogen levels (data not shown). Only the *amiC2* mutant of *N. punctiforme* resulted in a fragmentation phenotype and was also entirely incapable of differentiating heterocysts; however, this strain produced clusters of cells rather than filaments (24). Taken together, there seem to be two main developmental outcomes for improper peptidoglycan formation: (i) strains that differentiate heterocysts and fragment as a consequence of differentiation or (ii) strains that are fragmented and also cannot differentiate heterocysts. It is likely that as more research is done on the role of peptidoglycan formation and remodeling in heterocyst differentiation, causal relationships will inform this phenotypic dichotomy.

The majority of reported peptidoglycan synthesis/remodeling mutants that have decreased heterocysts and fragmented filaments are also impaired in the transport of molecules between adjacent cells. The primary defects responsible for restricted intercellular movement are the malformation of channels in septal junctions or the inability of septal junction proteins to localize for proper function. To assess intercellular movement, most studies observe the transfer of fluorescent molecules, such as 5-carboxyfluorescein, calcein, and esculin, using fluorescence recovery after photobleaching (FRAP) (26, 57, 59–61). These studies have demonstrated that intact copies of the genes that code for septal junction-associated proteins SepJ, FraCDE, FraH, and SjcF1 are required for movement between cells; their loss leads to the formation of insufficient or debilitated intercellular channels as assessed by TEM imaging. A recent study also provided evidence that the range of the HetN-dependent inhibitory signal, an inhibitor of cellular differentiation required for pattern maintenance, requires an intact copy of *sepJ* for optimal diffusion along filaments (19).

In this work, a wild-type pattern of *patS* expression was evident by 9 h after the induction of heterocyst differentiation, but after depletion of *murC* in UHM351, the pattern collapsed within 24 h. Based on the requirement of septal junction proteins for channel formation and the requirement of SepJ for optimal propagation of the HetN-dependent inhibitory signal (19), it is likely that the loss of patterned *patS* expression here was due to the inability of PatS to diffuse out of cells committing to a differentiated cell fate. Once cells commit to forming a heterocyst 9 to 13 h after induction (20), morphological changes begin, including changes to septal junctions, which must result in the continued movement of inhibitory signals to permit the completion of morphogenesis. If the channels within septal junctions fail to change accordingly, it is possible that the PatS inhibitor produced within patterned source cells would be unable to exit. Between 9 and 24 h after the induction of differentiation, the PatS gradient is lost in UHM351 due to the inability to diffuse from previously patterned source cells, resulting in a lack of PatS in the filament. Upon removal of the inhibitory signal, HetR is no longer actively degraded, and the developmental program of heterocyst differentiation is restarted. This scenario suggests two possible reasons as to why signaling is impaired in UHM351. (i) The incorrectly or incompletely formed peptidoglycan might impair the formation or rearrangement of channels necessary for movement of intercellular signaling molecules. (ii) Alternatively, correctly formed peptidoglycan could be required for the localization or function of proteins that regulate the expression of positive factors required for differentiation and patterning. Taken together, this posits an absolute requirement for peptidoglycan synthesis in the proper transfer of intercellular signals in *Anabaena* and the maintenance of a biological pattern through morphogenesis.

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